Richard A. Kaslow
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Viral Infections of Humans

Epidemiology and Control

Fifth Edition

Springer

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Preface to the Fifth Edition

It has been 17 years since the release of the last edition—an inordinately long interval in view of the dizzying pace of discovery in the biomedical sciences. That lengthy interval presented certain challenges in deciding whether and how to embark on the production of a new edition. An immediate reality was that a new volume had to be comprehensive enough to retain the durable parts of the increasingly obsolete earlier edition while capturing the myriad new advances. That significantly greater breadth and depth would require an expanded editorial team. We were fortunate that the three of us together were sufficiently familiar with current experts across our exciting and rapidly evolving field to recruit a stellar group of authorities in their respective specialties. Most auspiciously, although we had not worked so closely together before, we all brought our experience with the previous edition and a commitment to the high standards that Al Evans imparted to everyone involved in the four earlier editions of the text.

The time elapsed since the earlier edition also meant that many chapters and the roster of authors had to be entirely replaced or substantially modified by a largely new group of contributors since relatively few of those involved in the earlier chapters remained available. In the end, dozens of new authors were engaged.

Perhaps the most difficult decision had to be made primarily by Springer. Despite the fact that the publishing world was undergoing a revolutionary transformation to digital media, a determination had to be made on whether a printed book could still be both relevant to the discipline and financially viable. In that regard, during the several years since the first conversations about the new edition with William Tucker, Khristine Queja, and others at Springer, they have steadfastly supported the project even in such an uncertain environment surrounding print publications. To vindicate their judgment as well as our own leadership on the venture, we aspired to a product that would greatly surpass a typical new edition; we hope readers conclude that it meets that test by any reasonable measure.

The text now contains four sections. The first covers principles and methodology, including new chapters on methods of detection and modeling and a largely new perspective on surveillance and biomarker epidemiology. Astonishing technologic progress is reflected in the chapters discussing the proliferation of virologic and immunologic assay platforms, routine applications of RT-PCR, and more recent incorporation of nucleic acid sequencing into research and even clinical diagnostic identification. Some chapters also cite early dividends from the expanding disciplines of bioinformatics and computational biology. The chapter on disease surveillance systems and techniques highlights sophisticated approaches to data gathering, with ever faster reporting aided not only by conventional electronic transmission capability but also increasingly by the hardware and software on portable devices that power social media. Although surveillance still depends heavily on seroepidemiology, biomarker studies of viral infections in large populations now increasingly involve collection and storage of samples other than serum, opening further opportunities to study host-agent interactions at the cellular and molecular level. A new chapter on modeling focuses on the more theoretical aspects of transmission, particularly person-to-person transmission; on the other hand, reference to decision models on projected future numbers of cases, clinical utility of a diagnostic assay, or cost-effectiveness of interventions to combat particular infections appear in the chapters addressing those agents.

The remaining sections categorize viruses and the infections they cause into three groups: those involved primarily in acute disease, those involved primarily in chronic disease including malignancy, and those involved in both. Of the 47 chapters—13 more than in the previous edition—each has been substantially revised or written entirely anew. Where an earlier single chapter may have covered multiple viruses related to each other either clinically (e.g., hepatitis, gastroenteritis) or epidemiologically (e.g., vector-borne diseases), they are now treated more expansively. Four separate chapters cover hepatic diseases (hepatitis A, hepatitis B and D, hepatitis C, and hepatocellular carcinoma); three cover gastroenteritis due to a number of enteric viruses (noroviruses, sapoviruses, astroviruses, rotaviruses, enteroviruses, and hepatitis E virus); and six separate chapters cover countless viruses transmitted by arthropods or small mammal vectors. Although prion-associated spongiform encephalopathies are not viral diseases, the chapter on these conditions reflects recognition that many of their clinical and epidemiologic features resemble those of chronic viral infections closely enough to be instructive in this context.

Viral Infections in Humans has now been in print for more than 40 years. Many dozens of basic, clinical, and population scientists have contributed to one or more editions. And each successive edition has built on the solid foundations laid by colleagues who have preceded us. Of the 90-odd authors connected with this fifth edition, we three editors are among only 9 who also contributed to the fourth. Many who participated earlier are no longer alive, and we thought it was fitting at least to honor those listed below who have passed away during the years since the previous edition, including Caroline Breese Hall, who shared in preparing the chapter on HHV-6 included in this volume.

Abram S. Benenson	2003
Francis L. Black	2007
Floyd W. Denny	2001
Alfred S. Evans	1996
Clarence J. Gibbs Jr.	2001
Eli Gold	2011
Caroline B. Hall	2012
Joseph L. Melnick	2001
Robert E. Shope	2004
Thomas H. Weller	2008

We remember all of them here in gratitude and admiration. Each made noteworthy—some even monumental—contributions to our field, and each unquestionably enhanced the value of this latest edition.

Finally, we owe special thanks to our wives—Leanne, Elizabeth, and Maryellen—who have patiently endured the many annoying diversions of our attention.

Birmingham, AL, USA — Richard A. Kaslow
New York, NY, USA — Lawrence R. Stanberry
Galveston, TX, USA — James W. Le Duc

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Part I

Concepts and Methods

Epidemiology and Control: Principles, Practice and Programs

1

Richard A. Kaslow

1 Introduction: Infection and Disease

The epidemiology of infectious diseases is concerned with the circumstances under which both infection and disease occur in a population and the factors that influence their frequency, spread, and distribution. It is critical to distinguish between infection and disease because the factors that govern their occurrence may be different and because infection without disease is common with many viruses. Infection indicates the introduction and multiplication of a biological agent within a host, leading to an interaction often manifest as an immune response. It is determined largely by environmental factors that govern exposure to the agent and by both intrinsic susceptibility and personal behavior of the host. Disease represents the host response to infection when it is severe enough to evoke a recognizable pattern of clinical manifestations. The factors that influence the occurrence and severity of this response vary with the characteristics of virus involved, its portal of entry, and the dose or inoculum size; but the most important determinants for many common infections lie within the host. Of these, the age and general health at the time of infection, genetic background, and immune status of the host are the most crucial.

This chapter deals with the principles, observational methods, and control techniques applicable to viral infection and disease in general; these concepts and approaches are explored in greater detail in individual chapters concerned with specific viruses or groups of viruses. For broader and fuller presentations of the epidemiologic principles, see texts in Suggested Reading, and for widely accepted definitions, see Ref. [1].

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2 The Agent

This section addresses general properties of viruses that are important to an understanding of their epidemiology but not their basic genetic, chemical, or structural composition or details of their physiologic or multiplicative properties. Chapters of this book on specific viral infections provide some information on these topics. Other sources to be consulted include textbooks on basic virology, such as Fields Virology [2] and others, and books on the more general topic of microbiology and infectious diseases [3, 4].

Several intrinsic properties of all pathogens, including viruses, are of importance in the production of infection and disease; these properties can be quantified and permit comparisons among viruses. One property, *infectivity*, is ability to infect the preferred target cell in a standard assay, usually measured as the minimum number of infectious units or particles required. It may involve the capacity for attachment to, entry into, and multiplication within a variety of host cells. A second property, *pathogenicity*, is defined as the ability of an infectious agent to produce clinically significant disease. A third property, *virulence*, has been used synonymously with the previous two terms but more formally refers to the proportion of infections that produce serious disease, classically measured as a ratio of fatal cases to the total number of infections. A fourth property is what might be described as adaptability, a reflection of genetic responsiveness to external perturbations often determined by the type and organization of its nucleic acid. Over their long evolutionary history, most viruses have either gradually accumulated key mutations or even integrated entire host genes that alter their capacity to cause disease or permit them to evade or circumvent specific host mechanisms of resistance. The proper series of mutations may have enabled a virus to utilize an alternative biochemical pathway or conferred variable degrees of resistance to antiviral agents. Rapidly replicating RNA viruses like influenza, human immunodeficiency virus, and hepatitis C virus are more error (mutation) prone than many DNA viruses. More sophisticated mechanisms of evasive

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adaptation have been adopted by larger DNA viruses like the herpesviruses. One such mechanism utilized by human herpesvirus 8 is exemplified by the incorporation of several viral homologues of human genes (e.g., *IL6*, *IRF3*), which may mimic their human counterparts and/or usurp their function [5, 6]. All of these properties are factors that may influence not only the capacity to initiate infection and produce disease but also the transmissibility of the agent.

3 The Host

Characteristics of the host that influence the occurrence of both infection and disease are numerous and highly variable. Some host factors that alter resistance and susceptibility to infection are identical to those that modulate the development and natural history of disease, but many others affect only one or the other. Age, sex, and race are the most longstanding and obvious factors for both.

Most of the effect of age on resistance to infection either is attributable to the development of immunity over time or reflects the mode of transmission. Infants and young children are susceptible to a whole range of infections that confer lifelong immunity once acquired. Many viral infections that are now largely prevented wherever vaccines are available (e.g., measles, mumps, rubella, varicella, and rotavirus) and others like respiratory syncytial virus have historically caused major childhood morbidity and mortality. Acquisition of certain herpesviruses (herpes simplex virus, cytomegalovirus, and Epstein–Barr virus) begins in the perinatal period via congenital transmission followed by slow and steady increases until adolescence, most likely through relatively casual direct contact. Then occurrence accelerates somewhat for the next several decades during the period of more intimate oral or sexual contact before leveling off after middle age.

The severity of an acute infection and the course of chronic one infection may also differ by the age at acquisition. For reasons not well understood, hepatitis A in older children and adults can be much more clinically apparent than in younger children who are often asymptomatic. Similarly, poliovirus is much more likely to produce paralytic disease in older persons than in young children. The age at which an individual becomes infected with HIV-1 is a strong determinant of the natural history of that infection (see Ref. [7] and Chap. 43).

For most viral infections, especially those occurring principally in childhood, the two sexes appear approximately equally susceptible. In adolescents and adults, differential rates of acquisition by sex may be due to obvious differences in anatomy or in the mode of transmission, as in the case of human papilloma virus and HIV-1, respectively.

Differences in infection rate by race or ethnicity can be divided into those known or likely to have genetic or other biologic origins versus those most likely due to other factors that correlate closely with race. Genetic factors are discussed below. For the nongenetic explanations, race is usually a surrogate marker for some other often readily apparent factor. Geographic distribution is the most obvious reason for large racial differences. Viruses that have reservoirs or life cycles that involve nonhumans would be expected to occur only in regions of the world where the animal populations may coincide with humans of one particular racial group. Exposure may differ by race because of behavioral differences. New HIV-1 infections in young gay men in the United States have surged more in blacks because of their relatively exclusive exposure network (see Chap. 43). HIV-2 is heavily concentrated in individuals of West African and Portuguese ancestry because the virus probably originated and mainly spread in the former Portuguese colonies on the west coast of sub-Saharan Africa.

Other host biological and behavioral factors are known or likely to influence acquisition of viral infection: general nutritional status and specific micronutrient or vitamin deficiency, cigarette smoking, medication, use of alcohol and other non-medicinal substances, occupation, marital status, and sexual practices.

Many of the above-mentioned factors may not only predispose to or protect from infection but also modulate the nature, severity, or course of disease. Table 1.1 contains a list of the host factors implicated with varying degrees of certainty as modulators of occurrence, severity, pace, or duration of some viral diseases.

It is biologically based immunity that is the fundamental host characteristic governing not only acquisition but also, probably more profoundly than the other factors, the pathogenesis and evolution of infection. Indeed, the influences of age, sex, race, and some of those others reflect in part the accompanying biologic functions that vary with those characteristics. There are many examples in which these host factors are almost certainly operating as surrogates for immune

Table 1.1 Host factors that influence the occurrence, course, or severity of disease

1. Age at onset of infection
2. Sex
3. Race
4. Genetic factors controlling an enormous array of immune response elements
5. Preexisting level of specific or nonspecific immunity
6. Iatrogenic immunosuppression
7. Behavior and lifestyle: sexual practices, smoking, alcohol, and recreational drugs
8. Dual infection or superinfection with other agents
9. Preexisting chronic conditions and pregnancy
10. Nutritional status
11. Psychological status (e.g., motivation, emotional crises, attitudes toward illness) [8]

status. Age is prime example. Childhood respiratory and enteric viruses produce life-threatening disease in infants but little or no illness in adults who have developed natural immunity. Conversely, infections with hepatitis A and polio viruses are often asymptomatic in young children but may produce significant clinical hepatitis and paralytic poliomyelitis in older individuals. Aging adults develop increasingly severe infections as their immunity in general and specifically to previously encountered viruses wanes. Sex differences in disease expression are generally less obvious. Disease due to influenza virus is more or less equally common and severe in men and women in general, but pregnant women experience more serious illness [9].

Apparent racial disparities exist in rates of infection, disease frequency, or expression. Various sources of surveillance data suggest that persons of African ancestry in the United States have higher rates of influenza, HCV, HPV, and other viral infections, while those of Asian ancestry have higher rates of HBV infection. However, in each case these differences are readily attributable to geographic, socioeconomic, or behavioral influences on acquisition, as noted earlier, and race is simply a marker of some combination of those factors rather than signifying an underlying immunologic or other biologic basis for the difference. On the other hand, some racial or ethnic differences in the disease manifestations (severity or natural history) will undoubtedly prove to be due to underlying immunogenetic heterogeneity.

Immunity exists in a continuum ranging from lowest (i.e., complete susceptibility) to highest (i.e., complete resistance). Resistance is either intrinsic or induced, and it can be induced either naturally or artificially. The basis for natural resistance or immunity to viral infection is briefly discussed just below; the strategy and resources employed for inducing resistance by immunization are addressed later in this chapter.

Mediators of natural resistance include components of primary defense systems such as cilia, mucus, the integument, and other physical and chemical inhibitors. However, resistance is even more dependent on the principal organs involved in generating the immune response—spleen, bone marrow, lymph nodes, and other lymphoid tissue—the structures on which the highly complex cellular and humoral immune systems are founded. Multiple cellular arms of the immune system dedicated to defending against viral infection. They operate through dozens of known and as-yet-unknown interlocking pathways in which monocytes; macrophages; dendritic cells; a wide array of T-helper, suppressor, and regulatory cells along with B lymphocytes and plasma cells are all orchestrated to stimulate or respond to each other in a coherent and effective manner. They may do so by a variety of mechanisms. Communication and function may occur through direct cell–cell contact or binding between receptor–ligand pairs on cell surfaces. Alternatively or additionally, these and other specialized cells may secrete cytokines, chemokines, complement, peptides, or other immunoactive substances, for which their target cells carry surface or intracellular receptors. Thus, countless different cell types in elaborate interlocking pathways execute any of a variety of assigned functions—signaling, activating, inhibiting, attracting, killing, etc.

Humoral immunity is generated by a cascade of interactions among dendritic cells, T and B lymphocytes, and plasma cells culminating in the production of immunoglobulins that serve as antibodies to the virus that initiated the response. Once activated by a virus, the humoral immune system engages in sequential production of immunoglobulins of the IgM, IgG, and IgA classes in different proportions that bind to surface or intracellular antigenic components of the virus. This binding improves with avidity (tightness) and affinity (antigen specificity) that increase during the days or weeks following onset of infection. The antibodies, often in conjunction with complement or other proteins, may neutralize or kill the virus directly or recruit cells with cytotoxic capabilities into the vicinity to accomplish that task.

Both cellular and humoral systems demonstrate what are known as innate and adaptive responses. Certain effector cells and molecules preexist in the host before any pathogen is encountered. These relatively unvarying elements have been recognized as the "first responders" because they mediate intrinsic or innate functions that are automatically and uniformly invoked in the first hours and days after a viral infection or other foreign intrusion. Soon after the innate response is underway, the adaptive parts of the systems are activated. Dendritic and other cells use molecules on their surface to present antigenic fragments to effector T lymphocytes individually in a process exquisitely specific to the offending virus. This specificity is generated by the huge variation in the human leukocyte antigen (HLA) genes that encode those surface proteins used for microbial antigen presentation. These molecules not only activate helper and cytotoxic T lymphocytes but also interact with another set of cognate receptors on the surface of natural killer (NK) cells; these NK cell receptors are also encoded by highly complex multigenic systems such as the leukocyte receptor complex (LCR), the natural cytotoxicity receptor (NCR) family, and the killer lectin-like receptor (KLR) family. The antibody-mediated humoral response is likewise largely adaptive. As with cell-mediated antigen presentation and the cytotoxic systems, the organization of the genes encoding immunoglobulins are likewise programmed for genetically governed rearrangements to provide for maximum adaptability and specificity. Moreover, not only do most antibodies bind with specificity for a particular class of viruses or a single member of the class, they also undergo structural maturation over time to conform even more closely to the specific target antigen.

All of these processes depend primarily on differential expression of hundreds to thousands of genetic loci relatively directly involved in the immune response. Many of the simplest, often monogenic immunodeficiencies with Mendelian patterns of inheritance and the infectious diseases that complicate them have been well catalogued, although new examples continue to surface [10]. On the other hand, very little of the overall genetic contribution to more complex multigenic infectious traits have yet been elucidated, but research in this field is charging ahead. An example of very direct genetic mediation of resistance to viral infection is the remarkable protection against HIV-1 infection afforded by the deletion of a portion of the gene for the viral co-receptor (*CCR5*) in individuals of European ancestry, described further in Chap. 43. Other clear instances of genetic mediation are the increased susceptibility to norovirus infection among carriers of the O blood group antigen compared with carriers of A or B antigen [11] and the predisposition of children with polymorphisms in the Toll-like receptor 3 gene (*TLR3*) to herpes simplex viral encephalitis [12]. In the past two decades, the explosion of knowledge in immunology and genetics has paralleled the rapid advances in molecular and computational technology. A more detailed summary of the rapidly expanding knowledge of immune system function is beyond the scope of this overview. The reader is referred to textbooks and the most current reviews of specific subjects for more complete coverage [13–15].

4 The Environment

The external environment can exert its influences on the virus itself or its mode of spread or on the biological or behavioral aspects of the host response. Although viruses survive or die within defined ranges of such physical factors as temperature, humidity, and air currents, variability between viral groups is high. Chemicals may be used as antiseptics or microbicides to kill viruses prophylactically, and the list of pharmaceuticals now available for antiviral therapy has been growing rapidly (see Sect. 10). These environmental factors may enhance or diminish survival of viruses, but they probably have a greater impact on routes of transmission and on patterns of host behavior.

In addition to physical and chemical factors, biological variables play a role as well. For insect-borne agents like the equine encephalitis viruses, whose survival may depend on migration patterns of an avian host or on overwintering in a mammalian reservoir, the environment exerts an obvious role in restricting transmission of infection and occurrence of disease to those areas that have the proper temperature, humidity, vegetation, and other features necessary for the insects. For viruses potentially transmitted by water, such as hepatitis A virus and noroviruses, a warm environment with poor sanitation and fecal contamination clearly increases exposure and transmission efficiency.

Climate also alters the social behavior of the host. In settings where high temperatures promote not only contact of hosts with water through swimming or drinking but also viral replication, transmission of waterborne gastrointestinal diseases is increased in polluted areas. Warm weather also brings closer contact with insect vectors of arboviruses and with dogs and other animal sources of rabies. Conversely, in the cooler seasons, people collect indoors, where crowding promotes transmission of airborne and droplet-borne infections. Spread in indoor environments is amplified by relative concentration of military personnel in barracks, residents of assisted living facilities, and assembly and dispersal of students coinciding with the periodic openings and closings of educational institutions. Furthermore, the relatively hot and dry environment in many homes and commercial buildings may impair the protective mechanisms on mucous surfaces and promote entry and attachment of certain respiratory viruses.

In tropical settings, heavy monsoon rains bring groups indoors and closer together much as they do during winter in colder climates. The incidence of common upper respiratory diseases in college students was as high in the warm climate where the University of the Philippines is situated as in the intemperate winters at the University of Wisconsin [16]. Community studies in India [17], Trinidad [18], and Panama [19] have documented high morbidity from influenza and other respiratory diseases in tropical settings where people aggregate inside, as with heavy rainfall or school attendance.

Cultural as well as physical environment can contribute to the spread of infection, as exemplified by the patterns of spread of HIV infection among gay bathhouse patrons, injectable substance abusers in “shooting” galleries, women offering sexual favors in exchange for crack cocaine [20], and long-distance truck drivers patronizing commercial sex workers [21].

5 Agent, Host, and Environment Interaction

It is obviously the interplay of those three cardinal elements that dictates where, when, and in whom viral infection occurs. Their interaction further determines whether the infection produces disease, how soon it begins, how it manifests itself, how severe it becomes, and how long it lasts.

To initiate infection a virus must be in the appropriate structural and functional state, reflected in the intrinsic properties described above. It must also be present in a quantity sufficient to penetrate the target host. That quantity, the *inoculum*, is the amount of virus available to be transmitted; and the minimum size of a viral inoculum needed to initiate infection varies with infectivity,

a property discussed earlier, as well as with other features of those three cardinal elements. Upon introduction of the minimum inoculum and successful attachment to and penetration of its target cells, the infection is established. Then, again depending on various features of the agent, host, and environment, the virus elicits a response that may or may not manifest clinically.

The degree of response an agent can evoke in the host immune system is known as its immunogenic potential or *immunogenicity*. It commonly refers to the ability of the natural infection to confer protection against reinfection or significant disease upon reexposure. This protective ability has typically been measured as the quantity of antibody that neutralizes or incapacitates the virus, but viruses may generate antibodies that do not neutralize or protect. Many of the classic acute childhood infections (e.g., measles, mumps, rubella, chicken pox, and other exanthematous conditions) are not merely strongly immunogenic but also usually confer lifelong immunity. In contrast, other viruses can either readily alter their antigenic makeup like influenza and some flaviviruses or produce persistent chronic infection like herpesviruses; by a variety of mechanisms, they have ingeniously avoided generating highly neutralizing antibody. HIV-1 is the master of evasive and adaptive mechanisms that allow it both to evolve rapidly and to persist for years without stimulating antibodies that neutralize.

In the context of immunization, immunogenicity represents the extent to which a vaccine can provide the same or similar degree of protection achieved in the course of natural infection. Many of the regimens for vaccination against those highly immunogenic infections have succeeded in mimicking very high levels of neutralizing antibody if not lifelong protection. The more basic biologic characteristics of the agent and the host that determine immunogenicity are addressed in texts on virology and immunology (see Sect. 1).

6 Incubation and Latency Periods

The interval of time between the sufficient exposure and the appearance of the first symptoms in the human host is the intrinsic *incubation period*. Viruses transmitted by arthropods or other animals have their own (extrinsic) incubation periods that vary with both the response within the animal and the external environmental factors that affect the animal populations. However, here the reference is only to the intrinsic incubation period. The variation in this interval in different diseases is considerable (Fig. 1.1). Viruses that do not require distant spread but are able to produce disease through multiplication at the site of implantation, such as the respiratory tract, tend to have short incubation periods on the order of 2–5 days. Arthropod-borne viral infections like dengue and yellow fever may have onsets as early as 2–3 days after a mosquito bite [22], whereas inoculation by small mammals may lead to longer incubation periods [23]. Viruses that require hematogenous spread and involve distant target organs such as the skin or CNS have incubation periods of 2–3 weeks. Rabies virus, dependent on spread along nerves, has a long and variable incubation period ranging from 8 days to a year or more. Of course, transmission of the prion-associated kuru is exceptional with onset documented as long 34–56 years after exposure [24]. In some diseases (e.g., poliomyelitis, dengue, hepatitis, and infectious mononucleosis), early symptoms or even a rash may accompany the period of initial invasion or viremia. With these infections an early phase may not be clinically recognized or occurs before the patient seeks medical care.

Knowledge of incubation periods has many practical uses. Epidemiologically, the former helps define the period of infectiousness: a patient is not usually infectious until close to the time of the appearance of clinical symptoms. In epidemics, knowledge of the mean, minimum, and

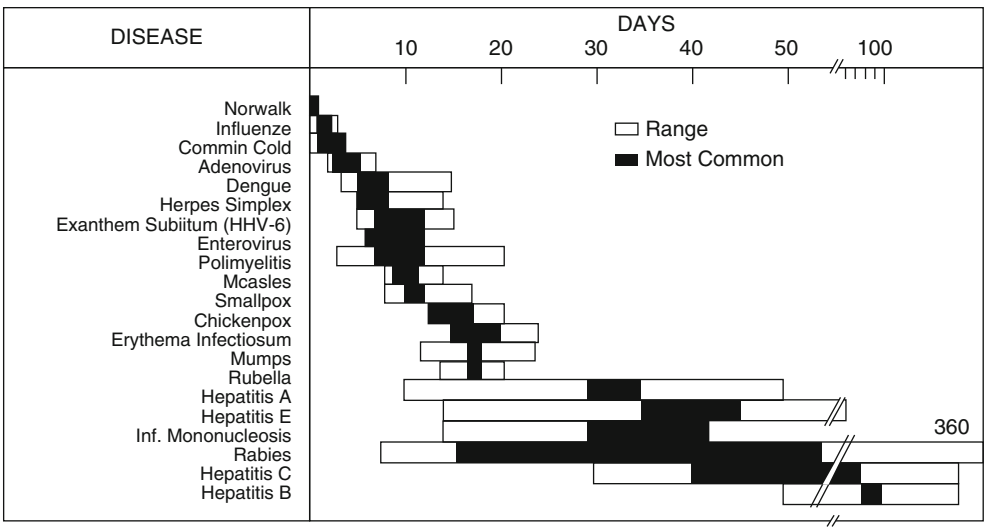


Fig. 1.1 Incubation periods in viral diseases (Based on data from Heymann [25]; Evans and Kaslow [210])

maximum intrinsic incubation periods can be used to identify the probable time of exposure to the index case or other source of infection. The duration of infectivity depends on the persistence of the virus and its exit into the environment. Clinically, the duration of the incubation period helps to identify the likelihood of viral exanthem after a known exposure or to differentiate hepatitis A from hepatitis B infections. For prophylaxis, it determines the feasibility of prevention of the clinical illness by immunoglobulin, as in hepatitis A and varicella zoster infections, rubella, and rabies, as well as the potential success of postexposure rabies vaccination.

In addition to the viruses that produce acute infections, there are delayed effects of certain common viruses in which the "incubation period" represents a true or apparent interval of "latency" lasting several to many years, during which there is little if any viral replication. Examples include the relationship of measles virus to subacute sclerosing panencephalitis, in which infection in infancy may be associated with involvement of the CNS some 5–10 years later [26]. Certain papovaviruses cause widespread inapparent infections in childhood. Rarely, reactivation occurs later in life in the form of progressive multifocal leukoencephalopathy. This phenomenon is seen in patients with Hodgkin's disease in association with depression of cell-mediated immunity and more recently in AIDS patients [27].

With HIV infection, a primary clinical response may occur within the first 2 months or so after infection in a substantial proportion of newly infected persons (see Sect. 7.1 and Chap. 43). During the subsequent clinically quiescent period, CD4 lymphocytes are destroyed at highly variable rates. Accordingly, clinical latency continues until immunosuppression is significant enough to permit new opportunistic pathogens to superinfect or long-latent agents to reactivate and produce clinical disease. The average time from initial HIV infection to that clinical event may be shorter or longer depending not only on the degree of viral control and immunosuppression under host genetic influence but also on the other cofactors required for any specific clinical AIDS outcome. For example, Kaposi's sarcoma tends to occur at a somewhat earlier stage of immunosuppression than cerebral atrophy with dementia or lymphoma for reasons presumably related to the unknown determinants of these conditions. Besides differences in the properties of the virus itself, such as the capacity to penetrate cells or induce syncytium formation in vitro, the route of transmission and host factors determine the rate of progression of HIV infection. The average AIDS-free interval is shorter for infants and children and for recipients of large volumes of blood products. The interval is longest among the youngest adults and then gradually shorter with increasing age [7]. There is also conclusive evidence for differential predisposition due to immunogenetic factors.

As with HIV infection, the latency period for different outcomes of HTLV-I varies. The interval between presumed infection via breast milk and onset of adult T-cell leukemia/lymphoma differs from the interval between presumed sexual transmission and onset of tropical spastic paraparesis or myelopathy. The concept of a latency period is also applicable to long-delayed virus-induced cancer as seen with an EBV-induced nasopharyngeal carcinoma KSHV-induced Kaposi sarcoma, HBV- and HCV-induced hepatocellular carcinoma, and HPV-induced cervical carcinoma (see Chaps. 34, 39, 40, 41, 44, and 45). In kuru, the latency period from exposure by ingestion of infected brain or other tissues or by absorption via abraded skin at a cannibalistic feast to the onset of disease can actually be as long as several decades (see Chap. 47) [24].

7 Patterns of Response

The response to viral infection varies along a biological gradient in terms of both the nature and the severity of the clinical syndrome produced. That variation is the product of the interaction of agent, host, and environment. The emphasis here is on the biological gradient as manifested in the human as a whole; qualitative and quantitative differences in responses that occur at the cellular and molecular levels are more properly the subject of basic virology and immunology texts.

7.1 The Biological Gradient

Response to a virus by an individual may range from completely asymptomatic to severe or fatal. The ratio of inapparent (or subclinical) to apparent (or clinical) responses varies from one virus to another; representative examples are shown in Table 1.2. The clinical response may be abrupt or more gradual, sometimes appearing long after the initial infection. It may be self-limited or arise from viral persistence or reactivation or both.

GBV-C infection increases with age but produces no known clinical illness [28]. Initial infection with BK and JC strains of polyomavirus, which show high prevalence and rates of acquisition of antibody in school children and adults [29, 30], is not known to affect them clinically. However, in immunocompromised patients (e.g., with AIDS, Hodgkin's disease, or renal transplantation), previously asymptomatic JC virus infection may evolve into progressive multifocal leukoencephalopathy[31]. Other primary or often reactivated infections are common in immunodeficient states; regardless of their clinical expression in normal hosts, most of the herpesviruses are more likely to cause mild to life-threatening complications.

Table 1.2 The gradient of subclinical/clinical ratio in selected viral infections (inapparent/apparent)

Virus	Clinical feature	Age at infection	Estimated subclinical/clinical ratio	Percentage of infection with clinical features
GBV-C	None known	Child to adult	∞	0
Poliomyelitis	Paralysis	Child	±1,000:1	0.1–1
Epstein–Barr	Heterophile-positive infectious mononucleosis	1–5	>100:1	1
		6–15	10–100:1	1–10
		16–25	2–3:1	35–50
Hepatitis A	Jaundice	<5	20:1	5
		5–9	11:1	10
		10–15	7:1	14
		Adult	2–3:1	35–50
Rubella	Rash	5–20	2:1	50
Influenza	Fever, cough	Young adult	1.5:1	60
Norovirus	Gastroenteritis	<2	1:5	60–90
HIV-1	Multiple	Any age	1:99	99
Measles	Rash, fever	5–20	1:99	99+
Rabies	CNS symptoms	Any age	0:100	100

A second group of viruses cause predominantly mild or asymptomatic infection when acquired in early childhood but symptomatic and sometimes severe clinical disease when infection is delayed. Examples of this are hepatitis A, poliovirus, and EBV.

At the other end of the spectrum are acute infections caused by agents like measles and rabies viruses or chronic infection with a virus like HIV-1 or the agents of spongiform encephalopathies, in which clinically recognized illness usually accompanies the infection but the course of infection may vary from days to decades. Rabies infection of man is the epitome of an infection from which death is virtually inevitable after characteristic symptoms develop.

The subclinical/clinical ratio for HIV-1 infection defies the more straightforward categorization possible for many other viral infections. A syndrome resembling mononucleosis with fever, fatigue, headache, lymph node swelling, joint and muscle aching, rash, sore throat, and other features occurs frequently in newly infected individuals. However, the proportion reported to have experienced this syndrome is higher or lower depending on the method of ascertainment (e.g., presentation at a sexually transmitted disease clinic, follow-up of cohort of initially uninfected homosexual men) and the clinical definition [32–34]. Likewise, the vast majority of HIV-1 infections follow a highly variable course—for a median of about 9 years in most studies of untreated individuals—free of the serious (AIDS-defining) illness; and on the average, HIV-2 infection progresses even more slowly. However, laboratory evidence usually indicates that immunologic deterioration is continuing at some rate even when the infection is clinically silent. The natural history of HIV infection and the factors that modulate it are addressed in detail in Chap. 43.

The biological gradient of many viral infections can be viewed as analogous to an iceberg in which clinically apparent illness represents only a small proportion of the response pattern and the usually larger amount represents unrecognized and inapparent infection. A similar analogy may exist at the cellular level. Figure 1.2 portrays these concepts, but Table 1.2 demonstrates that the precise shape of the iceberg for any given infection can vary considerably at the clinical level.

7.2 Clinical Syndromes: Manifestations, Etiologic Agents, and Frequencies

The tissue and organs targeted by human viruses can respond to infection in a limited number of ways; any of several viruses (or other causative agents) may trigger the same general response pattern. The clinician must often rely on epidemiologic and clinical features and simple laboratory tests in making a tentative etiologic diagnosis. This diagnostic reasoning may be based heavily on the known frequency of potential causative agents in the age group of the patient, the specific geographic or physical setting, the season, and their epidemic behavior. This section summarizes the major clinical syndromes produced by some of the more common human viruses. It includes (1) a very brief description of the clinical manifestations; (2) identification of major etiologic agents; (3) general frequency distributions by factors such as age and geography, not necessarily applicable at special times such as an epidemic or off-season or in settings like hospitals, nursing homes, and day-care facilities; and (4) selected other features relevant to the understanding of their occurrence in populations. For more detail, consult the chapter corresponding to the agent of interest or more specialized texts.

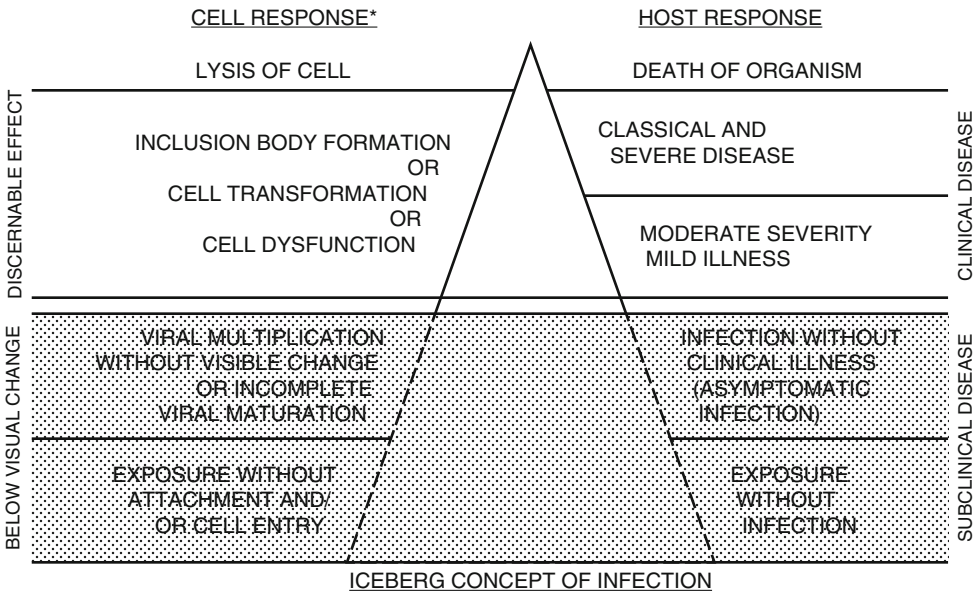


Fig. 1.2 "Iceberg" concept of infectious diseases at level of the cell and at level of the host. Within any cell population, varying patterns of cell response also occur. *Generic response [208] (Reproduced in Evans and Kaslow [210])

It is important to note that in recent years newer sample collection approaches, rapid immunologic assays, and highly sensitive molecular techniques have gradually supplemented and, in certain cases, supplanted the more traditional virologic and serologic tests, but they have come into use unevenly in different places (Chap. 2). Therefore, generalizations made in this discussion must be tempered by consideration of the methodology for sampling, detection, and identification that may have been used to generate the findings at different times in different settings.

7.2.1 Common Respiratory Tract Syndromes

Many viruses and viral groups can evoke symptoms and signs in the upper respiratory tract (e.g., eye, ear, nose, and throat symptoms; Viral infections of the lower respiratory tract trigger cough with little or no sputum production, shortness of breath, and changes in breath sounds on examination). Fever is less prominent than with bacterial or fungal infection.

Viral upper respiratory infection (URI) is extremely common in most temperate climates; it is the most frequent cause of absence from work or school in the United States [35]. A combination of rhinitis and pharyngitis (common cold) occurs most frequently in children under 5 years old, who may have multiple episodes in a single year. URIs are still frequent in older children, but incidence declines steadily through adulthood to greater than 60 years of age. Other manifestations of viral infection include sinusitis, which may be clinically or radiographically apparent in a very large proportion of those with viral URI [36].

Approximately nine of ten children under 2 years old have experienced an episode of otitis [37]. The usual viral etiologies are respiratory syncytial virus, parainfluenza (types 1–3), influenza (A and B), enterovirus, and rhinovirus [38]; however, the distribution of pathogens differs by age and location. Exposure to day care or a family member with a URI may be a predisposing factor.

Influenza virus is the leading cause of both upper and lower respiratory infections in middle-aged and older adults. Up to one-fifth of the entire US population may develop influenza in any particular season. Seasonal influenza typically lasts from November until March. However, in an atypical year, influenza activity may be present in summer and early autumn, as was true of H1N1 in 2009, when it overlapped with seasonal influenza, and of swine-associated H3N2v infection that occurred in 2012 in the setting of late summer county fairs [39].

Several viruses causing respiratory tract infection are relatively recent discoveries if not new to humans, and they are responsible for an appreciable proportion of viral respiratory tract infection (see Chaps. 10, 26, and 27). Human metapneumovirus can cause upper and lower respiratory tract infections (i.e., a cold, cough, bronchitis, pneumonia, and exacerbation of asthma) in people of all ages but more severe forms at the extremes of age and complicating immunosuppression. Coronaviruses, although known for years in animals and as the cause of more benign URI, came to attention dramatically during the large epidemic of severe acute respiratory syndrome (SARS) in 2003, and resurfaced again in the variant form producing Middle East Respiratory

Syndrome (MERS). Since then a succession of new human coronavirus strains has been associated with outbreaks of both upper and lower infections in different parts of the world. An even newer member of the parvovirus family, a bocavirus, has been at least presumptively linked with respiratory symptoms and otitis media [40].

Over the years, numerous investigators have tried to estimate the relative frequency of the different viral etiologies of clinical syndromes of acute respiratory diseases [40–48]. However, not surprisingly, the advent of newer more sensitive diagnostic techniques has made it increasingly clear that the distributions of viral pathogens vary greatly not only by age and season but also by geography and proximity to animal populations, previous human population experience, and other factors.

7.2.2 Gastroenteritis

This syndrome consists of nausea, vomiting, and/or diarrhea of varying severity, with relatively little fever or other features (e.g., significant abdominal pain or gross fecal blood) more typical of bacterial gastroenteritis. Worldwide, gastroenteritis causes serious morbidity and mortality, with the greatest toll in children under 5 years old, due to severe dehydration. In a study from the mid-1980s in the United States, diarrhea occurred in children under age 3 years in childcare at a rate of approximately three episodes per year [49]. Members of four families, rotaviruses, noroviruses, enteric adenoviruses, and astroviruses account for most of the disease caused by viruses, and each has epidemiologic characteristics that depend heavily on age and geography. Infections with these viruses tend to occur continually in children but only sporadically in adults. In the developed world, mortality is quite low although illness and hospitalization are more common.

Prior to the introduction of an effective vaccine, rotavirus infections were the leading cause of diarrheal illness in children under 2 years of age in the United States and many well-developed countries. The toll is still great in children under 5 years old in a number of places throughout the world, where it may account for as much as 30 % of mortality due to diarrheal disease [50]. As coverage with the vaccine expands, that picture is expected to change dramatically [51]. Caliciviruses and particularly the most notorious member of that family, norovirus, are now the leading cause of gastroenteritis among adults in the United States; these infections often occur in epidemic form as "winter vomiting disease" and are responsible for more than 90 % of outbreaks [52]. Facile interpersonal spread accounts for high secondary attack rates in family members and health-care personnel attending ill patients.

Viral gastroenteritis caused by adenoviruses and astroviruses has been studied more selectively, mostly in the United States. Specific serotypes of adenoviruses tend to cause disease in a year-round, endemic form, primarily in very young children. Up to 15 % of gastroenteritis in some settings may be due to astrovirus infection. Older children and adults are less affected. The infection is probably transmitted person to person, and it has caused infection in hospitalized and immunodeficient patients [11]. As many as another 10 % of cases in children may be caused by astroviruses, often in institutions like day-care centers and hospitals, where an even higher proportion of the transmitted enteric infections may be due to this agent [53, 54]. Elderly and immunocompromised patients appear to be at elevated risk [55, 56].

7.2.3 Common Central Nervous System Syndromes

Multiple viral agents are involved as causes of the syndromes of inflammation of the spinal cord (aseptic meningitis) and the brain (encephalitis) or a combination (meningoencephalitis). Comprehensive reviews of these infections can be found in Refs. [57–59]. The clinical features of these syndromes are quite variable, from mild headache and/or stiff neck with or without fever and subtle changes in cortical function (i.e., orientation, wakefulness, concentration, etc.) to more profound manifestations of spinal cord or brain dysfunction (including cranial nerve abnormalities; compromise of critical brain stem function; confusion, irritability, and poor feeding in very young children; and seizures). In younger children, long-term complications may include hydrocephalus, vision and hearing loss, weakness or paralysis, cranial nerve deficits, and learning and behavior problems.

The annual incidence of aseptic meningitis in the United States from all causes is approximately 11 in 100,000 persons, leading to 25,000–50,000 hospitalizations, mostly in children. However, many more mild and unconfirmed cases must go unreported. Again, the distribution of etiologic agents varies strongly with age, geography, and availability of vaccines.

The incidence of viral meningitis in infants may be as much as ten times that in older children. Worldwide, overall incidence is less easily estimated. Enteroviruses (echoviruses, coxsackieviruses, and others) are among the major causes in infants, and they may account for at least 80 % of cases in countries where vaccines against mumps, polio, and measles have significantly lowered the incidence of meningitis accompanying those conditions. On the other hand, in Asia, Japanese B encephalitis virus accounts for many thousands of meningitis cases each year and probably hundreds of thousands more that remain clinically inapparent.

Additional, less common causes include other flaviviruses (West Nile virus; see below), herpesviruses (VZV and others), lymphocytic choriomeningitis virus, other arboviruses (Eastern and Western equine encephalitis), and HIV-1. Because both enteroviral and arboviral infections show strong peaks in occurrence during the spring, summer, and early fall, the overall incidence in aseptic meningitis exhibits clear seasonality.

Encephalitis is less common than meningitis due to viral infection in the United States, with an annual incidence of about 20,000 cases or 3–8 in 100,000 persons. However, because many cases are not seen as part of an outbreak, an etiologic diagnosis requires a brain biopsy; therefore, only more severe and hospitalized cases are likely to receive such a specific diagnosis. HSV-1, the most frequent cause, is responsible for about 10 % of those cases. Arboviruses account for another 150 to several thousand cases, with variability according to the intensity of seasonal occurrence and striking peaks during sporadic epidemics. These have included Venezuelan equine encephalitis (1969–1971, several hundred cases), St. Louis encephalitis virus infection (1975, 3,000 cases), West Nile virus (encephalitis or meningitis, 2,800 cases in 2003 and 2,700 cases in 2012), and smaller numbers of La Crosse encephalitis and Western equine encephalitis cases [59]. In centers where concerted diagnostic effort is made, additional agents of sporadic cases of encephalitis and rarer central nervous system syndromes include other herpesviruses (EBV and VZV) and influenza A virus.

As with meningitis, accurate international estimates of the incidence of encephalitis are unreliable, but epidemic disease patterns similar to those in the United States occur throughout the world. For example, in a 1995 outbreak of Venezuelan equine encephalitis in Colombia and Venezuela, of the 75,000 humans estimated to have developed infection, neuroinvasive disease occurred in about 3,000, and 300 people died. Recently, henipaviruses in the paramyxovirus family have accounted for clusters of encephalitis and related neurologic disease in Australia along with Malaysia and Singapore (see Chap. 22).

7.2.4 Ocular Syndromes

Redness, watering, photophobia, feeling of irritation, and swelling of the membrane covering the eye (conjunctivitis) and the cornea (keratitis) are often severe symptoms epidemic keratoconjunctivitis (EKC) [60]. Outbreaks are commonly caused by very contagious human adenovirus infections. They are often nosocomial and have frequently occurred in such settings as the neonatal intensive care unit and ophthalmologic clinics, where contaminated contact lenses, eye drops, or instruments used for tonometry or slit-lamp examinations have been the modes of transmission (see Chap. 6).

Table 1.3 Viral causes of common exanthems

Type of rash	Examples[a]
Macular/papular	CMV, HHV-6, HBV, HIV
	Measles, atypical measles (vaccine)
	Rubella
	Echovirus
	Enterovirus 71
	Coxsackievirus
	Adenovirus
	Parvovirus B19 (erythema infectiosum)
Vesicular/pustular	Varicella zoster virus
	Smallpox
	Eczema herpeticum
	Eczema vaccinatum
	Coxsackievirus (esp. A16)
	Enterovirus 71
	Adenovirus [63]
	EBV [63]
	CMV [63]
Petechial or purpuric	Coxsackievirus (esp. A9)
	Echovirus, esp. 9
	EBV
	Atypical measles (vaccine)
	Parvovirus B19 [64]
Erythema multiforme	HSV-1 [65]
	Coxsackievirus A
	Echovirus
	Adenovirus
Others	Coxsackievirus A
	Echovirus
	HHV-8

[a]Examples of agents for each type of rash in table adapted from Cherry [66], except as noted by other citations [63–65]

7.2.5 Common Cutaneous Syndromes

Many viruses can produce one or more forms of eruption of the skin (exanthem). The most common acute viral infections involving the skin are listed in Table 1.3; many are classic exanthems of childhood including measles, rubella, varicella, erythema infectiosum or fifth disease caused by parvovirus B19, and roseola infantum (exanthem subitum) caused primarily by human herpesvirus type 6 (HHV-6) [61]. Rashes also occur in children or adults with coxsackieviruses and echoviruses [62], with certain adenoviruses (such as type 7), occasionally with EBV mononucleosis (often brought on by a reaction to ampicillin), and with other herpesviruses. HIV also causes a rash in a small proportion of newly infected patients. The distribution of these agents as causes of cutaneous conditions clearly varies by age, geography, vaccination status, and other host factors. The circumstances in which these many combinations of types of rash and agents are observed are not easily generalizable; the chapters on the individual agents should be consulted.

7.2.6 Hepatitis

Five principal agents are currently recognized as widespread causes of viral hepatitis: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis delta virus (HDV), and hepatitis E virus (HEV). However, viruses in a number of other families can produce hepatitis. In the parts of the world where yellow fever virus is prevalent, the syndrome is common. Less common agents are several herpesviruses (HSV-1, CMV, and EBV). All are associated with inflammation of the liver but with different severity, ranging from asymptomatic to acute mild liver tenderness and jaundice, often accompanied by malaise and anorexia, to fulminant hepatic failure in a fortunately small fraction of cases.

Two of the agents (HAV and HEV) produce more acute, usually mild to moderate self-limited illness, with milder illness in younger individuals. As enteric pathogens, they occur in geographic distributions that reflect socioeconomic conditions in those regions. Hepatitis A tends to occur sporadically in older individuals where immunity is high, often because poor sanitation permitted the virus to saturate the population at younger age. Conversely, epidemic HAV disease is more often seen in populations left susceptible as a result of better hygiene. In the United States and other countries where the vaccine against HAV is widely used, the decline in cases of hepatitis A has been striking. About 1.5 million cases occur annually worldwide. Hepatitis E is found worldwide, with different viral genotypes determining the disease distribution. Globally, genotypes 1 and 2 account for 70,000 deaths and 3.4 million cases often in outbreaks particularly in South and East Asia but in other developing countries as well, whereas genotype 3 occurs more sporadically in countries with better sanitation [67].

The three others (HBV, HCV, and HDV) have strikingly different clinical patterns. Both HBV and HCV may produce a range of symptoms including an acute syndrome much like that of HAV and HEV. However, a small proportion of HBV and the majority of HCV infections become chronic and often lifelong. In their chronic forms both infections may remain essentially quiescent for a lifetime or progress at quite variable rates to chronic inflammation and cirrhosis, with or without symptoms along the way, and both are responsible in some proportion for virus-induced hepatocellular carcinoma (see Chaps. 32, 33, and 34). Of the approximately two billion persons with HBV infection worldwide, 240 million have some degree of persistent infection of the liver, and 600,000 succumb to the sequelae [68]. The patterns of occurrence of these diseases are driven primarily by geographic and behavioral factors. Highly effective transmission by the parenteral, sexual, and perinatal routes accounts for the ubiquitous distribution of hepatitis B with particularly high prevalence in Asia. Until the introduction of vaccine, high prevalence in childbearing adults who transmitted the infection perinatally translated to high rates of chronic childhood infection and perpetuated the cycle. Throughout the world and in proportion to their representation in each population, hepatitis B has affected injection drug users (IDUs), recipients of contaminated blood products, those exposed to contaminated medical equipment, and men who have sex with men.

Because HCV is transmitted more by the parenteral and less by the sexual route, in the developed world, hepatitis C has been more confined to IDUs and others who have been exposed to contaminated blood and needles. Central Asia and East Asia along with North Africa and the Middle East have the highest prevalences of the HCV infection (>3.5 %) [69] as well as its incident sequelae hepatitis, cirrhosis, and cancer. Because of the highly variable absolute and relative prevalences of HBV and HCV infection and the other risk factors for those complications, it is difficult to estimate the incidence of HCV-induced cirrhosis or cancer or the risk attributable to HCV.

The delta virus (HDV) is an unusual partially defective RNA virus, closely dependent on the presence of HBV for its pathogenic expression if not for its multiplication. When HDV coinfects simultaneously with HBV, it may trigger an acute hepatitis syndrome but generally does not appreciably alter the course of disease. However, when HDV superinfects in a preexisting case of HBV infection, progression to cirrhosis is considerably more likely and often more rapid. The defective virus was first described in Europe, but it has been found at high prevalence in countries of sub-Saharan Africa, South America, and Asia where HBV is highly endemic, more often in parenterally infected blood product recipients or injection drug users and occasionally in men who have sex with men.

7.2.7 Perinatal Conditions

Infections of the fetus, neonate, and infant may be acquired from the mother in utero via placental transfer or during passage through the birth canal or from other individuals postpartum via nosocomial and other similar close contact. The major syndromes and their etiologic agents are tabulated below (Table 1.4). The specific clinical manifestations vary by agent and timing of infection relative to gestation. Vertically transmitted rubella, HSV-1/HSV-2, and cytomegalovirus infections have long been implicated as causes of especially severe congenital multiorgan anomalies [70]. Estimates of occurrence of perinatal infection vary according to location, personal hygienic and sexual activities, obstetric practices, utilization of vaccines, and other factors. Table 1.4 catalogues the major clinical consequences of the large number of agents responsible for perinatal infections.

The adverse outcomes of rubella during pregnancy, once numerically and clinically very important, have thankfully been virtually eliminated in the United States and other countries with effective vaccination programs. The fetal complications of the other vaccine-preventable diseases

Table 1.4 Effects of transplacental fetal infection

	Effect of infection on the fetus and newborn infant[a]				
Organism or disease	Prematurity	Intrauterine growth retardation and low birth weight	Developmental anomalies	Congenital disease	Persistent postnatal infection
Viruses					
Rubella	–	+	+	+	+
Cytomegalovirus	+	+	+	+	+
Herpes simplex	+	–	–	+	+
Varicella zoster	–	(+)	+	+	+
Mumps	–	–	–	(+)	–
Rubeola	+	–	–	+	–
Vaccinia	–	–	–	+	–
Smallpox	+	–	–	+	–
Coxsackie B	–	–	(+)	+	–
Echoviruses	–	–	–	–	–
Poliovirus	–	–	–	+	–
Influenza	–	–	–	–	–
Hepatitis B	+	–	–	+	+
Human immunodeficiency virus	(+)	(+)	(+)	(+)	+
Lymphocytic choriomeningitis virus	–	–	–	+	–
Parvovirus	–	–	–	(+)	–

Modified from Ref. [70]

[a]+, Evidence for effect; –, no evidence for effect; (+), association of effect with infection has been suggested and is under consideration

have also been drastically reduced. That has left CMV as the most common serious congenital disease due to viral infection in the United States [71]. It may infect more than 2 % of all pregnant women. About one-third of women with primary infection will transmit the virus to their newborn infants, but acquisition by mother and infant varies greatly with age, location, and socioeconomic conditions [72]. Both infection and serious sequelae are more common with primary than with recurrent infection. Congenital infection may be symptomatic in as many as 15 % of infected neonates [73]. Although these infections are usually benign, the virus can produce anomalies such as microencephalopathy, chorioretinitis, deafness, and mental retardation in a small proportion of those infected. Irreversible anomalies due to CMV infection occur in more than 5,000 children (0.1–0.2 %) each year.

Herpes simplex virus infections occur quite variably among pregnant women in different geographic, ethnic, and socioeconomic subpopulations. Infection in infants is almost always complicated by mucocutaneous (skin, eye, and mouth) lesions (75 %), encephalitis (57 %), pneumonia (18 %), or disseminated infection with combinations of the three (30 %). Prompt recognition is important because antiviral therapy is often effective in reducing morbidity. Estimates of incidence come from multiple sources. An early study from a single location found a frequency of 1 per 1,500 live births [74]. A more weighted population-based extrapolation from 2006 US hospitalization data yielded an incidence of 9.6 per 100,000 births [75].

The risk of perinatal HBV infection is primarily that of transmission of the virus from mothers who are chronically infected (i.e., HBsAg carriers). Although infection is not teratogenic and the reported impact on gestational age or birth weight must be quite infrequent [76], there are adverse consequences for the infected neonate who receives no immunoprophylaxis. About 5–8 % become hepatitis B surface antigen carriers during the first 6 months of life. Moreover, young adults infected in infancy are far more likely than those not infected until later to manifest not only persistent antigenemia but cirrhosis and, after an even longer latency period, hepatocellular carcinoma. Rates of infection in pregnant women show great geographic variation. Congenital and intrapartum HBV infection transmitted by maternal carriers has long been considerably more common in Asia and Africa than in Europe and the Western Hemisphere and has remained so. The pattern is changing in some places and will continue to do so wherever infant vaccination has been used routinely for more than two decades, and the earliest universally vaccinated birth cohorts are well into their childbearing years.

Untreated HIV-1 infection is transmitted from about 25–30 % infected mothers to their child/children. Adverse outcomes of pregnancy similar to those of perinatal herpesvirus and certain other infections have been reported with fetal and neonatal HIV-1 infection in some settings, but more recent systematic studies have not revealed clear causal associations [77, 78]. An accelerated course of infection and certain distinctive features (e.g., lymphocytic interstitial

pneumonitis) have been observed, potentially attributable to the unique attack on the immune system. Several regimens of antiretroviral agents alone or in combination have proved increasingly effective in interrupting perinatal transmission.

Parvovirus B19 has been repeatedly linked to anemia, neurological anomalies, hydrops fetalis, and fetal death (see Chap. 27). In an earlier prospective study of 156 mothers infected with this virus, 12 % delivered babies with some abnormality [79]. The investigators estimated the overall fetal risk to be 9 %, although higher in the second trimester, and the transplacental transmission rate to be 33 %. Adverse outcomes, particularly fetal loss and hydrops (a syndrome associated with destruction of red blood cells in utero), follow a proportion of the infections with this virus [79]. With a predilection for myocytes, it has also been implicated in myocarditis [80, 81].

7.2.8 Genital Tract Syndromes

Two families of viruses, HSV-1 and HSV-2, and several subtypes of HPV are common causes of conditions of the genital tract. Both HSV types can produce a spectrum of features ranging from no or minimal symptoms to an episode of fever; headache; myalgia; regional adenopathy; painful, tender blisters and ulceration on the epithelial surfaces of the genital organs and adjacent areas, including the anus and rectum; and dysuria. Small proportions of infected persons develop complications such as aseptic meningitis and proctitis (in men). Symptoms are more frequent and prominent with primary than with recurrent infection. Genital HSV infections are acquired through sexual contact, beginning with sexual debut and increasing through the earlier years of highest sexual activity in most areas of the world, where they are often the leading cause of genital ulcer disease. In recent years type 1 has matched or surpassed type 2 as a frequent cause of primary genital infection, particularly in younger persons. Infection with one of the two HSV types is extremely common; tens of millions of people in the United States have genital HSV infection [82]. Estimates from CDC indicated that in 2008, approximately 776,000 new HSV-2 infections occurred, with half being in persons under 25 years of age [83].

The two major manifestations of HPV infection are genital warts and squamous cell neoplasia. HPV types 6 and 11 and less frequently other types are responsible for genital warts (condyloma acuminata) on epithelial surfaces in the genital and anal area. The annual incidence of new cases of genital warts in the United States is about 360,000 persons [84]. Within the past decade, the cumulative incidence of genital warts in Scandinavian women up to age 45 years was about 10 % [85]. Among the unvaccinated group of women followed in a multinational trial of quadrivalent vaccine, approximately 1 % of the women developed warts each year [86]. HPV types 16 and 18 and less frequently other types cause a spectrum of cervical and vaginal cytological abnormalities that are largely benign but in diminishing proportions may progress to higher-grade neoplasia and culminate in invasive cervical carcinoma in a minority of women carrying those specific types. Annually worldwide, HPV causes about 500,000 cases of cervical cancer and ten million additional cases of premalignant neoplasia [87]. About 80 % of the cases and deaths occur in the developing world. The invasive cancer has a 50 % case fatality ratio. Analogous pathologic processes lead to anal and penile carcinoma, largely in men who have sex with men. The incidence rates of these cancers appear to be considerably lower than rates of cervical cancer in the same population [88].

7.2.9 Urinary Tract Syndromes

Acute hemorrhagic cystitis is the most prominent abnormality of the urinary tract caused by viruses. When the bladder inflammation is caused by the principal etiologic agents in children (adenoviruses, particularly serotypes 11 and 21 of subgroup B), hematuria may be recognized along with pain or burning, but the condition is often less symptomatic. Another possible viral etiology of a urinary tract syndrome (manifested as renal tubular damage) is BK virus, which, like other polyomavirus-related infections, tends to be pathogenic primarily in immunosuppressed patients.

7.2.10 Febrile Illness with Hemorrhage

Viral hemorrhagic fever may begin with a nonspecific prodrome of fever and chills, malaise, myalgia, headache, rash, flushing, and in some cases vomiting and diarrhea. These may rapidly progress to a multiorgan syndrome with petechial and more extensive bleeding but which is often less life threatening than vascular collapse with varying degrees of pulmonary edema, hypotension, shock, and renal failure. Variations of this syndrome are caused by a numerous and growing list of mostly vector-borne viruses in four families: arenaviruses, filoviruses, bunyaviruses, and flaviviruses (Table 1.5). Collectively, the viruses that produce this syndrome are found throughout the world although each individual agent is confined to the region where the principal arthropod vector or other animal reservoir lives. Certain of the diseases occur sporadically, for example, when a single individual has incidental contact with an infected animal. The occurrence of a cluster of cases is more ominous; because of the often fulminant illness and the unpredictable human-to-human propagation of cases, including nosocomial transmission, publicity about the event can have disproportional community impact. These phenomena have typified the ominously large outbreak of Ebola virus infection in West Africa. From time to time, one of these illnesses may present an immediate, major public health problem with hundreds of cases; there is, for example, concern about the potential for hemorrhagic dengue on a large scale in Southeast Asia.

Table 1.5 Families of viruses that may cause hemorrhagic fever

Arenaviridae	Bunyaviridae	Filoviridae	Flaviviridae
Argentine hemorrhagic fever	Crimean–Congo hemorrhagic fever (CCHF)	Ebola hemorrhagic fever	Dengue[a]
Bolivian hemorrhagic fever	Hantavirus pulmonary syndrome (HPS)	Marburg hemorrhagic fever	Kyasanur forest disease
Sabia-associated hemorrhagic fever	Hemorrhagic fever with renal syndrome (HFRS)		Omsk hemorrhagic fever
Venezuelan hemorrhagic fever	Rift Valley fever		Tick-borne encephalitis
Lassa fever			
Lymphocytic choriomeningitis (LCM)			

Adapted from [89]
[a]Dengue may occur in hemorrhagic form in certain circumstances (see Chap. 15)

Overall, the patterns of occurrence of these diseases are too variable to allow generalizations about their geographic distribution or the magnitude of the problem; they are addressed more individually in Chaps. 8, 9, 14, and 15.

8 Occurrence and Spread in Populations

In the broadest terms, viral propagation within an individual depends on properties such as the efficiency of spread from cell to cell, either by direct involvement of contiguous cells or by transport via body fluids to other susceptible cells; the number of cells infected; and the consequences of viral multiplication on the cell itself and on the organism as a whole. Further biochemical and physiologic details of these cellular processes are the subjects of more basic science texts. The next several sections are concerned with the occurrence and propagation of infection in populations, along with factors that influence those events.

Survival of human viruses depends on their long-term viability in human populations. Sustained viability, in turn, depends on the patterns of occurrence and propagation—within a host and between hosts. A virus must be capable of (1) infecting a host chronically without killing its cells; (2) infecting a host acutely and severely but escaping from the dying host cells as an intact, replicable entity in a manner that ensures its transport to a new susceptible host; or (3) infecting chronically or acutely but rapidly adapting to biological adversity such as exhaustion of susceptible hosts. Herpesviruses and other persistent viruses have evolved to establish durable relationships with their immunologically competent hosts. Arboviruses can destroy their hosts as long as the former can survive and replenish themselves in their nonhuman reservoirs and vectors. It is the viruses like influenza A and HIV, endowed with the greatest adaptability in the form of antigenic variation, that pose the greatest threat because they are not self-limited in their pathogenicity or in their dependence on favorable environmental conditions. Without its capacity for antigenic variation, influenza virus would, like measles or rubella virus, probably depend for survival on the temporal accumulation of new susceptibles. However, as noted earlier HIV has the powerful dual advantage of a retrovirus—capacity not only to vary its antigenic structure but also to establish latency for years.

8.1 Routes of Transmission

The major routes of transmission of selected viral infections are listed in Table 1.6. Viruses that have several alternate routes have an increased chance of infection. The sequence of events in transmission involves release of the virus from the cell, exit from the body, transport through the environment in a viable form, and appropriate entry into a susceptible host.

Some viruses are released from cells at the end of the cycle of multiplication. Others do not complete this cycle (incomplete viruses), and some are less effective at escaping (e.g., vaccinia). Many viruses are released from cells by budding, acquiring a lipoprotein coat or envelope as they go through the cell membrane; these include herpesviruses, togaviruses, myxoviruses, paramyxoviruses, and coronaviruses. Nonenveloped viruses not released by budding are the adenoviruses, parvoviruses, poxviruses, picornaviruses, and reoviruses. Some of these latter are released by cell lysis. Once released, viruses find their way to new hosts via one or more portals such as the respiratory tract (influenza, adenoviruses, RSV), skin (VZV and smallpox virus), blood (HIV, HTLV-I and HTLV-II, HBV, HCV, and arboviruses), gastrointestinal tract (enteroviruses, noroviruses, caliciviruses), genital tract (HIV, HTLV-I, HSV-2, and HPV), and placenta (rubella, HIV, CMV, HSV-1, and HSV-2). A more detailed presentation of these major routes of spread follows.

8.1.1 Respiratory

The respiratory route is probably the most important method of spread for most common viral diseases of man and is the least subject to effective environmental control.

Viruses transmitted principally by the airborne route include the agents of many classic childhood infections (e.g., rhinoviruses, measles, rubella, mumps, varicella, influenza, parainfluenza, respiratory syncytial virus). Of course, these

Table 1.6 Transmission of viral infections

Routes of exit	Mode of transmission	Example[a]	Factors	Routes of entry[b]
Respiratory tract	Bite	Rabies	Animal	Skin
	Saliva	EBV	Kissing	Mouth
			Prechewed food, infants	
		HBV	Dental work	
		HIV[c]	Sexual	
	Aerosol	Influenza, measles	Cough, sneeze	Respiratory
	Oropharynx to hands, surfaces	HSV, RSV, rhinovirus	Fomites	Oropharynx
Gastrointestinal tract	Stool to hands	Enteroviruses	Poor hygiene	Oropharynx
	Stool to water, milk food	HAV, rhinoviruses	Seafood, water, etc.	Mouth
		HAV, HEV		
	Thermometer	HAV	Nurses	Rectal
Skin	Air	Pox viruses	Vesicles	Respiratory
	Skin to skin	Molluscum contagiosum warts	Abrasions	Abraded skin
Blood	Mosquitos	Alphaviruses, flaviviruses	Extrinsic incubation period	Skin
	Ticks	Group B togaviruses	Transovarial transmission	Skin
	Transfusions of blood and its products	HIV, HBV, HCV, HTLV-I/HTLV-II, CMV, EBV	Carrier in plasma or lymphs	Skin
	Needles for injection	HIV, HBV, HDV	Drug addicts, tattooing	Skin
Urine	Rarely transmitted	CMV, measles, mumps, rubella	Unknown	Unknown
Genital	Cervix	HSV, CMV, HBV, HIV, HPV, rubella	Sexual, perinatal	Genital
	Semen	CMV, HBV, HIV	Heterosexual, homosexual	Genital, rectal
Placenta	Vertical to fetus	CMV, HBV, HIV, rubella	Infection in pregnancy	Blood
Eye	Tonometer	Adenovirus	Glaucoma test	Eye
Breast	Breastfeeding	CMV, HIV, HTLV-I	Maternal viremia	Mouth
Multiple organs Cornea, kidney	Transplant	Rabies, Creutzfeldt–Jakob disease	Surgery	

[a]*CMV* cytomegalovirus, *EBV* Epstein–Barr virus, *HAV* hepatitis A virus, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *HDV* hepatitis delta virus, *HEV* hepatitis E virus, *HIV* human immunodeficiency virus, *HPV* human papilloma virus, *HTLV-I/HTLV-II* human T-lymphotropic virus type I/human T-lymphotropic virus type II, *RSV* respiratory syncytial virus

[b]Transmission does not always follow standard routes (Modified table from Evans and Kaslow [210] (Table 3), Springer has copyright)

[c]Likelihood of transmission by this route is controversial

are also transmitted among adults by this route as well. Others are transmitted by more direct contact with the nose or mouth or their mucosal secretions (e.g., EBV, HSV, and rabies virus).

Various other factors that affect airborne transmission of respiratory viruses include the intensity and method of propulsion of discharges from the mouth and nose, the size of the aerosol droplets created, and the resistance to desiccation. Much of the early work on the transmission of respiratory viruses was done by Knight and his group [90]. Direct transmission of infection occurs via personal contact such as kissing, touching of contaminated objects (hands, handkerchiefs, soft drink bottles), and direct impingement of large droplets produced by coughing or sneezing. These last two behaviors are regarded as personal contact because of the short range of the heavy droplets formed.

They also create aerosols varying in size up to >20 μm that permit transmission of infection at a distance. Dispersion of an aerosol depends on air currents and on particle size. In still air, a spherical particle with a unit density of 100-μm diameter requires 10 s to fall the height of the average room (3 m), and 40-μm particles require 1 min, 20-μm particles 4 min, and 10-μm particles 17 min. This means that particles under 10 μm have a relatively long circulation time in the ordinary room. Particles 6–10 μm or larger in diameter are more readily trapped upon direct impact in the nose and nasopharynx. Further into the airway, flow diminishes to the point where smaller particles 0.5–5 μm in diameter settle on the tracheal and bronchial walls by sedimentation, and particles of 0.5 μm in diameter or smaller can enter and deposit in the alveoli. As infectious secretions are discharged in large numbers by coughing or sneezing, the initially hygroscopic particles of 1.5-μm diameter lose moisture and shrink in ambient air and then regain their original dimensions from the saturated air as they are again deposited in the respiratory tract. Of course, virus particles in an aerosol may not necessarily settle at a level in the respiratory tree with the optimally susceptible cells for that agent.

High concentrations of particles of rhinovirus (and other viruses, including influenza) on fingers, hands, and hard surfaces indicate that infection via hands may be at least as important a route of spread as aerosols containing lower concentrations. This is supported by the continual inadvertent

contact of hands with the nose or eyes [91]. Therefore, frequent hand washing, using antiseptic-containing fluids, sprays, or gels, is recommended for controlling the spread of rhinoviruses, respiratory syncytial virus, and influenza and other viruses [92–95].

Aerosolization of certain viral agents may occur from suction devices and from catheters in intensive care units and from blood products in dialysis units. These include not only respiratory and intestinal agents but also agents such as HBV that circulate cell-free in the blood and cell-associated viruses such as CMV, EBV, HIV, and HTLV-1. Viruses aerosolized from urine or fecal material can be inhaled; hantavirus and arenaviruses are examples of agents that may be spread by aerosols created from soil containing the rodent urine or feces in which those viruses are excreted.

8.1.2 Enteric (Oral–Fecal)

Transmission by the oral–fecal route via the gastrointestinal tract as the portal of entry is probably the second most frequent means of spread of common viral infections.

The major enteric viruses are enteroviruses, rotaviruses, and calici-, polio-, echo-, and coxsackieviruses, along with HAV and HEV. Adenoviruses and reoviruses also multiply in and shed from the intestinal tract, but this route of transmission is not usually of epidemiologic importance. Despite their name, although enteroviruses multiply in cells lining the gastrointestinal tract, they rarely produce local disease there; they rather target the central nervous system and skin and produce their major symptoms and pathology in those organs. Likewise, hepatitis A and E viruses attack the liver, not enteric mucosal cells.

Viruses can directly infect susceptible cells of the oropharynx, but to induce intestinal infection, virus-containing material must be swallowed, successfully resist the hydrochloric acid in the stomach and the bile acids in the duodenum, and access susceptible cells in the intestine. Viruses with envelopes do not normally survive exposure to these acids, salts, and enzymes in the gut.

Agents excreted via the gastrointestinal tract must successfully infect other susceptible persons via fecally contaminated hands, food, water, milk, thermometers, insects, or other vehicles. Both HAV and HEV are stable viruses in water and, when present in sufficient dosage, may not be inactivated by ordinary levels of chlorine. Outbreaks of viral hepatitis have occurred from sewage-contaminated water, as in the huge outbreaks in New Delhi, India, in 1955 [96], and subsequently often due to HEV and in less dramatic attacks elsewhere. Furthermore, HAV, at least, can persist over long periods in oysters and clams obtained from fecally contaminated waters. Milk and water have also served as vehicles of transmission of other viral agents: caliciviruses and poliomyelitis viruses. This is especially hazardous because these foods are so often eaten without having been cooked. Hepatitis viruses and the enteroviruses also flourish in certain institutional settings (mental hospitals, institutions for retarded children, some prisons) and in countries where personal hygiene is lacking or difficult to practice or where poor environmental control is present.

For viruses spread by enteric routes, environmental control is much more effective than for those transmitted by the respiratory route. Thus, good personal hygiene, especially washing of hands after defecation, proper cleanliness and cooking of food, pasteurization of milk, good waste disposal, and purification of drinking water supplies have all proved to be effective preventive measures. On the other hand, some enteroviruses may also multiply in the respiratory tract and be transmitted by the respiratory route; therefore, this alternate pathway is of epidemiologic importance even in the face of good personal and environmental hygiene.

8.1.3 Direct Cutaneous Contact

Direct penetration of intact skin is a less common route of viral entry, and when it occurs, the viruses are usually carried in fluid or some other vehicle. Human papillomaviruses, the agents causing warts, enter through abraded skin and anogenital epithelium; prions, the agents of kuru, may also gain access that way. With rabies, viral penetration of the skin invariably involves an animal bite or some other source of contaminated biological fluid.

The skin serves as a portal of exit for those few viruses that produce skin vesicles or pox lesions that release infectious particles on rupture. These include herpes simplex, smallpox, varicella zoster, and vaccinia viruses. The viruses of certain maculopapular exanthems may also be present in the skin, as in rubella, but this does not seem to be an important avenue of escape, since vesicles are not formed and skin involvement occurs late in the disease, when the virus may be bound by antibody; indeed, the antigen–antibody complex may be responsible for the rash itself.

8.1.4 Sexual

The genital tract serves as a portal of both entry and exit for viruses that infect the genital tissues themselves and more remote target organs. It is also the source of intrapartum infection as the fetus passes through the birth canal. Herpes simplex types 1 and 2 cause ulcerative lesions of the anal and genital mucosa and the surrounding skin. Sexual transmission of these viruses can produce oropharyngeal lesions as well. These and other viruses (most frequently, CMV, HIV, HBV, and HPV) can also be transmitted to their target cells in the anogenital epithelial layer (HPV), in nearby subepithelial lymphoid system (HIV and CMV), or in remote hepatic tissues (HBV). Receptive anal intercourse is a particularly important method of spread of these infections. Three of these four viruses (CMV, HIV, and HBV) along with rubella virus are present in cervicovaginal secretions and can infect

infants perinatally (see Sect. 8.1.5). Finally certain types of HPV, principally 16 and 18, cause a substantial proportion of cervical infection leading to a spectrum of mucosal abnormalities including cervical cancer.

CMV, HBV, and HIV are present in the semen and/or female genital secretions and can be transmitted during either heterosexual or homosexual intercourse [97–101]. Long-term asymptomatic cervical or semen carrier states exist and make recognition and control difficult.

The presence of other genital infections has been shown in repeated studies to predispose to transmission of HIV-1 by the HIV-1-infected partner and acquisition of HIV-1 by the susceptible partner. Most epidemiologic data indicate that the ulcerative lesions of syphilis, chancroid, and HSV-2 infection mechanically facilitate penetration of the epithelial barriers of the genital tract by HIV [102, 103]. However, suggestions of predisposition by non-ulcerative infections like gonorrhea, chlamydiasis, trichomoniasis, and bacterial vaginosis are consistent with an alternative to enhanced epithelial penetration, namely, recruitment and activation of macrophages and other inflammatory cells responsive to the mucosal breach (see Chap. 43).

8.1.5 Intrauterine and Intrapartum

Viruses may infect the fetus either by direct contact via the birth canal or by hematogenous spread via the placenta to the fetus within the uterus. Herpes simplex virus and CMV infections can initiate intrauterine infection by more direct local contact, whereas CMV, hepatitis B, rubella, varicella, and HIV all infect the placenta by the hematogenous route. CMV is the most common congenital infection, whereas congenital rubella has sharply declined wherever vaccination has become routine. Acquisition of EBV or HBV in the early postnatal period is associated with persistence of infection and substantially increased risk of subsequent cancer (see Chaps. 32, 34, 40 and 41).

8.1.6 Blood-Borne

Direct inoculation of viruses into the blood requires some object sharp enough to penetrate the skin and the wall of a blood vessel. That can happen naturally through the bite of an arthropod vector (see Sect. 8.1.7) or iatrogenically through an injection, either with an inadvertently contaminated needle (e.g., by an injection drug user or in medical practice) or for administration of medicinal blood products (e.g., a transfusion). The agents best known for transmission by this route include hepatitis viruses (HBV, HCV, and HDV), retroviruses (HIV and HTLV), and herpesviruses (EBV, CMV). The mechanism of transmission for each of these viruses is similarly straightforward, with the likelihood of infection dependent on a combination of the intrinsic viral properties described above (Sect. 2), the delivery mechanics, and the dose or the size of the inoculum delivered.

8.1.7 Vector-Borne: Insects and Mammals

Humans may be primary or incidental hosts for numerous vector-borne viral infections. They may be transmitted through direct injection by mosquitos, ticks, flies, and other arthropods; they may also be spread directly through saliva during the bite of larger mammal; or they may be spread more indirectly through aerosolization of urine from a rodent. For some viruses, their life cycle involves multiplication in their vector. In many arboviral infections, virus acquired from the human or animal host during viremia requires a period of multiplication in the vector before it is infectious. Examples of this include the transmission of yellow fever virus by *Aedes aegypti* mosquitos and of the seasonally epidemic St. Louis, California, and equine encephalitis viruses. The vector life cycles can be complex, including overwinter survival with transovarian or sexual transmission within mosquito populations.

A range of mammals (dogs, bats, raccoons, skunks, foxes, and others) carry rabies virus in their tissues and bodily fluids including saliva. They can transmit to a human during a bite, with the likelihood of transmission varying according to several factors: the mammalian species, the level of viremia, the duration of its infection, the nature and location of the bite, and others. Bats may also transmit henipaviruses and coronaviruses. Rodent species can also carry and transmit viruses in their urine or feces. Examples of rodent transmission include hantaviruses and Lassa fever virus. The viruses transmitted by vectors can vary greatly in their host range from a fairly high degree of species specificity (as with dengue in *Aedes aegypti* or Lassa fever virus in one or possibly more closely related rat species) to a much broader range (as with rabies in a number of different mammalian families).

Another kind of transmission by a vector is simply mechanical, involving adherence of material containing virus to an insect and transportation from one host to another. This type requires neither incubation time in the insect vector nor any specificity for either the arthropod host or the virus. Enteric viruses like polio and possibly hepatitis viruses may be carried in this way.

8.1.8 Urinary

Interestingly, although viruses such as CMV and measles are excreted in the urine, this portal of exit has not been established as being of epidemiologic or clinical importance. Considering the wide variety of viruses that can multiply in human kidney tissue cultures in vitro and the likely role they play in immune complex nephritis, it seems surprising that renal infections with these viruses are not observed more frequently in humans.

8.1.9 Nosocomial

The unique populations and physical circumstances found in hospitals and other health-care facilities lead to the

transmission of many viral infections by several of the foregoing routes. The more than two-dozen viruses that have been documented as being nosocomially transmitted include many viruses that affect the respiratory tract as well as those transmitted through the respiratory tract to other organs and cells (e.g., CMV, HSV-1, HSV-6, and VZV) [104], hepatitis viruses (including HAV, HBV, and HCV) [105], enteric viruses (mainly rotavirus [106] and caliciviruses [107]), the viruses of several exanthems (rubella and measles) [108], and picornaviruses [106, 109–111].

Respiratory Route

Respiratory tract infections spread by droplets or droplet nuclei like influenza may disseminate naturally through both the relatively vulnerable patients and the personnel in close proximity to each other. Nosocomial respiratory syncytial virus (RSV) infection in patients and staff has been a major concern in the pediatric nursery, intensive care wards, or other population groups [92] at high risk due to crowding and often an immature immune system. Outbreaks among adults and elderly patients in health-care facilities have also been reported, with influenza being a major concern [112–115]. In these settings, infections, diseases, and outbreaks of CMV, HSV, VZV, enteroviruses, myxoviruses, metapneumoviruses, and parainfluenza viruses have also occurred [116–118].

Although viruses may also theoretically be transferred from one patient to another by contaminated ventilating equipment, with the exception of occasional reports about herpesviruses (CMV and HSV-1) [119, 120], this type of nosocomial transmission has not been recognized as an important one. Lack of concerted effort to detect viruses rather than their actual absence may explain the dearth of information about this phenomenon.

Enteric Route

As noted above, enteric viral infections are very common in acute and long-term health-care settings, and outbreaks (mainly due to rotavirus [106] and caliciviruses [121]) can involve large numbers of patients. The circumstances in which they are transmitted within health-care facilities do not differ greatly from those operating outside them (Sect. 8.1.2), although they appear to have a tendency to attack immunocompromised patients.

Direct Contact with Biologic Tissue and Fluids

Transmission of cutaneous and mucosal viral infections in health-care settings by direct contact is not especially common (VZV, HPV, HSV). Although viruses naturally transmitted through the skin following bites (rabies, in particular) rarely propagate that way in hospital settings, contact by personnel with tissue, saliva, and other secretions from transplant patients infected with rabies virus has led to serious consequences [122]. Corneal transplant has also been associated with transmission of the slow (Creutzfeldt–Jakob) virus [123]. These incidents have prompted the transplantation community to adopt exacting protocols for excluding organs and tissue that may be infected with potentially harmful viruses. Lassa fever, Ebola, and Marburg viruses have often been transmitted as nosocomial infections [124]. Lassa fever, in particular, has infected patients and staff, especially in the obstetrical wards via infected placentas.

Blood-Borne Route

Blood transfusion has long been a potential source of viral infection, and with each significant pathogen discovered to be transmissible by transfusion (HBV, HIV-1/HIV-2, HCV, HTLV-I/HTLV-II, and West Nile virus), the US Food and Drug Administration has orchestrated the development and implementation of universal donor screening [125]. Other viruses that are capable of transmission by blood products (e.g., CMV, EBV, parvovirus B19) appear to be relatively harmless and/or ubiquitous enough that screening is not deemed necessary.

Of major concern for patients is accidental exposure during procedures where there is direct contact with a potentially contaminated tissue (e.g., transplantation) or instrument (e.g., needle, dental or dialysis equipment, or endoscope). In regions of the world with adequate resources and training, universal blood precautions and the use of safety measures for disposing or auto-inactivating contaminated needles have largely but not entirely [126] eliminated percutaneous transmission of HBV, HCV, HIV, and other viruses by needlestick injuries. During the first 20 years of the HIV-1 epidemic in the United States, 57 cases of infection were due to occupational injury in health-care personnel [127]. From 1999 to 2012, no further cases had been documented. The Centers for Disease Control (CDC) continues to collect data and inform personnel about those risks [128, 129].

8.2 Measures of Occurrence

Incidence is the number of new events (e.g., instances of infection or cases of disease) occurring in some time interval. Generally, the *incidence rate* is the number of new events divided by the number of people at risk. The incidence rate may be expressed more specifically as the number of events per unit of population per unit of time or as the number of events in a fixed total population during a fixed total time period. The latter is considered a cumulative incidence but is often called an "attack rate" in an epidemic setting, where the total time period under consideration is established by the circumstances.

In the public health context, incidence may refer to new infections or new cases of disease. In the past, *incident*

infection was usually documented by isolation or pathologic visualization of newly acquired virus or by an assay for seroconversion—the appearance of new specific antibody or a defined increase in the titer or concentration of preexisting antibody; occasionally, new infection would be recognized by a change in the concentration of a specific biomarker like an enzyme. In the current molecular era, a variety of techniques for detecting viral nucleic acid are being used to establish newly acquired infection (see Chap. 2). In calculating an infection incidence rate, the denominator would ideally consist of all individuals in a population considered to be both exposed to the agent and susceptible (i.e., lack antibody or other evidence of prior infection).

Incident disease has always meant the presence of clinical manifestations—some combination of self-reported symptoms, objective physical signs, and positive results of specific diagnostic tests; and the disease incidence rate, is based on all individuals in a population whether or not they are infected.

Mortality can be thought of as the incidence of death. In calculating mortality, because the number with infection is seldom known, the total population is customarily used as the denominator, even though that rate generally underestimates the actual rate of death due to a specific viral infection. The case fatality ratio, another measure of the deadliness of an infection, is the proportion of all persons with cases who have died.

In public health practice, the populations at risk or infected can only be estimated with variable degrees of accuracy depending on the circumstances. Evidence of prior infection is often not available. Commonly, the best estimate possible is simply the total population present during a particular time interval within the geographic area or physical space (e.g., hospital) encompassed by the detection or reporting system. Public health agencies generally tabulate statistics for infection or disease in the form of annual rates.

Prevalence is the number of persons with infection or cases of disease existing at a designated time or in a time interval. The *prevalence rate* is the number of such cases divided by the population at risk. The time period involved may be a given instant of time (point prevalence) or a fixed period such as a year (period prevalence). The term *period prevalence* thereby incorporates both the number of new (incident) cases and the duration of illness (number of old cases persisting from the previous reporting period). It is used most commonly for chronic diseases.

Prevalence of infection is usually measured by a test for the presence of a substance (e.g., antibody, antigen, fragment of nucleic acid, or other component) in biological fluid or tissue samples from a given population at the time of their collection. In calculating a prevalence, the denominator consists of persons whose samples were tested. For viral infections, the prevalence of antibody (i.e., seroprevalence) represents the cumulative rate of infection with the agent over recent and some years past depending on the persistence of the antibody. For neutralizing or other long-lasting antibody, it reflects the cumulative or lifetime experience with that agent. If the antibody measured is present only transiently, as is often true of immunoglobulin M (IgM) antibody, then its prevalence indicates infection acquired within a recent period and may even approximate incidence. As with antibody, the detection of nucleic acid could signify either recent or more persistent infection, depending on the natural history of the infection. In calculating a viral disease prevalence, the denominator is usually all individuals in the total population.

It should be clear that the number of new (incident) cases and the number of existing (prevalent) cases are related to each other. More specifically, the prevalence of infection or disease is determined by the duration of incident cases: the longer the duration of an incident condition, the higher the number of prevalent cases of that condition. That proportionality can be represented as prevalence = incidence × duration. More strictly speaking, the term in the equation should be average duration, which may vary considerably for more chronic infections. Two corollaries of this relationship are that the prevalence of acute self-limiting illnesses of short duration would closely mirror the incidence, and the more variable the duration of the condition, the less reliable the relationship in a particular population may be.

8.3 Patterns of Occurrence

Infections at the population level do not happen randomly; they occur in patterns, with different characteristics and in different magnitudes.

8.3.1 Secular, Periodic, and Seasonal Trends

Secular trends in occurrence with no obvious periodicity have been observed over longer intervals than a single year. Numbers may rise or fall depending on natural factors. Short- or long-lived climate changes can alter the balance of vector and host populations, leading to significant increases or decreases in incident arboviral infections. Local populations experience gradually declining rates of a particular strain of respiratory virus as population-level immunity develops following repeated exposures.

Other factors, natural or human in origin, may produce periodic fluctuations that are not strictly seasonal because they do not recur each year at the same time. In the absence of high levels of vaccination, herd immunity to a disease like measles may produce periodic but not strictly seasonal waves of new cases every 2–4 years depending on how fast new susceptibles are introduced into a population (see Sect. 8.3.2). Human migration may account for periodicity

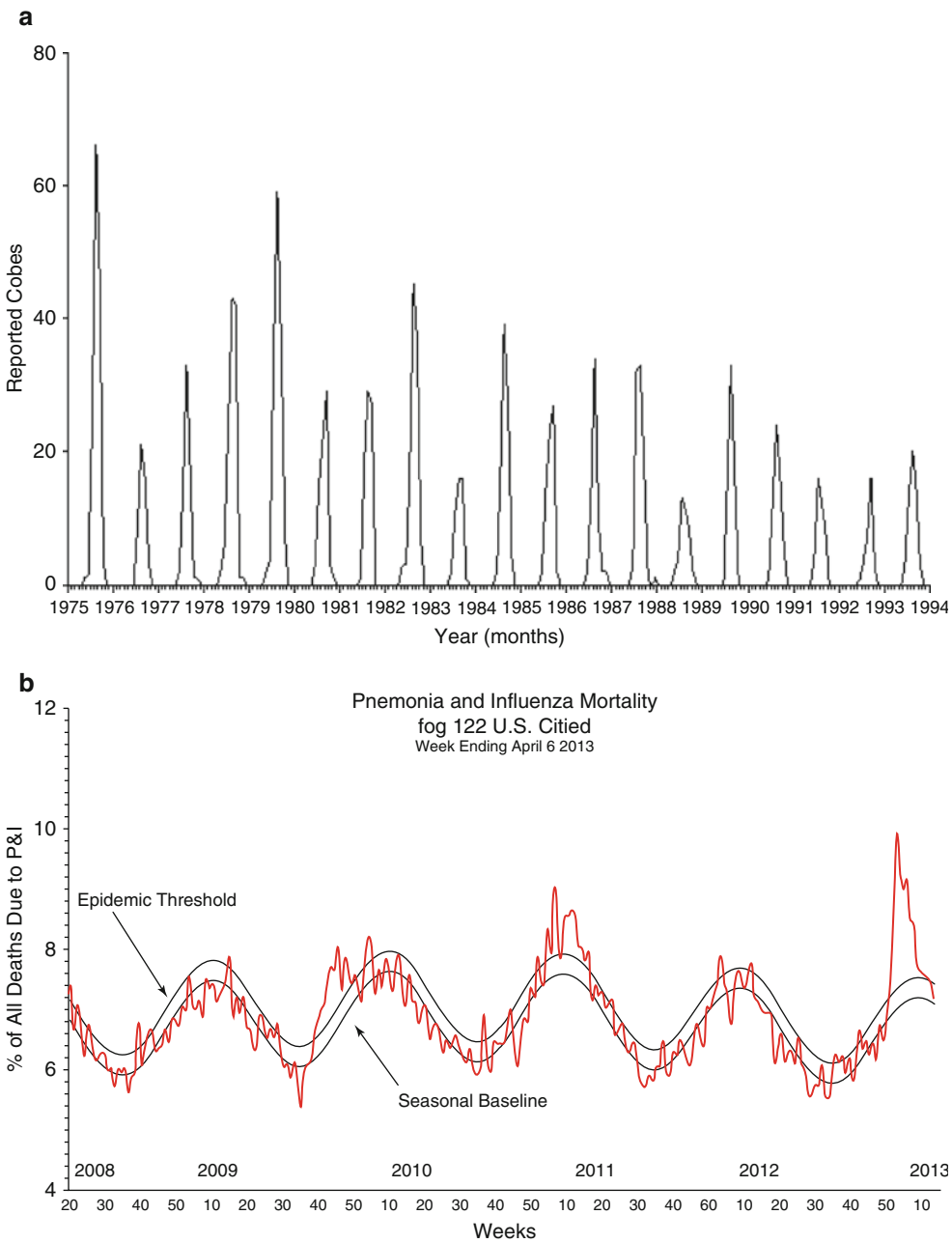


Fig. 1.3 Seasonal occurrence of selected viral diseases. Panel (**a**) Arboviral infections (of the central nervous system)—cases due to California serogroup viruses, by month, United States, 1975–1993 (Reproduced from Koo et al. [130]). Panel (**b**) Pneumonia and influenza mortality for 122 US cities, week ending April 6, 2013 (Reproduced from CDC [131])

that is predictable but might not occur every year in the same season.

Seasonality is one pattern that has been characteristic of many viral infections in the absence of widespread vaccine-induced immunity. Many of the childhood viral diseases (e.g., measles, rubella, mumps, chicken pox, polio, influenza gastroenteritis, etc.) and certain viral diseases of adults (e.g., vector-borne meningoencephalitis and influenza) show striking natural seasonal fluctuation.

For some of these illnesses, the explanation is obvious—rising incidence in summer parallels the resurgence of the vector population (e.g., for mosquito-borne encephalitis viruses, Fig. 1.3, Panel a) or the return of favorable conditions for replication (e.g., for enteric viruses) during warmer weather. For others (many respiratory infections), the conventional explanation is that increases in the winter reflect a combination of environmental factors (temperature and humidity) that both directly enhance the viral replication and transmissibility and indirectly lead to more conducive host behavior (more time spent indoors under artificial conditions of heat and ventilation). For a number of the diseases where vaccines have been effective at radically decreasing the incidence, the seasonality is barely or no longer discernible.

8.3.2 Endemic and Epidemic Occurrence

Infection and infectious diseases occur in populations in different patterns. Incidence rates may be constant or may show seasonal or secular trends. When the pattern of a given condition is stable or constant over a period of years, it is usually considered *endemic*. A number of chronic viral infections have affected certain populations in more or less the same pattern for decades; although their patterns may be distinctive in different subgroups, infections with herpesviruses, like cytomegalovirus, Epstein–Barr virus, and HHV-6, have largely remained endemic. Some could even be considered *holoendemic*—affecting entire populations, nearly universally and more severely in childhood and less so in older individuals. In contrast, an *epidemic* or outbreak of disease is recognized as the occurrence of cases in excess of the number expected for a population based on its past experience. The definition of "excess" is an arbitrary one and depends on the relative concentration of recent and previous cases in the place, time period, and population group of interest. In a country or region where a naturally occurring disease has not been seen for years, a few instances (e.g., encephalitis cases in summer) or even a single case (e.g., of poliomyelitis anywhere in the Western Hemisphere or Japanese B encephalitis in a country where none has been documented for years) could be deemed an epidemic. On the other hand, a large and rising number of cases of winter respiratory illness (e.g., due to the introduction of a new influenza strain) may signify normal seasonal fluctuation. For that pattern to be declared an epidemic usually requires application of more sophisticated criteria (i.e., deaths from influenza and pneumonia in 122 cities exceeding established threshold based on a 5-year average), for example, with the 2012–2013 season (Fig. 1.3 Panel b). When several continents are involved, as is the case with the global distribution of HIV/AIDS, the disease is said to be *pandemic*.

Three essential requirements for an outbreak of viral disease are the presence of an infected host or contaminated reservoir, an adequate number of susceptibles, and an effective method of contact and transmission between them. If the agent is not endemic within the community, then the introduction of an infected person, animal reservoir, or vector of transmission is needed to initiate a naturally occurring outbreak. While the infection may have long been endemic in some remote place, an accident of nature (e.g., importation of an animal carrying the agent) or change in human behavior (e.g., a lapse in hygienic practice in a hospital) triggers its emergence in a new location. This is particularly important in an island or isolated population group, where a virus disappears after no more persons remain susceptible, if persistent viral excretion does not occur to permit infection of newborns. Rubella, for example, disappeared from Barbados for 10 years despite an accumulation in the number of susceptibles to a level representing about 60 % of the population and despite the existence of a large tourist trade [132]. The introduction of more susceptibles or of more infected persons may tip this balance. On the other hand, antibodies to viruses characterized by persistent or recurrent viral excretion, such as herpesviruses and adenoviruses, have been present in every population thus far tested, no matter how remote or isolated [133].

Of recent, an increasing concern is a possible deliberate introduction of an agent capable of spreading mass illness and death. The leading viral candidates for use in an attack of bioterrorism (i.e., those causing smallpox, hemorrhagic fevers, arboviral encephalitis, or a severe pulmonary syndrome) are those for which the target population has little or no immunity. To be effective as an agent of bioterrorism, the proportion of susceptibles to infection with the candidate would need to be high, and the pattern and magnitude of the ensuing epidemic would therefore be particularly difficult to predict.

A critical characteristic of populations that strongly influences its experience with infectious diseases in general and the occurrence of an epidemic in particular is *herd immunity*. The herd immunity level is the cumulative proportion of persons immune to a given disease within a community. This proportion is, in turn, highly dependent on such variables as the probability of contact between a source of infection and the susceptible person, the portal of entry accessible, the contagiousness of the agent, and the degree of individual host immunity (all of which are often quantitatively summarized as the *basic reproduction number* (*or ratio*)). The term refers to the average number of new cases of infection transmitted by an index case during its interval of infectivity (see Chap. 5) [134, 135]. High prevalence of protective antibody to a virus among persons in a given community makes an outbreak with that virus most unlikely. Herd immunity to highly communicable infections such as measles, mumps, rubella, and influenza appear to require levels of antibody at least 75 % and even as high as 94 % to be effective (Table 1.7).

Table 1.7 Basic reproduction numbers and herd immunity thresholds for selected vaccine-preventable viral diseases

Disease	R_0	Herd immunity threshold (%)
HIV/AIDS	2–5	–
Influenza (1918 A/H1N1)	2–3	–
Measles	12–18	83–94
Mumps	4–7	75–86
Polio	5–7	80–86
Rubella	6–7	83–85
Severe acute respiratory syndrome (SARS)	2–5	–
Smallpox	5–7	80–85

Adapted from [136–140]

In an open college community, a preexisting level of immunity to rubella of 75 % failed to prevent an outbreak of this disease [141]. In a rubella outbreak among military recruits with a 95 % level of immunity, 100 % of the susceptibles were infected [142]. Close and prolonged contact is apparently extraordinarily efficient at spreading infection. Other principles are also worth emphasizing: (1) the concept of herd immunity is even less valid where several strains of virus exist and cross protection is not complete; (2) even the identical strain of virus that does not naturally confer complete immunity (e.g., certain herpesviruses) may reinfect; (3) reactivation of latent infection may produce disease, especially in immunocompromised hosts; and (4) the presence of antibody in the forms usually measured for such persistent viruses as HIV or hepatitis C indicates that humoral immunity is incomplete at best.

9 Investigative Approaches

9.1 Descriptive Epidemiology

The first steps in understanding infection and disease in populations constitute so-called descriptive epidemiology. It generally involves integrating the data that best characterize the phenomenon under investigation in the terms discussed in the preceding sections: the agent, environment, and host; the distinctive pathogenetic features; the patterns of occurrence in time and place; and the mode(s) of transmission. The technologies for detecting and defining the agent, quantifying environmental conditions, and profiling the immune status of the host have all been advancing at an exceedingly rapid pace. So have the noninvasive and imaging procedures for characterizing the clinical and pathophysiologic features of the disease. They have facilitated the pinpointing in time and location of cases of infection or illness, often revealing early key information about the origins and the mode of transmission of infection.

The next steps after cases are recognized involve systematically defining, counting, and reporting their occurrences; tabulating their frequencies; comparing their rates; graphing their trends; and evaluating and communicating the results of these efforts. The usual approaches for capturing and interpreting these events in the context of public health and disease control come under the broad heading of surveillance, which is covered in detail in Chap. 4.

9.2 Analytic Epidemiology

Beyond the collection of data for descriptive and surveillance purposes, insight into infectious disease at the population level also emerges from more sophisticated computational and pictorial representation of the salient events and relationships. Hypothesis testing and other formal evaluation of causal relationships, exploration of co-determinants of infection and illness, and recognition of patterns of occurrence all involve rigorous comparisons of data from exposed, infected, and/or diseased individuals with similar data from unexposed or unaffected individuals. That form of inquiry has come to be called analytic epidemiology, the basic methods of which are summarized below. Finally, in conjunction with these numerical and graphical methods, advances in the theory and methods of mathematical statistics have refined empirical and predictive models of virus–host adaptation, transmission and vector dynamics, incidence, epidemic trajectories, and other phenomena of infectious disease. Chapter 5 is a concise view of current approaches to modeling of viral transmission dynamics.

The analysis of the results assembled and integrated during the data gathering and descriptive phase primarily involves evaluating relationships or associations with standard measures. These measures and the investigative approaches to estimating them are summarized very briefly here; more elaborate treatment of the epidemiologic and statistical principles underlying them can be found in any of several textbooks on epidemiology as noted in Sect. 1.

9.2.1 Cohort Studies

The search for associations in epidemiologic data often consists of comparing rates of infection in cohorts of individuals exposed and unexposed to a specific risk factor or rates of disease in cohorts of infected and uninfected individuals. Cohort studies may be cross-sectional or longitudinal in design. In the former, the exposure and the outcome are measured in the population at a single time point. There are many relationships for which the timing of the exposure relative to the disease cannot be established through cross-sectional observation; however, there are many others for which the pathogenesis is understood or the exposure is unambiguously antecedent to the outcome (e.g., genetically mediated rapid progression of HIV infection or shingles in an individual with serologic evidence of VZV infection). In contrast, longitudinal cohort studies are often more powerful because the temporal relationship between the exposure and the outcome can be explicitly established. Although ulcerative inflammation due to HSV-2 is a risk factor for acquisition of HIV infection, it could also be a more prominent consequence of HIV-induced immunosuppression. Only longitudinal observation of precise temporal relationships between the onset of these two infections and the timing of their clinical manifestations would allow definitive inferences about causality.

The most informative measure of association in a cohort study is usually the *relative risk*, used synonymously with risk ratio or rate ratio. Relative risk is estimated as the ratio of the rate of the event (infected or ill) among exposed or infected persons, respectively, to the rate of such an event in the unexposed or uninfected persons. In cohort studies in which all or representative samples of individuals exposed and unexposed to a virus are observed for a period of time until some proportion of each sample develops disease, a

Table 1.8 Standard analytic table for estimating relative risk and relative odds

	Infected/ill	Uninfected/well	
Exposed/infected	a	b	$(a+b)$
Unexposed/uninfected	c	d	$(c+d)$
	Affected $(a+c)$	Unaffected $(b+d)$	Total $(a+b+c+d)$

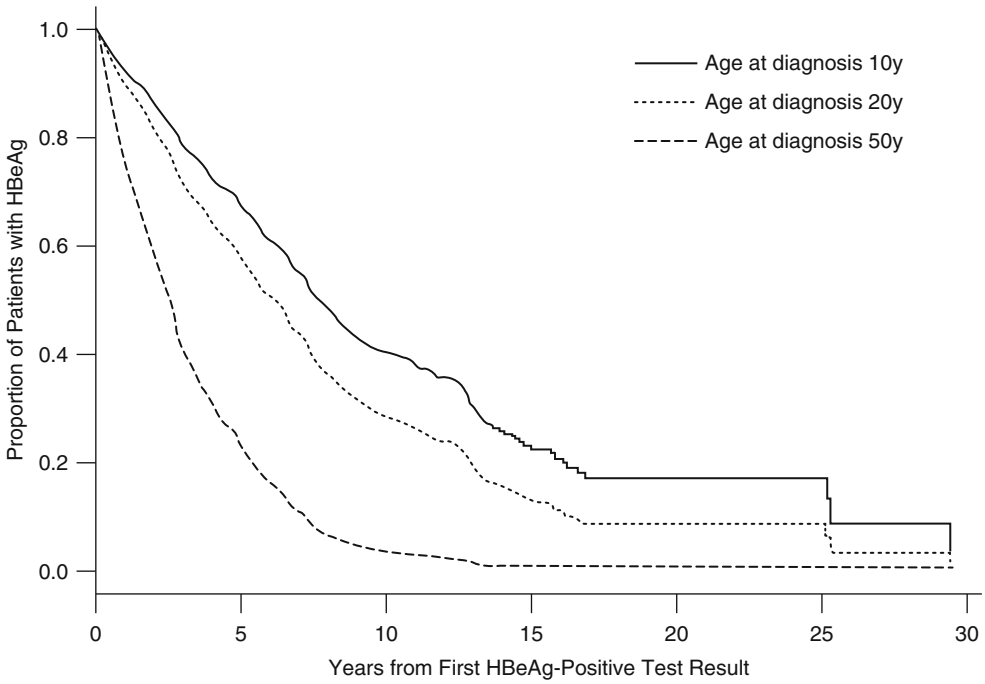


Fig. 1.4 Predicted survival curves (time from first HBeAg-positive result to clearance) at ages 10, 20, and 50 years. Significant covariates included in the model are age at diagnosis of HBsAg (including a nonlinear effect), an interaction of initial recorded status of HBeAg (positive or negative) and age, and the number of HBeAg measurements recorded. The overall probability of clearing HBeAg within 10 years was 72.5 %. In those who cleared HBeAg, the median time to clearance was 5.6 years (range, <1 to 29.4 years). Older carriers were significantly more likely than younger ones to clear HBeAg ($P<0.001$) (Adapted from McMahon et al. [143])

"true" relative risk is calculated as the ratio of the eventual cumulative incidences in the groups with and without exposure or $[a/(a+b)]/[c/(c+d)]$ (Table 1.8).

For conditions that develop over variable lengths of time, if the actual time between exposure/infection and disease is known, it is often preferable to incorporate that time element into the estimate relative risk. That can be done either graphically in survival (Kaplan–Meier) plots (Fig. 1.4) or numerically by application of statistical techniques of which a very useful and popular one is known as a (Cox) proportional hazards regression, yielding a *relative hazard*. Several useful pieces of information about the timing of events can be extracted from the survival curves. As in other regression models, a proportional hazards model can include multiple factors to be assessed for their independent contribution to the outcome (disease).

9.2.2 Case–Control Studies

When an investigation can only be done feasibly on infection or disease that has already occurred following presumed exposure to some etiologic agent in the past, properly designed comparison of those exposed and affected (cases of infection or disease) with those exposed and unaffected (controls) can provide a valid measure of association. When the available cases and, for efficiency, only a small subset of all the non-cases are compared, the putative causal relationship is measured as the *relative odds*, a term synonymous and interchangeable with the *odds ratio*. Based on mathematical principles, for diseases that are uncommon in the population, the relative odds, calculated as $[a\times d/b\times c]$, represent a close approximation to the relative risk (Table 1.8). The case–control study design is especially productive in epidemic situations where the investigation needs to be structured around cases that are reported or sought in the absence of a clear causal exposure. The analysis of case–control studies is usually performed with the technique of logistic regression, which can accommodate independent variables other than the putative causal factor. The major limitation is the many forms of bias that can distort the estimate of the strength of the key relationship, due to difficulties in selecting and assessing study subjects with the tight comparability necessary for proper analysis.

9.3 Investigation of an Epidemic

This brief introduction to the strategy and tactics of epidemic investigations is best supplemented by more extensive guidance found elsewhere [144–146]. From the earlier discussion of the origin and nature of epidemics, it should be clear that no two are exactly alike, but just

Table 1.9 Investigation of an epidemic

1. Determine whether an outbreak is occurring
2. Verify the diagnosis
3. Establish a case definition
4. Enumerate cases
5. Conduct descriptive analyses of the preliminary data
6. Develop hypotheses about the cause of illness and the source of infection
7. Evaluate the hypotheses with analytic methods
8. Conduct additional epidemiologic, environmental, or laboratory studies
9. Develop and implement prevention and control measures
10. Communicate the findings

Adapted from Dicker et al. [145] (CDC publication, no copyright)

as there are patterns to endemic occurrence of disease, there are patterns to epidemics. A systematic approach to their investigation should take account of those patterns by incorporating the sequence of steps outlined in Table 1.9, although this list is neither exhaustive nor in strict priority order of execution.

Outbreaks may come to attention in a variety of ways. An insightful clinician may suddenly see one or more unexpected cases of a syndrome or atypical presentations of infection, just as happened at the onset of the HIV/AIDS epidemic [147]; or a laboratory may identify an unusual viral strain in the course of routine testing (e.g., a novel influenza variant) [148]; or a health department officer may recognize a cluster of cases of, say, norovirus gastroenteritis as multiple entries in computerized syndromic surveillance system [149]. With any such initial event, it may not be possible to establish a diagnosis only at the level of a clinical syndrome and not with etiologic specificity. At this initial stage, a somewhat specific but simple working definition should include key epidemiologic and clinical features for the purposes of case finding. This definition can be altered in its sensitivity or specificity later, when the spectrum of clinical features has been clarified and laboratory studies have been completed. The definition is primarily for the purposes of beginning to enumerate cases. This enumeration is accompanied simultaneously with the organization and analyses of the clinical data and construction of a plot (epidemic curve) of cases according to onset or recognition and/or a plot of the location of cases. Categorization by occurrences by infection, case, or death may provide information about the seriousness of this outbreak relative to previous ones.

Even at this early point, if the pattern of cases or other information reveals a likely mode of transmission, control measures should be instituted. Not surprisingly many epidemics of viral infection result from person-to-person spread, particularly by the respiratory route, in open communities or in relatively closed populations like health or extended care facilities. Vector-borne spread also accounts for a significant portion of epidemic viral infection throughout the world (see Chaps. 7, 8, 9, 14, 15, and 16,). Common source outbreaks of viral infections from water, food, milk, or other environmental sources have not historically been as common as those due to bacterial infections. However, such outbreaks have been recognized increasingly in recent years. Some examples include spread of adenoviruses by tonometers in eye clinics or in swimming pools, hepatitis A by public water supplies or by seafood, or enteroviruses by fecally contaminated water, food, or milk (see Chap. 17).

As the nature, magnitude, mode of transmission, and likely etiologic agent of the outbreak become clearer, the analyses can be refined and focused on more conclusive tests of hypotheses about individuals or other sources and the modulating factors such as age, sex, occupation, recreation, and other geographic or social characteristics of cases. The results of these analyses may assist in excluding or adding cases and focus control measures on individuals most directly affected as well as those at continued risk. The epidemiologic discovery of the cause and other features of the outbreak along with the implementation of control measures will further oblige and enable the investigative team to communicate effectively with the numerous stakeholders in a typically quite visible public health event.

10 Control and Prevention

The basic strategy for controlling a disease is to break one or more links in the chain of causation or pathogenetic continuum. Any such a strategy must rest on the fundamental knowledge of the agent, host, and environment for the particular infection; the control may be physical, chemical, or biological in nature. In the simplest terms, control at the level of the agent usually means incapacitating or destroying it with a physical force as with ultraviolet or light or freezing. A variety of chemicals can also be used externally, particularly on inanimate surfaces (i.e., virucidal disinfectants such as detergents, methylene blue, and chlorine or other compounds that depend on the activity of hypochlorite). At the level of the environment, the strategy might involve an engineering solution such as improving water supplies or sewage disposal or installing negative-pressure ventilation systems, or it might involve avoiding, suppressing, or eradicating the vector population using a widely sprayed insecticide or some other intervention that alters the vector habitat. Effective prevention can also be achieved at the personal level with behavioral intervention to prevent contact (e.g., isolating or cohorting infected individuals in separate rooms, wearing protective clothing such as masks or gowns, applying a topical insect repellant, or washing hands with any of a variety of antiseptic agents). Because of space limitation, for more information on the principles and practices of isolation and barrier, sanitation, disinfection and antisepsis, environmental engineering,

and vector control, the reader is referred to more general texts and references on selected topics here; many others on these numerous topics are available [95, 150–153].

The remainder of this section will concentrate on strategies that depend on biological activity of pharmaceutical agents that can be injected, ingested, or topically applied in humans. These are antiviral products that target specific strains or broad classes of viruses and vaccines and immunobiologics that capitalize on specific host responses to particular viruses.

10.1 Therapy and Chemoprophylaxis

Strategies for designing antiviral agents focus on the numerous vulnerable points in the replicative cycle of a virus that are, ideally, distinct from any point along the synthetic pathways of the human host. There is a growing list of candidate drugs with established clinical antiviral efficacy, and new compounds can now be designed in silico with computer programs that can produce exquisitely precise models of protein–protein interactions that help predict their likely biologic effects. Of course, as promising as these advances are, the ideal is rarely achieved. Limitations still include the poor correlation of effects predicted by in silico, in vitro, or animal studies with those observed in humans, strain differences in response, easy emergence of resistance, and unforeseen acute toxicity. Delayed adverse consequences like oncogenicity and teratogenicity are also a concern.

Each chapter on individual viral infections provides more information about the antiviral pharmaceuticals in use or in trial for those viruses. For another review of antivirals used in specific infections, refer to Ref. [154]. Here simply for rapid reference and comparison is a summary tabulation of the diseases and viruses (other than HIV/AIDS, which is covered in greater depth in Chap. 43), the corresponding antiviral drugs available at the time of publication, their mechanism of action and/or stage of replication affected, and their use for treatment and/or prophylaxis (Table 1.10).

In addition to the antiviral agents covered here, several new candidates in familiar classes as well as new classes of drugs are under experimental and in some cases human investigation. They include numerous new drugs of several classes for HCV; cyclopropavir for CMV infection; a methanocarbathymidine compound active against several herpesviruses; a drug similar to cidofovir with activity against polyoma-, adeno-, pox-, and herpesviruses; favipiravir, which inhibits replication of influenza and probably arena-, bunya-, and hantaviruses; pleconaril and other drugs that inhibit picornaviruses; drugs targeting kinase pathways; and other agents with a broader antiviral spectrum, such as those that bind to the uniquely viral intermediary double-stranded RNA while initiating apoptosis [155–157].

10.2 Immunization

Each chapter on a viral infection for which an effective or promising immunizing agent is available includes details of that virus-specific immunization. This section presents

Table 1.10 Antiviral drugs licensed/generally available for human use

Condition	Virus	Agent	Mechanism of action	Treatment (Rx), prophylaxis (Px)
Cold sores, fever blisters, labial herpes	HSV-1	Acyclovir, ganciclovir, famciclovir, zanamivir, valacyclovir, vidarabine[a] foscarnet, and others	Nucleos(t)ide analogue, inhibits deoxypyrimidine kinase as a competitive substrate	Rx, Px
Genital herpes	HSV-2			
Chicken pox, zoster	VZV			Rx
Cytomegalovirus	CMV	Cidofovir, fomivirsen, foscarnet		
Influenza A	Influenza A virus	Amantadine, rimantadine	Interferes with ion channel protein (M_2) and uncoating	Rx, Px
Influenza A	Influenza A virus			
Respiratory syncytial virus	RSV	Rimantadine		Rx
Influenza A and B	Influenza	Oseltamivir, zanamivir	Blocks release by inhibiting neuraminidase	Rx, Px
Chronic hepatitis B	HBV	Adefovir, emtricitabine, lamivudine, tenofovir	Nucleos(t)ide analogue	Rx
Chronic hepatitis C	HCV	1. Interferon-α	1. Cytokine initiating intracellular immune response cascade	Rx
		2. Ribavirin	2. Nucleoside analogue	
		3. Boceprevir, telaprevir, simprevir	3. Inhibits replication by binding to the nonstructural protein, serine protease	
		4. Sofosbuvir	4. RNA polymerase	
Respiratory syncytial virus infection	RSV	Ribavirin	Nucleoside analogue	Rx

Reproduced and adapted from [155]
[a]Now only for ophthalmic use

general perspectives and more concrete information, including the objectives of vaccination, types of immunization for viral infections, schedules for immunizing target populations with agents licensed in the United States, prospects for eradication of viral diseases, and national and international programs concerned with immunization. For deeper treatment of the principles and practice of vaccinology, consult Ref. [158].

10.2.1 Active Immunization

Active vaccines are administered with the goal of stimulating antibody production by the host to provide a high degree and long duration of protection but with no or minimal accompanying illness. The full set of objectives for creating a good vaccine for active immunization is listed in Table 1.11. Both live and killed vaccines are used, and Table 1.12 compares the two types. Active viral vaccines containing live attenuated virus (measles, mumps, rubella, and smallpox viruses, poliovirus, adenovirus, and VZV) have generally been more immediately, broadly, and durably protective than killed vaccines or other constructs, especially when they are administered by the natural portal of entry to produce local immunity. However, there are a growing number of exceptions to that rule—live virus preparations that are less fully successful (e.g., VZV vaccine against herpes zoster, nasally administered influenza vaccine) while certain non-replicating types formulated as recombinants that appear to provide unexpectedly high protection (e.g., HBV and HPV vaccines). In the case of hepatitis A, both inactivated and live attenuated formulations are highly effective, although immunity generated by the latter may be somewhat longer lasting [159].

Limitations to the wide applicability of live vaccines include the difficulty of successfully attenuating the candidate strain without reversion to virulence, the avoidance of viral persistence and the risk of reactivation, and the elimination of possible oncogenicity. These have been major hurdles for vaccines against herpesviruses, and it is difficult to measure some of these attributes in the laboratory. Efforts to produce live vaccines with temperature-sensitive mutants that replicate in the upper respiratory but not in

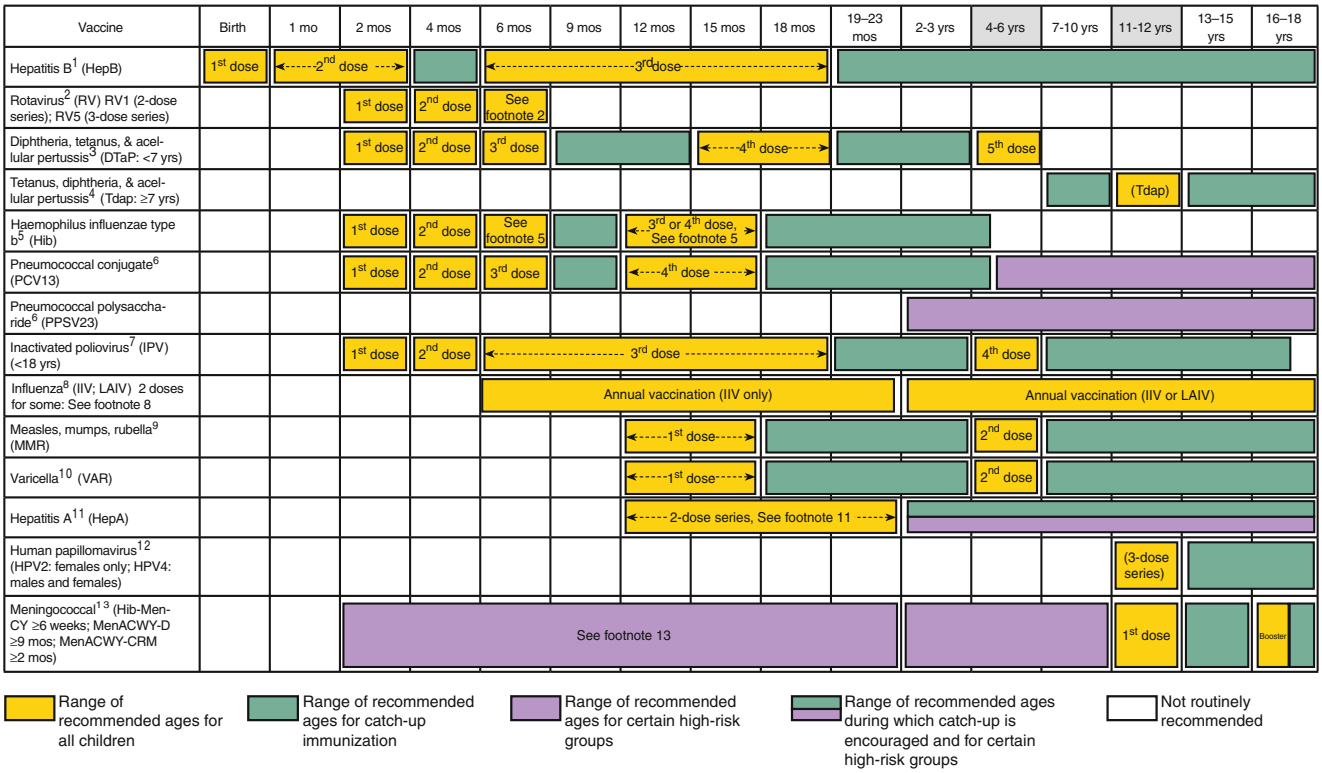
Table 1.11 Objectives for a vaccine

1. Elicit a good humoral, cellular, and/or mucosal immune response that correlates with protection against clinical disease and reinfection
2. Provide protection lasting at least several years and preferably a lifetime, similar to that induced by natural infection that leads to lifetime immunity
3. Result in minimal immediate side effects or adverse reactions or mild disease and result in no delayed effects (e.g., late reactivation, CNS involvement, or cancer)
4. Require a simple regimen for administration in a form and a schedule acceptable to the target population and the general public
5. Pass a favorable cost/benefit analysis, where the health and economic benefit of administration clearly outweighs the cost and risk of natural disease and the adverse consequences of administration

Table 1.12 Comparison of live and killed vaccines

	Live	Killed
Immune response		
Humoral antibody (IgG)	+++	+++
Local antibody (IgA)	+++	+
Cell-mediated immunity	+++	+
Duration of response	Longer	Shorter
Epidemiologic response		
Prevents reinfection by natural route	+++	++
Stops spread of "wild" virus to others	++	+
Some vaccine viruses (polio) spread to others	+++	0
Creates herd immunity if enough persons are vaccinated	+++	0
Characteristics of the vaccine		
Usually heat stable, may need to keep below freezing point until just prior to administration	++	0
Vaccine virus may mutate or increase in virulence	+	+
Antigenic site limited or lost during preparation (e.g., formalin treatment)	0	+
Contraindicated in immunosuppressed persons	+++	0
Side reactions:		
Systemic (viremia)	+	0
Local	0	++
Number of doses for successful take	1	2–3

The table is a simplification and may not apply to all vaccines. Some live vaccines, e.g., polio, are relatively heat stable. Knowledge of the presence of and degree of protection by cell-mediated immunity is inadequate for many vaccines



This schedule includes recommendations in effect as of January 1, 2014. Any dose not administered at the recommended age should be administered at a subsequent visit, when indicated and feasible. The use of a combinationvaccine generally is preferred over separate injections of its equivalent component vaccines. Vaccination providers should consult the relevant Advisory Committee on Immunization Practices (ACIP) statement for detailed recommendations, available online at http://www.cdc.gov/vaccines/hcp/acip-recs/index.html. Clinically significant adverse events that follow vaccination should be reported to the Vaccine Adverse Event Reporting System (VAERS) online (http://www.vaers.hhs.gov) or by telephone (800-822-7967).Suspected cases of vaccine-preventable diseases should be reported to the state or local health department. Additional information, i ncluding precautions and contraindications for vaccination, is available from CDC online (http://www.cdc.gov/vaccines/recs/vac-admin/contraindications.htm) or by telephone (800-CDC-INFO [800-232-4636]).

This schedule is approved by the Advisory Committee on Immunization Practices (http/www.cdc.gov/vaccines/acip), the American Academy of Pediatrics (http://www.aap.org), the American Academy of Family Physicians (http://www.aafp.org), and the American College of Obstetricians and Gynecologists (http://www.acog.org).

Fig. 1.5 Recommended immunization schedule for persons aged 0 through 18 years—United States 2013. These recommendations must be read with the footnotes that follow [161]

the lung have culminated in the licensure of a cold-adapted nasal influenza vaccine recommended for use in healthy 2–49-year-olds.

A comprehensive overview of general immunization principles and recommendations can be found in Ref. [160]. The schedule for immunization of immunocompetent infants, children, and adolescents is shown in Fig. 1.5 [161], and for immunocompetent adults in Fig. 1.6 [211]. They reflect recommendations that took effect in 2013 and will obviously be modified as new agents and regimens are found to be effective.

The above pages of the CDC website are the definitive sources of information about the requirements for immunization and details of administration, precautions, contraindications, and other aspects in the United States. References to CDC recommendations are also available for immunization in other specific situations: health-care personnel [162], special health conditions [163], pregnancy [164], and international travel [165].

10.2.2 Passive Immunization

Passive immunization with an Ig preparation is an expedient useful in short-term prevention primarily when it can be administered soon (preferably within hours) after exposure and when it contains a sufficiently high titer of antibody that will be effective against the agent.

Some preparations are derived from persons known to be convalescent from the disease and from persons hyperimmunized against it or by selecting only donors shown to have high antibody titers. Passive immunization is generally limited to well-defined exposures to rabies virus or in immunocompromised patients who are susceptible to HAV, HBV, VZV, CMV, and vaccinia (unlikely in the absence of smallpox immunization but potentially useful if vaccinia virus gains acceptance as a carrier for other antigens).

10.3 Disease Eradication and Elimination

Smallpox was the first and only disease to have been officially declared eradicated from the earth. From the moment of that historic accomplishment in 1977, this consummate success of the WHO eradication program has inspired initiatives to replicate it with other diseases. The definition of *eradication*, telegraphed in Table 1.13, can be more fully understood in

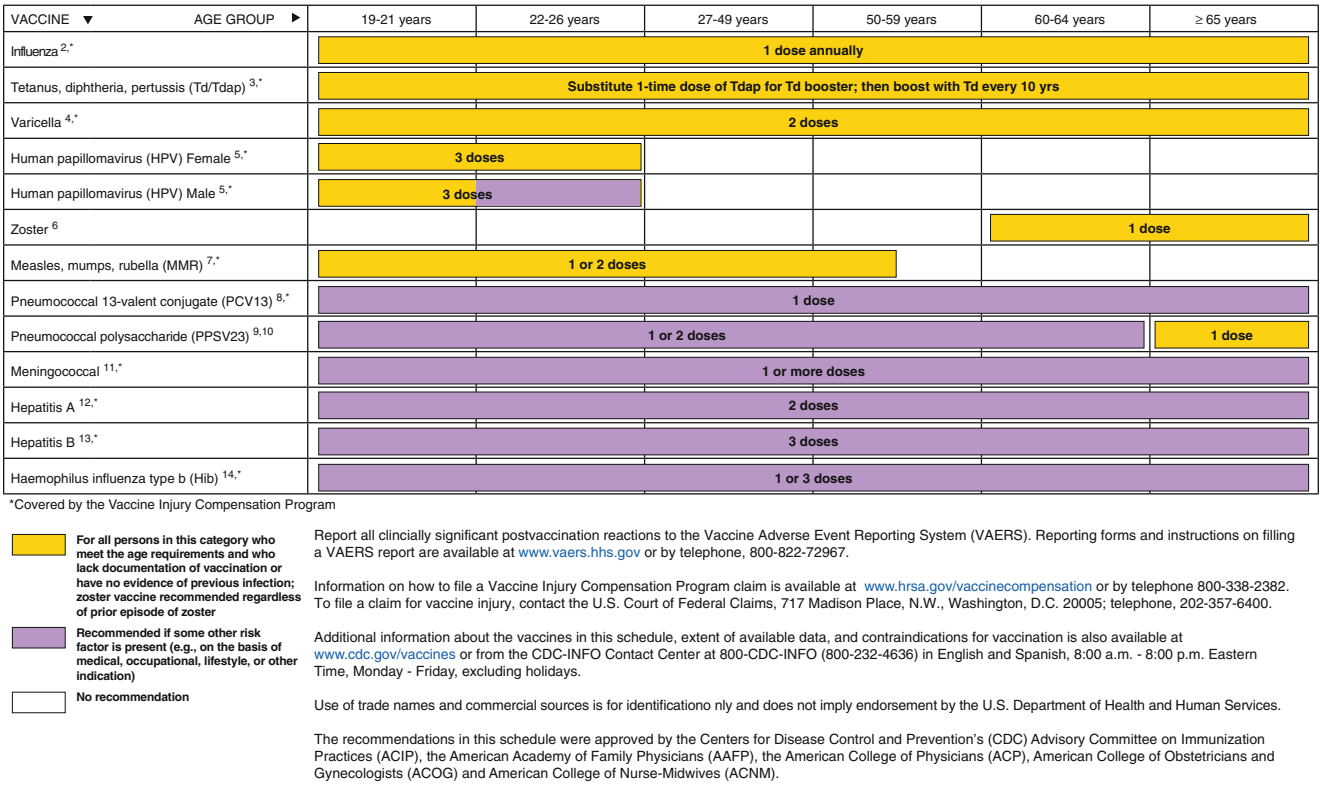


Fig. 1.6 Recommended adult immunization schedule by vaccine and age group [211]

the context of viral infection as "…disappear[ance] from all countries of the world because transmission of the causative organism has ceased in an irreversible manner" [166]. A less ambitious goal, *elimination*, denotes disappearance of the causal agent within a large geographic area; this goal has often been viewed as an intermediate step toward eradication. The critical distinction made in Table 1.13 is that once a disease is eradicated, it is no longer necessary to maintain the elaborate public health apparatus required for ongoing surveillance and control. The implications for achieving either result in practice are that (1) every real or potential occurrence in every location within the target area must be taken seriously, (2) the infection and the interventions to control it must be carefully monitored, (3) program structure and function must be nimble and thorough in response to any suggestion of difficulty or failure, and (4) the rising cost of preventing each successive case cannot be allowed to justify diverting resources away from the goal [167].

Only a few other viral diseases have come close enough to meeting the criteria for potential eradication or elimination to receive serious consideration. In 1993 the International Task Force for Disease Eradication promulgated its list of recommended target diseases for eradication or elimination, and the Task Force recommendations and assessment were updated

Table 1.13 Disease eradication

Definitions
Eradication
Zero disease globally as a result of deliberate efforts
Control measures no longer needed
Elimination
Zero disease in a defined geographic area as a result of deliberate efforts
Control measures needed to prevent reestablishment of transmission
Criteria for assessing the eradicability of a disease
Scientific feasibility
Epidemiologic susceptibility (e.g., no nonhuman reservoir, ease of spread, naturally induced immunity, ease of diagnosis)
Effective, practical intervention available (e.g., vaccine, curative treatment)
Demonstrated feasibility of elimination (e.g., documented elimination from island or other geographic unit)
Political will and popular support
Perceived burden of the disease (e.g., extent, deaths, other effects; relevance to rich and poor countries)
Expected cost of eradication
Synergy of eradication efforts with other interventions (e.g., potential for added benefits or savings)
Need for eradication rather than control

Reproduced from Ref. [167]

Table 1.14 Diseases considered as candidates for global eradication by the International Task Force for Disease Eradication

Disease	Current annual toll	Chief obstacles for eradication	Conclusion
Measles	780,000 deaths, mostly among children	Lack of suitably effective vaccine for young infants	Potentially eradicable
Mumps	Unknown	Lack of data on impact in developing countries; difficult diagnosis	Potentially eradicable
Poliomyelitis	2,000 cases of paralytic disease; 200 deaths	Insecurity; low vaccine coverage; increased national commitment needed	Eradicable WHA 41.28 (1988)
Rubella	Unknown	Lack of data on impact in developing countries; difficult diagnosis	Potentially eradicable
Diseases/conditions of which some aspects could be eliminated			
Hepatitis B	250,000 deaths	Carrier state, infections in utero not preventable; need routine infant vaccination	Not now eradicable, but could eliminate transmission over several decades
Rabies	52,000 deaths	No effective way to deliver vaccine to wild animals that carry the disease	Could eliminate urban rabies

Reproduced from Ref. [169]

in 2008 (Table 1.14) [168, 169]. Global efforts against both poliomyelitis and measles have been the most concerted. The successes and setbacks are covered in the respective chapters on the two conditions (Chaps. 13 and 23).

Although the original Task Force and those who have updated their work acknowledged certain key obstacles, in reality, even when the commitment is strong, funding is seldom entirely sufficient for the tasks at hand, motivation of all key stakeholders is difficult to sustain, and political and social insecurities remain an unpredictable threat. The intensive effort to eradicate polio has been a test case. In 1988 WHO formally initiated its campaign to eradicate poliomyelitis. Under the overall leadership of WHO, it and numerous public and private organizations (e.g., CDC, Rotary International, UNICEF, and the Bill and Melinda Gates Foundation) have persevered in this mission for more than 20 years [170]. It is noteworthy that even before the inception of this more coordinated effort, in 1979 Rotary International began its remarkable commitment to polio eradication, and for three decades, through its worldwide chapters, it performed tireless vaccine campaign work that has recently captured the attention of major news organizations [171, 172, 176]. By 2006 polio remained endemic in only four countries, whereupon gains continued but began to be punctuated by periodic setbacks. Then new blows began to be struck in late 2012 as a result of lethal attacks on healthcare workers conducting polio immunization campaigns in Pakistan and Nigeria and even on their police escorts [173], as well as introductions into countries where war had degraded the public health infrastructure. Continual political and social unrest has fueled the spread of lies about motives of the health workers and the consequences of immunization [174]. All of this turmoil has seriously threatened the eradication program.

Unfortunately, even in the United States, where widespread if not universal elimination of other childhood viral diseases is at least imaginable, there have been very recent resurgences of measles [175], mumps [176], and chicken pox [177], most likely for a combination of reasons: waning immunity despite what had been deemed adequate vaccination recommendations and coverage, gaps in coverage among minority and other populations, and more general resistance to vaccination stimulated by negative publicity in social media along with pockets of objection on religious grounds.

10.4 Programmatic Approaches to Immunization

10.4.1 United States

Creating, testing, producing, licensing, recommending, and monitoring vaccines share many aspects with analogous steps involved in pharmaceutical development and marketing. Descriptions of those elaborate processes are beyond the scope of this chapter. On the other hand, because vaccines are administered almost entirely to basically healthy individuals, often on a global population scale, various distinctive programmatic features of their use are worth considering here briefly. The following summary of the major domestic and international programs involved in vaccine development and delivery may help the reader to appreciate the enormous commitment to immunization for prevention and control of viral infections in general and to understand how a vaccine for any specific infection covered in this text fits into this larger context.

In the United States, besides certain private sector pharmaceutical manufacturers, the National Institute of Allergy and Infectious Diseases [a component of the National Institutes of Health within the Department of Health and

Human Services (DHHS)], the Department of Defense, and other agencies conduct and/or support a broad variety of basic microbiological and immunological research fostering the creation of new and better vaccines. The Food and Drug Administration oversees the development and licensure of all vaccines for human use; through many years of legislation, regulation, and policy-making, this agency has built an elaborate system for ensuring that the products of vaccine manufacturers are safe and effective. Such pre-market design, development, and production activities are beyond the scope of this chapter. Although these Federal agencies remain closely involved with monitoring and reviewing the safety and effectiveness of vaccines once they are licensed and distributed, the National Vaccine Program and CDC are the DHHS components in the US Federal government that have primary authority for implementing and monitoring their use in populations. The DHHS and particularly the CDC are engaged in other activities related to vaccine use, but the following paragraphs highlight its principal responsibilities.

National Vaccine Program Office

This group oversees and coordinates all of the DHHS agencies and offices involved with vaccine development and utilization. In 2010, in part with a view toward achieving the immunization-related objectives established by the Federal government in *Healthy People 2020*, the NVP/DHHS published *the 2010 National Vaccine Plan*, a carefully articulated set of goals and objectives and recommendations on how to reach them [178]. The five overarching goals of the plan are to (1) develop new and improved vaccines; (2) enhance the vaccine safety system; (3) support communications to enhance informed vaccine decision-making; (4) ensure a stable supply of, access to, and better use of recommended vaccines in the United States; and (5) increase global prevention of death and disease through safe and effective vaccination. There also a separate implementation plan, which details the tactics to be pursued in meeting the goals [179].

Surveillance of Vaccine-Preventable Diseases

Through its long-standing relationships with state and local health departments, CDC has implemented increasingly elaborate systems and methods for reporting of selected infectious diseases, a number of which are viral and a subset of which are vaccine preventable. These systems are reviewed in detail in Chap. 4.

Vaccines for Children Program (VFC)

In 1994 the Federal government initiated and began to fund this program to provide free vaccines to children for whom they would otherwise be unaffordable [180]. As administered by CDC, the program currently provides vaccines against the following viral infections: hepatitis A, hepatitis B, human papillomavirus, influenza, measles, mumps, poliomyelitis, rotavirus, rubella, and varicella zoster. There is general agreement that VFC had been a vital force in ensuring relatively high levels of immunization in vulnerable populations, thereby contributing to the major successes in reducing vaccine-preventable diseases in the past two decades.

Advisory Committee on Immunization Practices (ACIP)

This committee is chartered by Federal law [181] to advise CDC and, in effect, all government agencies and the public at large about the appropriate use of vaccines to control those diseases for which immunizing agents are available. The committee meets regularly to review statistics on vaccine-preventable diseases; new experimental and other research findings relevant to vaccine safety and efficacy; information about current vaccines, including labeling and package inserts; newly licensed products; policies and guidelines of other organizations; cost considerations; and other aspects of immunization policies and programs. Recommendations may be forthcoming on any of those topics; in addition, recommendations may also cover for modification of schedules, for administration of vaccines in the Vaccines for Children Program, or for introduction of new vaccines into the program. This advice carries heavy weight throughout the public health, health-care provider, public and private health insurance, and legal communities. The primary legal authority for matters of public health and disease control is vested in the states, and most of them follow ACIP guidelines closely.

Vaccine Adverse Event Reporting System (VAERS)

In 1986, congressional legislation required CDC and FDA to develop this system for receiving, monitoring, and responding to reports of potential side effects and complications of immunization [182, 183]. Every effort is made to document any such untoward event following administration of a licensed vaccine, regardless of the degree of certainty about the causal relationship to the vaccination. Each year, the system receives some 30,000 reports of events, the vast majority of which are mild.

10.4.2 International

WHO

In 1974 the World Health Assembly, encouraged by the success of the smallpox eradication campaign, created the Expanded Program on Immunization (EPI) [184] to ensure that children everywhere would receive routine immunizations. The early goals of the program were to assist in developing the appropriate immunization policies and systems. In its evolving role, it has promoted the key objectives of service delivery, vaccine storage with temperature control (cold chain maintenance), timely vaccine distribution, surveillance of disease and vaccination rates, health-care personnel training, and efficient program management.

More recently (2006), WHO and UNICEF produced the Global Immunization Vision and Strategy (GIVS) [185] aimed at reducing morbidity and mortality from vaccine-preventable diseases during the decade ahead, not just in children but in all segments of the population. The strategy includes immunizing more broadly, introducing a range of new vaccines and technologies, integrating vaccination and other preventive health care, and coordinating programs on a global level. The overall strategy contains numerous goals from which countries can select to tailor their own specific programs. Within only a few years after its adoption, GIVS has been successful in stimulating the establishment of a number of national immunization plans.

GAVI

As a culmination of the World Economic Forum in Davos, Switzerland, at the beginning of the new millennium, major stakeholders in the global immunization (UN agencies, donor governments, vaccine industry leaders, aid organizations, and others) formed a consortium called the Global Alliance for Vaccines and Immunization (GAVI). The mission of this entity is to bring new vaccines to all children of the developing world. The specific goals include intense focus on the more than 20 million children worldwide in poor areas who would otherwise remain unvaccinated against vaccine-preventable diseases, acceleration of delivery of new vaccines to the poorer countries as soon as possible after they are available in the wealthier ones, and channeling support for academic and industrial research on new vaccines targeted to the developing world.

Early GAVI efforts in the realm of viral diseases concentrated on vaccines against hepatitis B and yellow fever. Lately, attention has turned to delivery of rotavirus, the second dose of measles, human papillomavirus, Japanese encephalitis, and rubella vaccines. Two examples of current initiatives include the intention to immunize 700 million children against measles and rubella by 2020 and plans for administering HPV vaccine to 180,000 preadolescent girls in the first phase of a campaign to protect girls in many developing countries against cervical cancer. The alliance has depended heavily on grants and donor government pledges along with private philanthropic contributions, but it has substantially capitalized on more innovative financing for purchases of existing vaccines and for mutual assurances about the future availability of vaccines and the funds needed to purchase and deliver them. More details about the goals and accomplishments of GAVI to date can be found at Ref. [186].

Bill and Melinda Gates Foundation

As a powerful force in the quest to control vaccine-preventable diseases, for years this foundation has supported both traditional and innovative approaches to the development and delivery of vaccines for the places in greatest need [187]. The strategy incorporates five themes: making routine vaccines available, introducing new vaccines as they become available, using innovative and market-based approaches to financing and implementing immunization programs, promoting decisions about the deployment of vaccines based on scientifically sound evidence, and advocating for the support for vaccine programs by other stakeholders. The Supporting Independent Immunization and Vaccine Advisory Committees Initiative is one of those funded by the Gates Foundation to promote National Immunization Technical Advisory Groups (NITAGs) [188]. These groups provide recommendations on immunization policies and programs (e.g., schedules for administration of existing vaccines, improved coverage, and introduction of new vaccines) [189].

10.5 An Emerging Challenge to Immunization

Government and nongovernment organizations alike are engaged in an enormous multipronged worldwide immunization enterprise. There are many natural and legitimate concerns about the prospects for continuing success with each vaccine-preventable disease. The obstacles in every domain—scientific, political, economic, cultural, and others— are formidable. It was especially distressing to learn that one of those obstacles, opposition on religious/ethnic grounds, had motivated the murder of innocent Pakistani and Nigerian health workers in 2012–2013 [190, 191]. In retrospect, such extreme but, hopefully, isolated acts should not be all that surprising as part of the spectrum of social or religious opposition to vaccination campaigns in countries with poorly educated populations. However, on a more ominous note, beginning in the 1990s, the United States and other developed countries have witnessed a gradual increase in the numbers of parents who are refusing to permit their children to receive required routine immunizations. Because the refusals have tended to concentrate among certain subsets of the population who may be clustered geographically [192, 193], the relatively higher proportions of children whose parents have denied them vaccination have led to outbreaks of vaccine-preventable disease [194]. The resulting increased risk of such diseases as measles engenders particular concern because children unvaccinated against them not only are vulnerable in their own right but also pose significant risk to their contacts who may remain unprotected—because of exemption from vaccine requirements, a medical precaution or contraindication for live vaccine, vaccination exclusion for young age, or inadequate response to vaccine [195].

Religious beliefs have often been cited reasons for requesting exemption, but other anti-vaccine forces are at work too [196]. Although incidents with contaminated products had raised largely transient concern in the more distant past [197],

it was the reported but now thoroughly discredited research on the role of vaccination as a cause of autism that probably accounted for the first more serious and lasting rupture in public confidence in the benefits of immunization in general [198, 199]. The news media, in their predilection for controversy, have not always presented a properly *iunbalanced* view of the facts and claims [200]. Mass migration to the Internet as a primary source of health information has led to a proliferation of online websites highlighting anecdotal attributions by parents of various other adverse events to vaccines. While unsupported allegations are unfortunate, their appeal to parents of ill children as easy explanations or as sources of comfort or even justifications for tangible compensation is also understandable. However, more disturbing are print- and web-based testimonies by supposed experts in field of vaccines including radical, unfounded assertions about research that links vaccination to various deleterious biological and clinical consequences. Some of these arguments appear to have arisen from distrust of government [201]. Other claims are likely motivated by anecdotal experiences, favorable publicity, and/or financial benefits that may accrue from sales of books and other materials [202–205]. Regardless of the origins or current forces driving this anti-vaccine sentiment, the recent experience with deliberate discontinuation or refusal of routine immunization has provided ample forewarning of how this growing multifaceted anti-vaccine movement could reverse decades of progress.

As one reaction to this movement, some clinicians have discontinued or have considered discontinuing their provider relationship with patients who refuse vaccines. However, the American Academy of Pediatrics Committee on Bioethics has advised against this and recommends that clinicians address vaccine refusal by respectfully listening to parental concerns and discussing the risks of non-vaccination to the health of their patients and to the health of their community [195, 206, 207]. While neither confrontation nor rejection is an acceptable response, health professionals must devise effective countermeasures against this emerging challenge to the most fundamental strategy for control of viral infections.

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Diagnosis, Discovery and Dissection of Viral Diseases

2

W. Ian Lipkin and Thomas Briese

Only a few years ago, viral diagnosis was largely an exercise for academic researchers and public health practitioners with focus on epidemiologic analyses and outbreak prevention, detection, and control. Opportunities for therapeutic intervention were limited to only a few applications such as herpesvirus infections, influenza, and HIV/AIDS; hence, once a bacterial or fungal infection was excluded, clinicians were limited to providing supportive care for what was presumed to be a viral syndrome. Public health organizations tracked the incidence of viral infections and the development of resistance to the few antiviral drugs in use and provided input to governments and the pharmaceutical industry regarding selection of vaccine targets. More recently, interest in viral diagnostics has burgeoned with the advent of new tools for detection and discovery, global recognition of pandemic risk, high-throughput drug screening, rational drug design, and immunotherapeutics. An additional impetus has been the implication of viruses in chronic illnesses not previously attributed to infection. The objective of this chapter is to review the factors responsible for the rise in awareness of viral infections, methods for diagnosis and monitoring viral infections, and future prospects for improvements in discovery, detection, and response to the challenges of clinical virology.

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1 Emerging Infectious Diseases and Biodefense

In an era when travel and trade are increasingly global, patients with what were once considered exotic infectious diseases restricted to the developing world, like dengue fever, Ebola, or chikungunya, now present in clinics and emergency rooms in North America and Europe. Nonstop flights of less than 24 h connect the world's major airports; hence, physicians must be prepared to expect the unexpected. In New York City, for example, more than 12 million passengers annually pass through John F. Kennedy (JFK) airport from more than 100 international destinations. With this traffic volume in one metropolitan airport alone, it is not surprising that human and stowaway passengers like mosquitoes have been implicated in the transmission of West Nile virus, HIV, influenza virus, *Mycobacterium tuberculosis*, SARS coronavirus, and chikungunya virus. Exotic agents can also transit internationally in legal and illegal (bushmeat) food products and companion animals. The annual traffic in bushmeat through Charles de Gaulle airport in Paris is estimated at 273 tonnes [1]. In work with nonhuman primate, rodent, and bat bushmeat seized at JFK by the Wildlife Conservation Society, EcoHealth Alliance, and the Centers for Disease Control and Prevention, we have found evidence of infection with retroviruses, herpesviruses, and pathogenic bacteria [2]. Illegal importation of companion animals such as birds, primates, and rodents has been linked to outbreaks of poxviruses and Salmonella [3, 4].

Approximately 70 % of emerging infectious diseases are zoonoses—infections that are transmitted to humans from wildlife or domestic animals [5, 6]. The majority of zoonotic diseases can be attributed to anthropogenic change. Loss of wildlife habitat to development and consumption of bushmeat necessitated by poverty or due to cultural preference increases opportunities for cross-species jumps. Global warming may also increase the geographic range of phlebotomus insects like mosquitoes and ticks that serve as reservoirs and vectors for infectious agents [7]. Given that there

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are more than 50,000 vertebrate species, if we assume an average of 20 endemic viruses per vertebrate species, the potential reservoir of vertebrate viruses can be estimated at one million. Although it is unlikely that all of them can be transmitted to humans and cause disease, it is sobering to consider the challenge of detecting and responding even to 1 % of them (10,000 novel viruses).

In the aftermath of the fall of the Twin Towers on September 11, 2001, in New York City, and the anthrax attacks that followed, many western governments became concerned about bioterrorism. Early investments in surveillance for biological weapons gave way to surveillance for emerging infections when sober reflection led to recognition that the latter were more likely threats to public health. However, advances in synthetic biology over the past decade have been so dramatic that clinicians and public health practitioners must again consider the possibility that high-threat known and novel pathogens may arise through deliberate genomic manipulation either in the form of bioweaponeering, inadvertent release of high-threat human pathogens, or legitimate gain-of-function research, whereby low-risk agents become high risk. The scientific and larger communities are currently grappling with the implications of gain-of-function research in the context of experiments designed to understand virulence and transmission of H5N1 (avian) influenza viruses [8–13].

2 Impact of Mechanisms of Pathogenesis on Viral Diagnostics

Establishing a causative link between a virus and disease can be straightforward or complex. In some instances, the virus responsible for the induction of disease is present at a site of organ pathology, and there is precedent for the same or a related virus causing similar disease. A classic example is herpes encephalitis where the detection of herpesvirus sequences by polymerase chain reaction (PCR) in the cerebrospinal fluid (CSF) of a patient with encephalitis provides a clear diagnosis and suggests a specific therapeutic intervention [14]. PCR alone can be inadequate. In West Nile encephalitis, PCR of CSF is less reliable than assays of CSF for IgM antibodies to the virus [15, 16]. In some instances, the footprints of an agent cannot be found in or adjacent to the affected organ but can be detected in other compartments. PCR detection of enterovirus, for example, in the feces of a patient with aseptic meningitis, provides strong evidence of enteroviral meningitis [17]. Despite these examples of success, an etiological agent is not identified by any test in up to 70 % of what is presumed to be viral encephalitis. Similar figures pertain in viral pneumonias.

There are several explanations for surveillance and diagnostic failure. In some instances, the problem is simply lack of access to the appropriate sample. Infectious agents that do not shed into saliva, nasopharyngeal secretions, blood, urine, CSF, or feces may be detected in tissue biopsies. Alternatively, pathogenetic mechanisms may be indirect, or consequences of infection may be delayed obscuring the relationship between the causative agent and the disease.

The most straightforward mechanisms for viral pathogenesis are cellular damage due to replication and lysis, apoptosis, autophagy, or immune responses to proteins expressed on infected cells. However, viruses can also induce systemic damage through cytokine storm resulting in shock, acute respiratory distress, and/or organ failure, cause immunosuppression resulting in opportunistic infection, or break tolerance for self, resulting in autoimmune disease. Infection can be cryptic, impairing differentiated cell functions like hormone secretion, inducing neoplasia, or impairing developmental programs that may not become apparent for months or years. In summary, the challenge in viral diagnostics is to develop strategies for not only detecting footprints of the agent itself in target tissues but also enduring shadows of infection in accessible compartments.

3 Culture

Once the mainstay of viral diagnostics, culture now receives less emphasis in clinical microbiology, chiefly because assays require days rather than hours; thus, information obtained is unlikely to directly impact patient management. Culture nonetheless continues to play an important role in public health as well as basic and clinical research because it enables insights into pathogenesis and the efficacy of drugs, antibodies, and vaccines. The presence of virus can be detected by changes in cell morphology at the level of light microscopy including lysis, rounding, and syncytia formation—fusion of cells as revealed by an increase in size and the presence of more than one nucleus—visualization of pathognomonic structures by electron microscopy; or viral proteins that bind antibodies as revealed through immunohistochemistry or immunofluorescent microscopy. A wide range of cell lines has been established for culturing viruses. Some are immortalized; others are primary cultures that can only be propagated for a few generations. Although some viruses can grow in many cell types, others have fastidious requirements. Some viruses have never been cultured despite implication in disease. In some instances, propagation failure may be overcome by adaptation with serial passaging in the presence of a second, permissive type of cell (cocultivation), the use of antibodies or RNAi to suppress innate immune responses, or cells obtained from genetically modified animals. However, serial passaging can lead to adaptation, including changes in virulence (the capacity of the virus to cause disease) or tropism (the cells and organs the virus

can infect). Indeed, serial passage may be utilized to develop less virulent strains that can be used as vaccines. A potential confound in characterizing samples that may contain more than one virus is that the culture environment can select for the agent that is more fit to replicate—that may or may not be the agent of interest. In an attempt to address this potential confound as well as to propagate viruses that fail to grow in simple cultures, investigators have developed cultures that include more than one cell type. In some instances these complex cultures are designed to replicate the architecture of an organ like the respiratory tract. An alternative to culture in cells is animal inoculation. An advantage of animal inoculation is that the presence of a wide range of cell types is associated with expression of a wide range of receptors that may allow virus entry. Most investigators use suckling mice because their innate immune responses are immature. Others use mice genetically modified to abrogate immune responses.

4 Molecular Assays

Nucleic acid tests (NATs) have largely replaced culture in viral diagnostics due to advantages in cost, speed, and ease of use. Common NAT platforms include polymerase chain reaction (PCR), in situ hybridization, microarray, and high-throughput sequencing.

4.1 Singleplex Assays

These assays, designed to detect individual viruses, are the most common NATs employed in clinical microbiology. They take several forms, but quantitative real-time PCR, wherein nucleic acid replication results in either cleavage or release of a fluorescence-labeled probe oligonucleotide that binds to a sequence region between the regular forward and reverse primers, is the most popular. The continuous ("real time") reading of the reporter fluorescence signal affords these systems with unprecedented dynamic range and low false-negative rate. The required equipment, thermal cycler with fluorescence detector and (laptop) computer for data analysis, is cost competitive, and rugged battery-powered instruments are available for field use. Loop-mediated isothermal amplification (LAMP) tests do not require programmable thermal cyclers [18–20]. In the laboratory, LAMP products are detected in conventional dye-stained agarose gels, but in field applications the estimation of product accumulation through turbidity or dye reading by the naked eye is also possible [21]. The sensitivity of all such assays is highest when primers and/or probe sequences perfectly match the selected single genetic target. Fluorescence-based TaqMan or molecular beacon assays, for example, typically have detection limits of <10 molecules per assay. Although ideal for detecting and quantitating a specific known agent [22, 23], these assays may nonetheless fail with templates of variable sequence composition, especially if this affects the region of reporter molecule binding. This can be particularly challenging in the diagnosis of RNA virus infections as RNA viruses are characterized by high mutation rates and include species with high genetic strain variability. In comparison, consensus PCR assays are less likely to be confounded by sequence divergence but are also less sensitive than the specific PCR assays. Nested PCR tests that can employ consensus or specific primers in two sequential amplification reactions with either one (hemi-nested) or two (fully nested) primers located 3′ with respect to the first primer set may both accommodate sequence variation and be more sensitive than fluorescent or beacon-based singleplex assays. However, whereas in quantitative fluorescence- or beacon-based real-time assays reporter readings are taken indirectly without opening the reaction vessels ("closed system"), nested PCR systems bear a high risk of contamination because of the transfer of (amplified) material from the first to the second, nested reaction [24, 25], even if scrupulous experimental hygiene is observed. Recently, automated (closed) systems have been developed that allow contamination-free transfer between separate reaction compartments of single-use cartridges that may present new opportunities for nested assay design.

4.2 Multiplex Assays

As signs and symptoms of disease are rarely pathognomonic of a single agent, particularly early in the course of an illness, many microbial candidates must be entertained simultaneously. Multiplex NATs provide such an opportunity. The number of candidates considered may range from 10 to 50 with multiplex PCR systems to thousands with microarray platforms to the entire tree of life with unbiased high-throughput sequencing approaches. However, genetic targets compete for assay components in multiplex assays, and thus they may be less sensitive than a singleplex assay. In compensation, multiplex assays provide the advantage of consistently interrogating each sample for a wide range of agents without the selection bias introduced by singleplex testing. This comprehensive coverage is particularly important for surveillance and applications.

4.2.1 Multiplex PCR

Multiplex PCR assays are more difficult to establish than singleplex assays because primer sets may differ in optimal reaction conditions (e.g., annealing temperature or magnesium concentration). Furthermore, complex primer mixtures are more likely to result in primer-primer interactions that reduce assay sensitivity and/or specificity. To advance multiplex

primer design, we developed Greene SCPrimer, a software program that automates consensus primer design over a multiple sequence alignment with customizable primer length, melting temperature, and degree of degeneracy [26].

Gel-based multiplex PCR assays are limited by size differentiation of the amplification products in agarose gels and the concomitant requirement for short product sizes (approx. 90–250 base pairs) to ensure high sensitivity and fidelity [24, 25]. Multiplexing can be achieved in fluorescence- or beacon-based real-time assays to the degree by which different fluorescent reporter emission peaks can be unequivocally separated. At present up to five fluorescent reporter dyes are detected simultaneously, although multiplexing may be increased to some extent by double-labeling strategies and/or melting curve analyses. "Sloppy Molecular Beacons" address this limitation in part by binding to related targets at different melting temperatures [27]; however, they are not suited to detect targets that differ by more than a few nucleotides.

The Bio-Plex (or Luminex) platform employs flow cytometry to detect multiple PCR amplification products bound to matching oligonucleotides that are attached to differently colored fluorescent beads [28, 29]. By combining multiplex PCR amplification systems with various protocols for direct or indirect (tag-mediated) bead hybridization of the products, assay panels have been developed that permit detection of up to approx. 20 genetic targets simultaneously [30–32]; the most commonly used respiratory panels range from 9 to 20 plex [33–37]. Like real-time PCR, these assays rely for assay specificity on a three-oligonucleotide interaction with the target sequence. They are thereby limited in their tolerance for mutated or variant templates when compared to mass spectroscopy (MS)-coupled platforms that require only two oligonucleotide-binding sites, such as MassTag PCR or the Ibis T5000 system.

Two platforms are established that combine PCR with MS for sensitive, simultaneous detection of large numbers of targets. The Ibis T5000 system uses matrix-assisted laser desorption/ionization (MALDI) MS to directly determine the molecular weights of the generated PCR products and to compare them for identification with a database of known or predicted product weights [38–40]. MassTag PCR uses atmospheric pressure chemical ionization (APCI) MS to detect molecular weight reporter tags attached via a photo-cleavable linkage to PCR primers [41]. Whereas the Ibis system or the subsequent electrospray ionization (ESI)-based Plex-ID system requires analytical MS to determine the exact weight of the PCR products and thus depends on advanced mass spectroscopic data analysis, MassTag PCR can be performed using smaller instruments and does not require sophisticated analyses because it only records the known masses of the 40–80 reporter tags used in a given multiplex test. The Ibis system may be able to alert of variants of known organisms via a divergent PCR product weight, but like MassTag PCR, it too requires subsequent sequencing of the product for detailed characterization. A wide variety of syndrome-specific MassTag PCR panels have been developed and applied to the detection of viruses, bacteria, fungi, and parasites associated with acute respiratory diseases, diarrheas, tick-borne diseases, encephalitides/meningitides, and hemorrhagic fevers [41–50].

Although multiplex PCR methods are designed to detect known agents, they can nonetheless facilitate pathogen discovery. MassTag PCR requires only two differently tagged primers per target that may include degenerate positions to address genetic variation of larger taxonomic groups such as a whole species or genus, and its use to investigate influenza-like illness in New York State revealed the presence of a novel rhinovirus clade by the employed conserved enterovirus/rhinovirus primer set [42]. This discovery enabled follow-up studies across the globe wherein this third species of rhinovirus, rhinovirus C, was implicated not only in influenza-like illnesses but also in asthma, pediatric pneumonia, and otitis media [44, 51–63].

4.2.2 Microarray Assays

Whereas multiplex PCR systems support rapid high-throughput diagnosis with highest sensitivity for a limited number of agents, microarray-based systems provide detection of all known pathogens for which sequence information is available, but at the expense of some degree of sensitivity. Modern printing technologies can generate high-quality arrays with several million features, a printing density that enables not only detection of a wide range of infectious agents but also discrimination of medically important types or subtypes. Examples of the latter application include respiratory virus resequencing arrays that identify the different influenza virus HA and NA subtypes [64–68].

The discovery array platforms currently in use are the GreeneChip and the Virochip [69, 70]. The panmicrobial version of the GreeneChip, addressing viruses and in addition pathogenic bacteria, fungi, and parasites, led to the recognition of *Plasmodium falciparum* infection in a case of unexplained fatal hemorrhagic fever during the 2004–2005 Marburg virus outbreak in Angola [70]. A variant of the GreeneChip facilitated recently the implication of Reston Ebola virus in a respiratory disease outbreak on pig farms in the Philippines [71]. In 2003, the Virochip supported the characterization of the SARS coronavirus and was also used subsequently to diagnose parainfluenza virus 4 and infection with a human metapneumovirus variant in cases of acute respiratory disease [69, 72, 73].

Both platforms rely on random PCR strategies to amplify and label nucleic acids for detection. In comparison to multiplex consensus PCR methods employed with some targeted array applications or resequencing arrays, this limits

sensitivity especially with complex sample types. In tissue specimens, for example, the sensitivity may not exceed 10^6–10^7 copies per assay because host and pathogen nucleic acids compete for PCR reagents. Thus, these platforms have been more successful with samples containing comparatively low levels of competing nucleic acid, such as virus culture supernatant, serum, respiratory specimens, spinal fluid, or urine. Improvements in sensitivity to a range of 10^3–10^4 copies per assay have been achieved with methods for host DNA digestion and/or the depletion of host ribosomal RNA (rRNA) prior to amplification through subtraction or use of random primers selected for lack of complementarity to rRNA [74].

In current array platforms, virus detection is achieved via fluorescent reporter systems—either through direct incorporation of fluorescent nucleotides into the PCR product that is bound to the array or with a "sandwich approach" whereby fluorescent-branched chains of DNA are added to the product after it is bound to the array [75, 76]. However, new arrays are in development that will detect viral sequences through changes in electrical conductance. Such platforms would enhance portability by eliminating the need for fluorescent scanners. They may also increase sensitivity and reduce costs by eliminating the need for PCR amplification.

5 High-Throughput Sequencing

High-throughput sequencing has transformed microbiology by enabling discovery as well as diagnostics. Unlike PCR or array methods where investigators must choose the pathogens to be considered or are limited by known sequence information, high-throughput sequencing has the potential to simultaneously detect not only all viruses but also bacteria, fungi, and parasites. Although the technology is presently limited to specialized laboratories, sequencing is becoming increasingly accessible as instruments become smaller, methods become more user friendly, and costs decrease. Over the past 10 years, the cost has decreased 10,000-fold from $5,000 per 1,000 nucleotides in 2001 to $0.5 per 1,000 nucleotides in 2012 [77]. Even more impressive perhaps is the time required to generate sequence data. Projects that required weeks only a decade ago are now completed in hours [78].

Current sequencing platforms analyze libraries of amplified nucleic acids. However, some platforms in development will have the capacity to directly sequence nucleic acid. Irrespective of the platform, raw sequence reads are filtered for quality and redundance before assembly into contiguous sequence streams. These streams, known as contigs, as well as reads that cannot be assembled, are aligned to databases using bioinformatic algorithms that examine homology at the nucleotide and deduced amino acid levels in all six potential reading frames [79]. The alignments allow identification of known and novel agents, as well as detection of genetic features that may be associated with drug or vaccine resistance, or provide insight into provenance and evolution.

6 Proof of Causation

Finding the nucleic acid footprint of a virus is frequently only the first stage in implicating it in disease. There is no functional equivalent in viruses to the pathogenicity islands found in bacteria, wherein specific sequences acquired through horizontal gene transfer confer specific pathogenic properties. The best established criteria for proof of causation in infectious disease were developed in the late 1800s by Koch and Loeffler [80]. Known as Koch's postulates they stipulate that an agent be present in every case of the disease, be specific for the disease, and be sufficient to reproduce the disease after culture and inoculation into a naive host. In the 1930s, Rivers suggested that the development of specific immunity to an agent following the appearance of disease could be used in demonstrating causation [81]. Adapting the original postulates to the molecular era, Fredricks and Relman later established that microbial sequences may be used as surrogates for culturing the actual organism [82]. Lipkin and colleagues recently established levels of confidence in the strength of association between an agent and a disease that considers viral burden and distribution, specific immunity, and prevention or amelioration of disease with use of specific drugs or vaccines [12]. Given the sensitivity of molecular methods, it is imperative that physicians and researchers consider the biological plausibility of an assay result and, where feasible, pursue confirmation with an independent assay, particularly when engaged in pathogen discovery.

7 Future Perspectives

NATs are rapidly replacing classical culture methods in clinical microbiology laboratories. Although some NATs, such as microarrays and high-throughput sequencing, still require substantial investment in equipment and personnel, diagnostic platforms are becoming more accessible and less expensive through miniaturization and improvements in methods for bioinformatic analysis. Systems using handheld microarrays, for example, are in development that will ultimately enable diagnosis at the bedside or in the field. Benchtop sequencers are also in production. It is inevitable that as sequencing costs continue to decrease, clinicians will seek information concerning not only the presence of a single candidate organism but also the predisposition of the host to disease based on genetic factors and coinfections with other microflora. These improvements will bring dramatic benefits to medicine and public health.

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Immunological Detection and Characterization

3

Robert L. Atmar

1 Introduction

Immunological methods are valuable strategies for the diagnosis and characterization of viral infections. These methods rely on antigen–antibody interactions, and they can be adapted to allow direct detection of the virus (antigen detection) or to identify the host's immune response to the virus infection (antibody detection). The methods are also used to identify and characterize virus isolates following in vitro or in vivo propagation. Although molecular detection is often more sensitive than antigen detection, immunological methods still have an important role in the study of the epidemiology, pathology, and assessment of clinical disease associated with viral infection.

The development of simple, rapid, and often relatively inexpensive antigen detection test kits has revolutionized both clinical care and laboratory practice. An understanding of various detection methods is increasingly important in the design and interpretation of epidemiologic studies. The vast array of laboratory tests now permits enhanced detection of viral antigens, although the clarification of the classic issue of "causation" of disease remains blurred.

The significance of detection or lack of detection of a virus or viral antigen remains difficult to interpret. Isolation of a virus from a normally sterile site, such as tissue, cerebrospinal fluid (CSF), or blood, is generally highly significant and usually establishes the etiology of the infection. The identification of certain viruses, such as influenza or respiratory syncytial virus (RSV) in respiratory specimens, also is diagnostic because an asymptomatic carrier state has not been shown to exist. However, prolonged and generally asymptomatic excretion or shedding of other viruses can make the determination of the effect of a particular virus on the disease process very difficult. Viruses such as cytomegalovirus (CMV), adenoviruses, and enteroviruses are shed in disease states but also may be shed asymptomatically for prolonged periods of apparent good health. An additional complicating factor is the differentiation of primary infection from reactivation of disease, a problem common to the study of infections with viruses such as herpes simplex (HSV) or CMV, especially in immunocompromised hosts such as transplant recipients or patients infected with HIV. Interpretation of laboratory results in such situations requires a thorough understanding of the pathogenesis and epidemiology of the virus.

Failure to detect a virus or viral antigen does not necessarily mean that the virus was not present previously or did not cause disease. Although failure to detect a virus may be a result of inappropriate or inadequate specimen collection and handling, it may also be a function of the time course of the disease, the age or antibody status of the patient, and the technical resources available to detect or cultivate the virus. The investigator today has many options to diagnose the presence or past presence of a viral infection, but careful epidemiologic and laboratory studies are still required to ultimately link the viral agent to a specific disease process.

2 Historical Background

The recognition of immune-mediated virus neutralization dates back to the late 1800s when Sternberg extended observations of other scientists of the time and described the neutralization of vaccinia infectivity using serum from a recently vaccinated calf [1]. It was another several decades before diagnostic viral serological methods were developed, including complement fixation in the 1930s and hemagglutination inhibition in the 1940s [2]. Immunofluorescent methods for detecting virus antigen were developed in the 1950s by labeling virus-specific antibodies with a fluorescent reporter such as fluorescein isothiocyanate [3]. A little more than one decade after the initial description in 1959 of the

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radioimmunoassay [4] for detecting human plasma insulin, the method was adapted for virus antigen detection [5]. The radioisotope used as a reporter was replaced later during the 1970s with enzymes such as alkaline phosphatase and horseradish peroxidase that allowed colorimetric detection [6]. The method was also readily modified (see below) to allow detection of antibody. A further modification of immunoassay detection methods followed with the use of substrates that allowed detection of the antigen–antibody interaction via measurement of a chemiluminescent reaction.

The diagnostic procedures used for evaluation of specific viral infections are presented in individual chapters of this book. However, certain common aspects of immunological methods used for virus diagnosis are important for the design and implementation of epidemiologic studies. These general issues are addressed below.

3 Principles of Antigen–Antibody Interactions

An antigen is defined as a substance capable of stimulating an immune response when introduced into the body (e.g., of an immunized animal). When the immune response is the production of antibody, the generated antigen-specific antibody can bind directly to the antigen. The portion of the antigen recognized by and specifically interacting with the antibody molecule is called an epitope. An antigen may have more than one epitope. When this occurs the epitopes either may be distinct molecular structures (and thus distinct epitopes) or they may be the same molecular structure (the same epitope) repeated many times. The portion of the antibody interacting with the antigen is called a paratope. The ability of the epitope and paratope to bind to each other is the basis of the specificity of an antigen–antibody interaction.

The binding of antibody to antigen is thought to be the result of electrostatic and van der Waals bonding over short distances, with the kinetics of the reaction following the law of mass action. The law can be represented mathematically as follows:

$$K_d = [Ag \cdot Ag] / [Ag]$$

where K_d is the dissociation constant, [Ag · Ag] is the concentration of the antigen–antibody complex, [Ag] is the antigen concentration, and [Ab] is the antibody concentration.

From this law, the formation of antigen–antibody complexes reaches equilibrium, and the amount of complex formed is proportional to the concentration of the antibody and antigen present. The binding constant is a measure of the strength of interaction between one epitope and one paratope and is also referred to as the affinity of the antibody. The higher the affinity of a paratope for a corresponding epitope, the greater the strength of binding and the lower the K_d value.

A related concept is antibody avidity, which is a measure of the overall binding strength of an antibody to the corresponding antigen. Antibody avidity is correlated to its affinity, but it is also affected by the number (or valency) of interactions between the antibody and antigen. For IgG antibody, the valency is up to two. Since the antigen–antibody interaction is in equilibrium, the presence of multiple binding interactions can maintain the antigen–antibody complex once formed during periods of dissociation between an epitope and paratope.

There are many factors that affect the interaction between an antigen and antibody and the ability to one or the other in a diagnostic assay. These include the temperature of the reaction conditions and the ionic strength of the solutions used in the assay. As noted above, antibody affinity and concentration are particularly important when developing an antigen detection assay. The relative concentration of antigen-specific antibody is lowest in postinfection sera. It can be increased through hyperimmunization with the antigen to generate polyclonal antisera, and the relative concentration can be increased further by affinity purification of the antibody. The highest relative concentrations are reached through the generation of monoclonal antibodies, and selection of high-affinity monoclonal antibodies for use in diagnostic assays is now widely used in the development of antigen detection assays [7].

Another factor that can influence the assay development is nonspecific interactions between the antibody and other substances in the reaction mix (e.g., attachment to the reaction vessel, other microbial antigens). Although these are generally low-affinity interactions, the concentrations of the competing substances can be high enough to affect the readout of the assay. Many of the low-affinity interactions can be removed by washing steps, use of blocking reagents and use of lower concentrations of antibody, but each antibody used in a diagnostic assay should be evaluated for the presence of these nonspecific reactions. In addition, the use of appropriate controls can identify problems with assay performance [8].

4 Specimen Collection

The appropriate collection of specimens is of the utmost importance for the successful identification of viruses in clinical samples. The source of the specimen, the timing of collection in relation to onset of symptoms, the rapidity and method of delivery to the laboratory, and the clinical and epidemiologic data provided to the laboratory all are important variables that relate to the likelihood of successful identification of a viral pathogen. Knowledge of the restrictions of the diagnostic assay to be used is also important; many antigen detection assays are only approved for use when applied to a limited number of clinical sample types. For example, some influenza antigen assays should only be used with nasal swabs while other assays are approved for detection

of influenza in a broad array of respiratory samples types, including nasal aspirate, nasopharyngeal swab, throat swab, and bronchoalveolar lavage [9].

4.1 Source

The clinical syndrome caused by a virus and its pathogenesis of infection determine the specimen(s) that is most appropriate for virus identification. Viruses that primarily cause disease at a mucosal surface or cause vesicular skin lesions generally can be identified in specimens taken from those sites. However, viruses causing generalized or congenital disease or causing symptoms in an internal organ (e.g., central nervous system) often can be identified in specimens taken from multiple different sites. Viruses that cause respiratory tract disease such as influenza viruses, RSV, and rhinoviruses are most frequently identified in samples of respiratory secretions; viruses that cause gastroenteritis, such as rotaviruses, caliciviruses, and astroviruses, are identified in fecal specimens; and viruses that cause generalized or congenital diseases, such as measles, CMV, and mumps, are identified from respiratory secretions, urine, and blood. However, antigen detection methods may not be available for some of these different sample types. Furthermore, some samples may be more likely to yield a positive sample than others (e.g., fecal specimen vs. rectal swab for rotavirus and bronchoalveolar lavage for respiratory viruses in immunocompromised patients) [10, 11]. The reader is referred to specific chapters for information on the ideal specimen type for specific viruses.

4.2 Timing of Collection

Specimens to be used for virus identification should be obtained early in the course of the illness. For many viral infections, viral shedding begins before the onset of symptoms, peaks during the illness, and disappears around the time that symptoms resolve. There are notable exceptions; enteroviruses and adenoviruses may be shed in the feces for weeks to months, and congenitally acquired CMV is shed in the urine for prolonged periods. Some factors that influence the likelihood of successful virus identification include the type of virus, the site from which the sample was obtained, the test being used, the age of the patient being sampled (e.g., younger children shed influenza viruses longer than adults), and the immune competence of the patient (e.g., immunocompromised hosts shed HSV from genital lesions longer than do immunocompetent adults) [12].

A serum sample should be collected early in the course of illness for potential use in identification of a viral infection. For some viral infections, the identification of IgM antibody or the presence of high titers of antibody is sufficient to confirm a virus infection. A second, or convalescent, serum sample should be obtained 2–4 weeks later to look for a rise in virus-specific antibody titer.

4.3 Clinical Data

Clinical information may be useful in helping one choose the types of diagnostic assays that should be performed on a clinical specimen. The time of year and age of the patient are examples of epidemiologic information that will influence the likelihood of identifying certain viral infections. For example, rotavirus infections occur seasonally and are more common in young children, while norovirus infections often occur in outbreaks and are more common in older individuals. Enteroviruses are the most common cause of viral meningitis and tend to occur seasonally in epidemics, whereas HSV type 1 is the most common cause of sporadic viral meningoencephalitis. Knowledge of the pertinent epidemiologic information will permit the use of appropriate enzyme immunoassays (EIAs), immunofluorescence assays, and other diagnostic methods to identify a potential viral pathogen in the clinical specimen.

5 Detection of Viral Antigens

The detection of viruses or viral components is the foundation of diagnostic virology. Although the detection of antibodies to specific viral proteins remains an important element of viral epidemiology, the ability to isolate and/or characterize viral pathogens initially is critically important. The performance of any diagnostic test in a reproducible, sensitive, and specific manner is crucial in the study of viral diseases. Combinations of various techniques, including centrifugation-enhanced tissue culture, antibody–antigen detection, and detection of viral nucleic acid, can be used to supplement classic tissue culture methods.

Methods for the detection of virus-specific antigens have allowed rapid identification of a wide variety of viruses (Table 3.1). Specific monoclonal antibodies conjugated to biochemical markers may provide high levels of sensitivity and specificity [13]. The key to the success of the assays outlined below is the use of reliable virus-specific antibody. Molecular biological techniques that permit the production of relatively large quantities of avid monoclonal antibodies have facilitated viral antigen detection.

5.1 Latex Agglutination Techniques

Viral-specific antibodies linked to latex beads can be used to detect viral antigens in a clinical sample. The presence of viral antigen in the sample results in cross-linking of the beads that can be identified by visual inspection. This

Table 3.1 Antigen detection methods used for diagnosis by virus group

Virus group	Immunofluorescence	Enzyme immunoassay	Immunohistochemistry
Enteric			
Adenovirus		++	
Astrovirus		+	
Norovirus		+	
Rotavirus		++	
Respiratory			
Adenovirus	++	++	+
Coronavirus	+		+
Human metapneumovirus	++	++	+
Influenza	++	++	+
Parainfluenza	++	++	+
Respiratory syncytial virus	++	++	+
Herpes viruses			
Cytomegalovirus	++		+
Epstein–Barr virus			+
Herpes simplex	++	+	
Human herpesvirus 8			+
Varicella–zoster virus	++		
Others			
Arenavirus		++	
Filovirus		++	+
Hantavirus			+
Hepatitis B virus		++	
Hepatitis D virus			+
Human papillomavirus			+
Rabies	++		+
Rubeola			+

+ occasionally used, ++ commonly used

strategy has most commonly been applied to detection of viral enteric pathogens like rotavirus and adenovirus, and it has the advantage of low complexity and providing rapid results (in less than 15 min). However, a major disadvantage is its relatively lower sensitivity (<70 %) compared to other antigen detection methods (>80–90 %) or culture [14, 15].

5.2 Immunofluorescence Techniques

Viral-specific antibodies conjugated to a fluorescein-labeled moiety have been used to identify viral pathogens since the late 1950s [16]. Immunofluorescence (IF) assays are widely used for the rapid detection of viruses in clinical samples and for definitive identification of a virus in tissue culture that may allow viral antigens to be sought before or after cytopathic effect (CPE) is evident. In the direct IF test (Fig. 3.1), the virus-specific antibody labeled with a fluorescent dye such as fluorescein isothiocyanate or, less commonly, rhodamine isothiocyanate is allowed to react for a short time with cells obtained from a clinical specimen or from an inoculated cell culture. After allowing time for an antigen–antibody reaction to occur, the slides are washed and examined microscopically for direct visualization of the fluorescence of the infected cells in the specimen. In the indirect IF test, two different antisera are used: an unlabeled virus-specific antibody capable of binding to a specific viral antigen is used first and is followed by a fluorescein-labeled, species-specific antibody directed against the species in which the first antibody was raised. If a reaction occurs between the first antiserum and the clinical specimen, the second antibody will bind to the antigen–antibody complex and fluorescence of the virus-infected cells can be detected.

The two IF methods each have advantages and disadvantages. Both tests allow an assessment of the quality of the sample in that samples that do not contain cells are poor quality and cannot be interpreted. The indirect IF is usually more sensitive because several fluorescein-conjugated molecules potentially are able to bind to each virus-specific antibody molecule attached to the viral antigen, resulting in amplification of the fluorescence. The direct IF test may offer enhanced specificity due to lower background fluorescence. The indirect IF method requires more reagents and more time to perform. Whether monoclonal or polyclonal antisera are optimal for use in either test method is still debated. The use of monoclonal antibodies generally

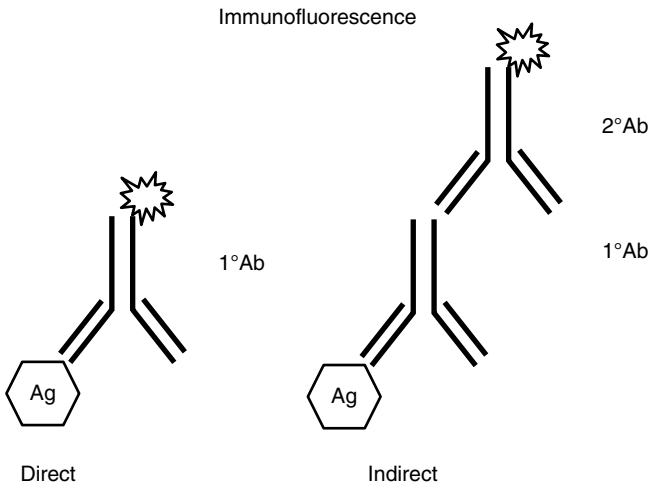

Fig. 3.1 Schematic of immunofluorescence. *Ag* antigen, *Ab* antibody

provides the lowest background but may be limited by the high specificity of these reactions. This problem can usually be overcome by using a pool of monoclonal antibodies.

The use of IF for the direct detection of viral antigens in clinical samples and the confirmation of viral growth in cell cultures has increased with the widespread commercial availability of relatively inexpensive antibodies specific for many of the herpesviruses and respiratory viruses. The IF method has the advantage of allowing rapid viral diagnosis in properly obtained specimens [17–20]. When working with large numbers of clinical specimens, the time required for sample collection, processing, and interpretation of the stained slide becomes substantial. The enthusiasm for this technique in clinical specimens has varied due to the time and degree of technical competence required to read such samples, the availability of other, the less labor-intensive antigen detection methods, and the frequency of false-negative results obtained because of the dependence of the assay on having a high degree of viral antigen expression in the clinical sample. Nevertheless, the appropriate use of this test can result in reliable and sensitive rapid diagnosis from clinical samples. The use of IF for the detection of RSV in pediatric patients by an experienced laboratory can detect up to 90–95 % of the samples positive by culture [21, 22], although many laboratories report rates of 60–80 % [23–25].

The combination of IF techniques with cell culture has increased the sensitivity of cell culture while providing a positive result in a shorter time period. With the use of centrifugation or other methods of enhancement of viral replication and pools of varying antibodies, cell cultures can be incubated between 1 and 3 days and then stained for a variety of virus antigens using indirect or direct IF methods. For some viruses, such as CMV or VZV, specific antibodies directed toward early or nonstructural antigens permit the rapid diagnosis within 48 h, well before CPE would be visualized under routine cell culture conditions [26, 27]. Disadvantages of IF techniques include the need for fluorescent microscopes, difficulty in the interpretation of clinical specimens that have a high level of nonspecific fluorescence, and the fact that prepared slides are not generally stable over periods longer than 1 month [20].

5.3 Immunocytochemical Staining

Immunocytochemical staining is a sensitive and specific method for detecting viral antigens with labeled antibodies. This technique, pioneered by Coons [28] in 1942, has been used to study the structure and function of a variety of viral proteins and continues to be utilized in both the research and clinical laboratories. It has been used both for detection of viral infection of a monolayer prior to the appearance of cytopathic effect and in rapid screening assays for drug resistance [29, 30]. This method utilizes reagents similar to those used in the IF assay except that the fluorescent marker is replaced by an enzyme. When enzyme-specific substrates are provided, a colored precipitate forms at the site of reaction. Typical enzymes used to detect viral antigens include alkaline phosphatase and horseradish peroxidase. A major drawback of alkaline phosphatase-based reagents is their lack of stability; a major drawback of peroxidase as a marker is the fact that this enzyme is endogenous to some mammalian tissue, thus requiring either elimination of the endogenous enzyme or use of a nonmammalian enzyme, glucose oxidase [31].

Advantages of immunoenzymatic staining compared to IF staining include the virtual permanence of stained preparations and the ability to view slides using an ordinary light microscope. Both direct and indirect staining with immunoperoxidase and other enzymes have been utilized to detect many viruses. Refinements have been developed that allow even greater sensitivity than that seen with indirect staining without the need to conjugate enzyme to an antibody. For example, a modification of these techniques has been a four-layer sandwich technique involving (1) virus-specific antibody raised in species X, (2) an excess amount of a second antibody raised against the species X antibody, (3) a complex of peroxidase and antiperoxidase antibody (raised in species X), and (4) reducing substrate for peroxidase [32]. The second antibody acts as a bridge, binding to both the virus and the antiperoxidase antibody. Similar unlabeled assay methods have been described for alkaline phosphatase–antialkaline phosphatase and glucose oxidase–antiglucose oxidase [33, 34]. The sensitivity of antigen detection has been further improved by more recently developed signal amplification methods, including avidin–biotin complexes (binding of 4 biotins per streptavidin), chain polymer-conjugated technology where multiple enzyme and antibody molecules are

attached to an inert molecule such as dextran, and the use of tyramine conjugates as substrates for horseradish peroxidase that allow signal amplication as much as 100-fold [35].

5.4 Radioimmunoassay

Radioimmunoassay (RIA) techniques have been valuable for the detection of many compounds in laboratories and clinical medicine. Initially, the technique was developed for the determination of endogenous human plasma insulin levels [4]. The first important use of RIA in diagnostic virology was for the detection of hepatitis B surface antigen [5]. The original RIA described by Yalow and Berson [36] was a competitive binding assay in which the competition between an unlabeled antigen and a radiolabeled antigen reacting with a limited amount of antibody over a short period of time was monitored. Variations in RIA methods have been developed, with the most common being the direct and indirect solid-phase RIA. In the direct solid-phase RIA, antigen or antibody are captured on a solid support and detected by radiolabeled (usually ^{125}I) antibody or antigen, respectively. The amount of signal increases proportionally to the amount of antigen or antibody present in the sample. In indirect assays, the capture of antigen or antibody to the solid phase prevents the binding of labeled antibody or antigen, respectively, so that the amount of signal detected is inversely proportional to the amount of antigen or antibody present. RIA methods currently are utilized mainly for the detection of antigens and antibodies of viral hepatitis [37]. The use of RIA for the detection of various hepatitis markers has demonstrated the assay's high degree of sensitivity. For the most part, RIA methods have been replaced by EIA for routine diagnostic purposes due to the complexity of the assay, the use of radioisotopes, lack of standardized commercially available reagents, and high equipment costs.

5.5 EIA

Enzyme immunoassays, or EIAs, have gained widespread acceptance in virology laboratories for the detection of a variety of viral antigens and antibodies. The assays used in this method rely on antibodies directed against a specific virus or viral antigen that are adsorbed or directly linked to polystyrene wells in microtiter plates, plastic beads, or membrane-bound material. When viral antigen is present in a specimen, it binds to the immobilized "capture" antibody and a second "detecting" antibody conjugated to an enzyme such as horseradish peroxidase or alkaline phosphatase then attaches to the antigen, forming a three-layer "sandwich" consisting of the immobilized antibody, the antigen, and the detecting antibody with enzyme attached (Fig. 3.2). A substrate

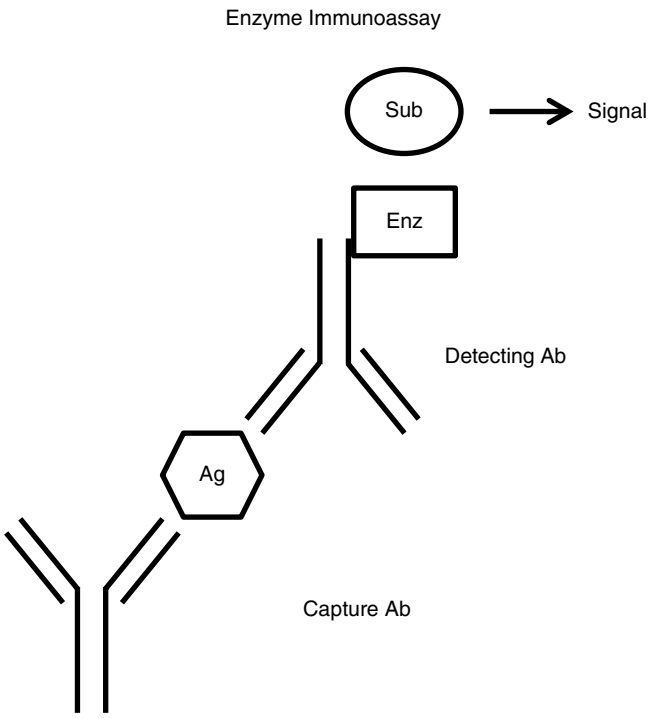

Fig. 3.2 Schematic of sandwich enzyme immunoassay. *Ag* antigen, *Ab* antibody, *Enz* enzyme, *Sub* substrate

specific for the enzyme is added and a color reaction occurs that can be monitored by spectrophotometry or by direct visualization. The test is quite simple to run, requiring only standardization of reagents and techniques such as dilution, incubation, and washing. The principles involved in EIA are similar to those involved in immunofluorescence and RIA, but the EIA test has the distinct advantages of being simple to perform, utilizing reagents that have long shelf lives, are inexpensive, and do not require sophisticated technical evaluation to determine results. Advantages of the EIA technique also include sensitivity (less than 1 ng/ml), specificity, rapidity, safety, automation potential, and low cost, particularly when many specimens require evaluation.

Variations in the methodology for EIA testing include the materials used, the procedures for incubation and detection, and the interpretation of results. Many different test kits are commercially available and in widespread clinical use for the detection of common viral pathogens such as RSV, influenza, adenovirus, HIV, norovirus, and rotavirus; EIA tests have been devised and reported for nearly all virus groups and continue to be used widely for clinical and research purposes.

5.6 Optical Immunoassay (OIA)

The OIA utilizes a virus-specific antibody coated onto a thin molecular film on a silicon wafer surface. The clinical sample

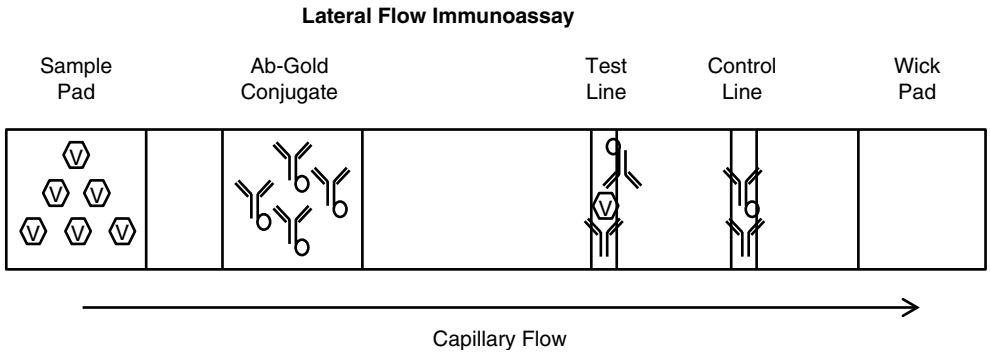

Fig. 3.3 Schematic of lateral flow immunoassay. *V* viral antigen, *Ab* antibody

is treated to extract and expose any viral antigens present and is then placed on the surface of the chip. Viral antigen is captured and the resulting antigen–antibody complex changes the optical thickness of the film on the chip. The change in the surface thickness is magnified through addition of a second virus-specific antibody conjugated to horseradish peroxidase followed by addition of a substrate such as tetramethylbenzidine (TMB). The presence of virus antigen is then detected by a change in the color of reflected light from gold to purple. Kits have been developed for influenza and respiratory syncytial virus detection [38, 39].

5.7 Lateral Flow Immunoassay

The lateral flow immunoassay, also called the immunochromatographic assay, is an immunoassay that is performed on chromatographic paper along a single axis (Fig. 3.3). The clinical sample is applied to an absorbent pad and then is drawn by capillary action through a conjugate pad. If viral antigen is present in the clinical sample, it will interact with a virus-specific antibody conjugated to a colored particle (often colloidal gold). The fluid in the sample carries the antigen–antibody complex to a reaction membrane to which another virus-specific antibody has been immobilized in a line perpendicular to the capillary flow direction. The antigen–antibody–conjugate complex is captured and can be observed as a colored line on the membrane. The sample is carried further across the reaction membrane to a control line. Antibody specific for the antibody–conjugate is immobilized along the control line, and visualization of the control line indicates that the sample migrated across the membrane and picked up the antibody–conjugate as designed. An absorbent pad is beyond the reaction pad and acts as a waste reservoir, drawing the clinical sample across the other pads by capillary action.

The simplicity of the lateral flow immunoassay design allows the use of these assays as point-of-care tests. Results can usually be obtained within 15 min of sample collection. Tests have been developed for detection of respiratory and enteric virus as well as dengue viruses [40–43].

5.8 Time-Resolved Fluoroimmunoassay (TR-FIA)

The TR-FIA is an immunoassay that replaces the reporter molecule with a lanthanide metal. When exposed to the appropriate wavelength of light, the lanthanide will fluoresce [44]. Compared to fluorescein and background autofluorescence, which have fluorescence decay times of less than five nanoseconds, the lanthanides have much longer decay times of 1,000 to 1 million nanoseconds [8]. The format of the antigen detection TR-FIA is similar to that of a sandwich EIA, where a microtiter plate is coated with a virus-specific capture antibody and is then blocked. The clinical sample and antibody conjugated to the lanthanide is added next, and after a suitable incubation period, the unbound components are removed by washing. An enhancement solution is added and the well is exposed to the appropriate wavelength of light. A fluorometer is used to measure fluorescence for 1 s, and the pattern of fluorescence allows the separation of antigen-specific signal from background fluorescence. Several different lanthanides are available for use, but europium is frequently used because of its long fluorescence decay time and the difference between its excitation wavelength (~340–360 nm) and emission wavelength (~615 nm) [8]. TR-FIA has been developed for detection of a variety of viral pathogens [45].

6 Laboratory Methods for Virus Characterization

Further characterization of a virus obtained from a clinical specimen is frequently desirable once an agent has been isolated. This can be done in a variety of ways, depending on what is known about the virus and what additional information is being sought. For example, if a previously unrecognized virus is recovered, characterization of its physicochemical as well as biological, antigenic, and genomic properties would be useful. Various immunological methods can be used for this purpose because of the general availability of immune reagents for most human viruses. Immunofluorescence,

radioimmunoassay, and enzyme immunoassay formats may be used in a fashion similar to that described for the virus detection in clinical specimens (Sect. 5). Other methods for virus identification and characterization include virus neutralization assays, hemagglutination-inhibition assays, and epitope-blocking enzyme immunoassays using monoclonal antibodies.

6.1 Neutralization Assays

Virus neutralization assays detect the loss of virus infectivity that results from the interaction of virus with specific antibody. Unknown viruses may be identified using virus-specific antisera, and antibody to a specific virus present in a serum sample can be detected or quantitated (see Sect. 7.1). The loss of infectivity can be measured in a number of ways, depending on the biological systems capable of supporting virus growth, the types of viruses being sought, and the capabilities of the laboratory performing the studies. The principal biological systems used for neutralization assays are tissue culture, embryonated chicken eggs, and adult and suckling mice [46]. Cell culture systems are used most commonly because they support the growth of a large number of viruses, are widely available, are easier to work with than the other two systems, and lack an immune system (that may influence test results). Embryonated hen's eggs and mice are used as for primary isolation. Neutralization cannot be measured for some viruses (e.g., norovirus) because their infectivity cannot be measured in currently available culture systems.

Pools of virus-specific antisera have been used to decrease the number of neutralization assays needed to serotype enteroviruses [47]. Each serum pool contains antisera to a discrete number of enteroviruses, and antiserum to a given enterovirus is present in one to three pools. Thus, the pattern of neutralization obtained from the use of only eight intersecting serum pools allows the identification of 42 different enteroviruses [48]. Methods for the production of intersecting serum pools have been published [49].

6.2 Hemagglutination and Hemagglutination-Inhibition Assays

The ability to agglutinate erythrocytes, a property shared by many viruses, can be used for the identification of some of these viruses. The differential hemagglutination of rat, human group O, and monkey erythrocytes by different adenovirus serotypes allows their separation into groups so that fewer type-specific sera need to be used in neutralization or hemagglutination-inhibition assays [50]. Type-specific antisera can be used to prevent hemagglutination (hemagglutination inhibition) and permit the identification of influenza A and B viruses, parainfluenza viruses, and adenoviruses [51].

6.3 Agar Gel Immunodiffusion

Agar gel immunodiffusion, or agar gel precipitation, has been used for the characterization of a variety of viral antigens using standard, or reference, antisera. A thin layer of agarose is made in a plate or on a slide, and small wells are cut into the agarose. The unknown antigen and known antiserum are placed in separate wells, and the proteins in the wells diffuse through the agarose. If the antiserum reacts with the virus antigen, a precipitation band appears. Though less sensitive than other methods and largely replaced by molecular assays, this method offers high specificity and is simple to perform. It has been used for the identification of orthopoxviruses, typing of influenza viruses, and subtyping of hepatitis B viruses [51–53]. It also has been used to characterize unknown sera with known virus antigens [54].

6.4 Antigenic Characterization

The antigenic differences or similarities between vaccine and wild-type strains or among virus strains that have been isolated from different geographic locations or at different times may be examined in a number of ways. The availability of monoclonal antibodies permits the examination of these relationships and may detect differences or similarities that cannot be detected by polyclonal antisera [55, 56]. These assays examine the ability of a given monoclonal antibody to interact with a particular virus strain and are performed using the same formats used for polyclonal antisera: RIA or EIA, neutralization (if antibody is neutralizing), immunoprecipitation, hemagglutination inhibition (if the virus has hemagglutination activity), and so forth.

Monoclonal antibodies also have been used to map antigenic sites on virus proteins. When a virus is grown in the presence of a monoclonal antibody that normally neutralizes it, the only progeny virus will be escape mutants, or viruses that are no longer neutralized by the antibody. Frequently, escape mutants arise after substitution of a single nucleotide, resulting in a single amino acid change, and the location of the change can be determined by sequencing the virus gene(s) encoding the viral protein(s) important in neutralization (e.g., rotavirus) [57, 58]. A less precise map of antigenic sites can be obtained through the use of a panel of monoclonal antibodies by determining whether an individual monoclonal antibody competes with other monoclones for an antigenic site and whether the antibody has activity against the escape mutants raised by a different monoclonal antibody [59].

7 Serological Diagnosis

The detection of newly developed, virus-specific antibody or the detection of an increase in titer of preexisting antibody is important in viral diagnosis and is one of the most commonly used methods in epidemiologic studies of viruses. Most primary infections or reinfections result in the production of specific antibodies. In addition, viruses such as EBV, HIV, rubella, hepatitis A and B viruses, and arboviruses are difficult to detect directly and the serological diagnosis may be the only practical means of identifying the particular agent.

The detection of specific IgM antibody may be used to suggest a recent infection in a single serum specimen. Detection of specific IgM antibody in the neonate is useful to diagnose congenital infections, because maternal IgM antibody does not cross the placenta. IgM antibody also is useful to detect acute disease in a variety of other clinical situations, including infection with CMV, rubella, hepatitis A and B, and EBV. Limitations to the use of IgM detection include the following: (1) IgM-specific antibody is not restricted to primary infection and may be seen with reactivated disease, particularly with herpesviruses such as HSV or varicella–zoster; (2) false-positive responses may occur in the presence of rheumatoid factor or false-negative results from competition by IgG antibody for binding sites on the antigen; (3) IgM antibody may persist for months to a year or more after an infection occurred; and (4) heterotypic reactivation of IgM may be found with some infections (such as CMV or EBV). For example, removal of Coombs antibody from sera is necessary for the EBV–VCA–IgM test; otherwise, false-positive results may arise. Methods useful for the detection of viral-specific IgM will be described below, but, in general, diagnosis using a single IgM sample needs to be carefully controlled to exclude the detection of IgG or other interfering substances.

Many different serological techniques have been used in the diagnosis of viral infections (Table 3.2). Factors involved in the selection of a specific antibody assay include specificity, sensitivity, speed, technical complexity, cost, and availability of reagents (Table 3.3). All antibody assays rely on the proper collection and storage of sera and, ideally, the comparison of acute and convalescent specimens collected at an interval of at least 2 weeks. The development of newer techniques, such as EIA, for antibody determination is replacing some of the older methods, such as complement fixation, but an understanding of the available methods is important prior to choosing a laboratory test to evaluate a specific question.

Table 3.2 Serological diagnosis of detected viruses

	Serological method[a]			
Virus	Neutralization	Complement fixation	Hemagglutination inhibition	Immunoassay (EIA, IF)
Adenoviruses	+	+		+
Arboviruses	+	+	+	++
Coronaviruses	+		+	+
Cytomegalovirus	+	+[b]		++
Enteroviruses	+		+	
EBV[c]				++
Hepatitis B and C				++
HSV	+	+[b]		++
Influenza	+	+[b]	++	+
Measles	+	+[b]	+	++
Mumps	+			++
Norovirus/rotavirus			+[d]	++
Parainfluenza	+	+[b]	++	+
Parvovirus				+
Rabies	+			+
RSV	+	+[b]		++
Retroviruses	+			++[e]
Rhinoviruses	+			+
Rubella			+	++
VZV		+[b]		++

[a]+ Method used in research setting, ++ method in use and readily available in virology laboratories
[b]Complement fixation method may lack sensitivity for these viruses
[c]The absorbed heterophile test is commonly used for infectious mononucleosis, with the EBV–VCA–IgM needed if that test is negative
[d]Selected strains only
[e]Western blot commonly used as confirmatory test

Table 3.3 Comparison of serological methods used to detect viral antibodies

Method	Sensitivity[a]	Specificity	Cost	Time to Dx	Availability
Neutralization	+++	++	Expensive	>1 week	Research
Complement fixation	+	+/++	Inexpensive	<1 day	Widely available
Hemagglutination inhibition	++	++	Inexpensive	<1 day	Research/reference labs
Enzyme immunoassay	+++	++	Inexpensive	<1 day	Widely available
Immunofluorescence	++/+++	++	Moderate	<1 day	Research/reference labs
Radioimmunoassay	+++	++	Expensive	1–3 days	Research
Immunoblot (Western blot)	++/+++	+++	Expensive	<1–3 days	Research/reference labs

[a]+ relatively low, ++ moderate, +++ high

Problems specific to serodiagnosis of a viral infection include the broad cross-reactivity among some virus groups, such as the coxsackie A viruses that cross-react with antibodies to coxsackie B and echoviruses. Another serious limitation of this approach to diagnosis is the failure of some individuals, particularly young children or immunocompromised patients, to mount a detectable antibody response to a specific infection. However, serological methods remain extremely important in epidemiologic studies because results are not dependent on obtaining a specimen at the peak of illness, tests can be performed retrospectively for a variety of agents simultaneously, and large-scale studies can be conducted in a timely and cost-effective manner.

7.1 Neutralization

Serum specimens may be assayed for neutralizing antibody against a given virus by testing serial dilutions of the serum against a standard dose of the virus. The antibody titer is expressed as the highest serum dilution that neutralizes the test dose of virus. As a bioassay, neutralization assays are highly specific and quite sensitive. For many viral agents, the neutralizing antibody level is directly correlated with immunity, an important clinical and epidemiologic endpoint. Disadvantages of the assay include the time required to obtain a result and the relatively high cost, due to the labor intensity and requirement for cell culture and titered viral stocks. Neutralization assays may be carried out in a variety of systems and the endpoint measured by a number of different procedures. Different neutralization systems include plaque reduction neutralization, sometimes using complement enhancement, where the number of virus plaques in control wells are compared with the number seen in cultures inoculated with the virus–serum mixture; microneutralization, an assay performed in microtiter plates requiring small amounts of sera; and colorimetric assays. Colorimetric assays rely on markers indicating metabolic inhibition of the virus in cell cultures or on antigen–antibody reactions with antibody tagged or reacted with enzyme-linked antibodies. Colorimetric assays are generally analyzed by measurement of optical density and have the advantage of being less time-consuming and costly to set up and analyze than other assays. Colorimetric assays often are more sensitive than assays relying on inhibition of CPE [60].

The type of assay used to assess antibody is very important, particularly in the evaluation of susceptibility to vaccine-preventable or epidemic viruses such as measles or rubella, where low levels of antibody may be predictive of protection. Direct comparison of various laboratory methods used for the detection of virus-specific antibody may be important in designing studies or evaluating study results. For example, analysis of CMV antibody using neutralization by plaque reduction has shown poor correlation with CMV antibody using EIA [61]. Different neutralization methods also may give differing results, as has been shown in the analysis of antibody to RSV, where microneutralization assays appear to be more indicative of biological protection than either direct or competitive ELISA methods or complement-enhanced plaque reduction [62].

In general, measurement of neutralizing antibody is the most specific method that reflects immunity, although other tests for some viruses may be surrogate markers for this. However, neutralization tests are rather expensive since a demonstration of inhibition of viral replication in cell culture, embryonated egg, or laboratory animal (such as the suckling mouse) is required.

7.2 Complement Fixation

The complement fixation (CF) test is a relatively simple technique that may be used successfully with a large variety of viral antigens. First developed in 1909 by Wasserman and coworkers [63, 64] for the detection of syphilis antibodies, CF has been adapted to test for antibodies to many bacterial and fungal pathogens of animals and man. The CF test relies on competition between two antigen–antibody systems for a fixed amount of complement, with the result ultimately demonstrated by the lysis of erythrocytes. The serum is heated at 50 °C for 30 min to inactivate any complement that may be present. Antigen and a known amount of complement are added to dilutions of serum. The complexes formed between the initial antigen and antibody bind the available free complement, thus preventing further reaction of the complement in the second step of the assay. In the second step, a

hemolytic indicator system using red blood cells (RBCs), which have been reacted with hemolysin to sensitize them to complement, is used to detect the free complement. The RBCs are reacted with hemolysin, or antibody to the RBC, and added to the assay. Lysis of the RBCs occurs if free complement is present. Thus, the presence of hemolysis at the conclusion of the assay is indicative of the absence of specific antibody, whereas the formation of clumped RBC (often referred to as a "RBC button") indicates a positive test reaction. Antibody titers can be calculated using standard endpoint determinations. Antibodies detected by CF are primarily of the IgG class and develop during the convalescent stages of illness. The greatest application of the CF test lies in the demonstration of a rise in antibody in convalescent compared with acute sera. The CF test has been widely used for the serodiagnosis of many human viral pathogens because of its broad reactivity and effectiveness in detecting changes in antibody titers and it is often the standard against which new methods are compared. The CF test has largely been replaced in many laboratories by enzyme immunoassays, but it continues to maintain its usefulness in some circumstances because reagents are relatively inexpensive and readily available and the test is rapid, reliable, and relatively easy to perform [65]. Another advantage of this assay is that many antigens can be easily tested in the same sera samples simply by changing the antigen but keeping all other reagents and conditions the same. The CF test is also adaptable to microtiter and automated methods.

Despite the widespread use of the CF assay over time and in many epidemiologic studies, the assay has some unique problems: (1) the test relies on a cascade of interactions involving multiple biological reagents that must be carefully monitored; (2) it is relatively insensitive because high concentrations of antigen are required to produce CF complexes and the assay is unable to detect small changes in antibody concentrations or low levels of antibody that may be predictive of protection in other assay systems (such as VZV or measles) [66, 67]; and (3) sera containing antibodies to host cell components or anti-complementary sera will not give a valid result. Newer laboratory methods, such as EIA methods, are now available commercially and have largely replaced CF tests for many viral pathogens because of their ability to discriminate between IgG and IgM antibody, increased sensitivity, and enhanced specificity at a similar cost.

7.3 Hemagglutination Inhibition

The hemagglutination-inhibition test (HAI) is based on the ability of some viruses to attach to receptors on certain species of erythrocytes and cause hemagglutination (Sect. 6.2). While HAI may be used to identify an unknown virus with virus-specific antisera, it also is useful for the detection of virus-specific antibodies in the serum. HAI may be used to evaluate antibody titers of influenza viruses, parainfluenza viruses, adenoviruses, rubella, arboviruses, and some strains of picornaviruses and noroviruses [68, 69]. In this assay, serial dilutions of sera are allowed to react with a defined amount (4 HA units) of viral hemagglutinin. Subsequently, agglutinable RBCs are added and the ability of the virus to agglutinate the RBCs is measured. Properly treated sera containing antibody specific to the virus will prevent agglutination of the RBCs, resulting in formation of RBC buttons in the test wells; sera lacking specific antibody will result in RBC agglutination. Nonspecific viral inhibitors can give rise to false-positive results in some systems, requiring that the sera be properly prepared prior to use. The specificity of the assay varies somewhat with the particular virus, with influenza and parainfluenza virus systems being more specific than the arbovirus system [69]. For example, the HAI test can identify specific strains of influenza viruses, whereas it identifies only the group-specific antigens of the arboviruses (with neutralization tests required for strain identification). Advantages of the HAI assay are its simplicity, the low cost for reagents and equipment, and speed of the assay. Disadvantages of this assay include the fact that the system only works with those viruses that cause hemagglutination and that nonviral-specific serum components may also inhibit hemagglutination, thereby invalidating the test results.

The immune adherence hemagglutination method is another method that has been used in the clinical laboratory. After an initial reaction between viral antigens and specific antibodies is allowed to occur, complement is added and binds to the antigen–antibody complex, if present. Human erythrocytes then are added and reaction to the antigen–antibody–complement complex with the C3b receptor causes hemagglutination. This method, commonly used as a microtiter procedure, has a well-defined endpoint and is rapid, inexpensive, and more sensitive than CF [70]. Disadvantages of this method include the inability to differentiate between different immunoglobulin subclasses and its difficulties with viruses that themselves agglutinate RBCs.

The direct agglutination of sheep or horse RBCs by serum is a diagnostic test for the heterophile antibody of infectious mononucleosis. Removal of an inhibitory (Forssman) antibody by adsorption of the sera with guinea pig kidney extracts is required before testing. The hemolysis of beef cells by sera from patients with acute infectious mononucleosis due to EBV is another diagnostic test that does not require adsorption but is less sensitive (see Chap. 37).

Red blood cells or latex particles can be coated with viral antigens and used to determine the presence or absence of viral antibodies in reactions called passive hemagglutination and passive latex agglutination, respectively. If viral antibodies are present, the antigen-coated red blood cells or latex particles are agglutinated. This assay is subject to a prozone effect, in which an excess of antibody reduces or eliminates agglutination leading to a false-negative result. To circumvent

this problem, negative samples can be diluted and the assay repeated. Commercial kits are available for the qualitative identification of antibodies to VZV and rubella virus [71].

7.4 Immunoassay Techniques

7.4.1 Enzyme Immunoassay

Enzyme-based immunoassays (EIAs), sometimes called enzyme-linked immunosorbent assays or ELISA, are widely used for many purposes, including the detection of antigen-specific antibody. This methodology has replaced other methods in many laboratories in part due to the commercial availability of test materials and complete test kits. The most commonly used immunoassays employ a four-layer approach: antigen is bound directly to a surface, the unknown serum sample is then added, an enzyme-conjugated antihuman IgG or IgM is added next, and an indicator system is used to determine the amount of reaction between the enzyme-linked antibody and the antigen–serum reaction. As discussed in Sect. 5.5, this method has gained widespread use due to its sensitivity, specificity, safety, simplicity, low cost, and ability to be automated. The system lends itself to automation because of readily available microtiter diagnostic systems and because multiple tests for different antigens may be run by varying only the initial antigen in the system. Clinical specimens from various sources besides serum or plasma, such as respiratory secretions, cerebrospinal fluid, and breast milk, also may be tested in this system. The EIA test may also be read visually and so is useful in developing countries unable to afford the photometric reader used in developed countries to accurately quantitate the antibody content.

Difficulties inherent in immunoassay techniques include those associated with obtaining and standardizing purified, sensitive, and specific IgG and IgM reagents. Although many of these reagents are commercially available, there can be variability in the specificity of the reagents so that during assay development the reagent specificity should be assessed. In general, results from different laboratories using different reagents are not directly comparable. Specific analysis for subclasses, particularly IgM, requires careful standardization and quality control to assure reliability and specificity of the assay. Attention to such detail is critically important in assays with life-threatening implications, such as the EIA assays currently used in blood banks to detect the presence of antibody to HIV or hepatitis B and C. Evaluation and standardization of tests, including commercially available kits, and comparison among different products prior to use in research and clinical settings remain an important part of EIA.

The lateral flow immunoassay described in Sect. 5.7 can be modified to detect viral antibody. Antihuman immunoglobulin or protein A labeled with conjugate is used in the reagent pad in place of viral antibody conjugates, and the labeled conjugate can interact with virus-specific antibodies in the clinical sample. Recombinant viral antigen is used to capture the virus-specific antibody–antibody–conjugate complex in a "test" line, and a "control" line containing antihuman immunoglobulin is present to indicate that the assay performed as intended. The test format provides a rapid qualitative answer with sensitivity and specificity similar to that obtained by more complex laboratory-based enzyme immunoassays [71, 72]. These assays can be applied to serum as well as to saliva, and they have been developed for several different viruses including HSV, HIV, hepatitis C virus, and chikungunya virus [72–75].

Epitope-blocking assays using monoclonal antibodies have been used to examine serological responses to a number of viruses [76–81]. These assays measure the ability of a test serum to block the binding of a monoclonal antibody to a virus antigen. They have been particularly useful in determining serotype-specific immune responses to multivalent vaccines and in determining which of a number of cross-reactive virus strains is responsible for a natural infection in a given host.

7.4.2 Other Immunoassays

Other variations of the immunoassay include isoelectric focusing and affinity immunoblotting. These techniques are useful because of enhanced sensitivity, particularly for diagnosis in the congenitally infected infant or for the differentiation of passively acquired antibody from endogenous antibody. Isoelectric focusing relies on the separation of serum antibody in thin-layer gels, with subsequent antibody detection by a reaction with antigen-coated membranes [82]. Clonal-specific antibodies can be detected by this method, which may discriminate, for example, between unique maternal and fetal antibodies.

The TR-FIA assay can be modified for detection of virus-specific antibodies by coating the well of the microtiter plate with viral antigen and labeling antihuman immunoglobulin with the lanthanide reporter. The availability of lanthanides with distinct emission spectra allows multiplexing of the assay such that different antibody isotypes (e.g., IgG and IgA) can be measured in the same well [83, 84].

Radioimmunoassay techniques for the sensitive detection of antibody to viral antigens, pioneered in the 1970s for the serodiagnosis of hepatitis B, are used by research laboratories but otherwise are not widely utilized [5]. Immunoassays that do not require radioisotopes and require less technical expertise, such as the EIA and IF tests, are more commonly available.

7.4.3 Immunohistochemical

The most commonly used immunohistochemical technique to detect antibody is the immunofluorescence technique (IF). Whereas direct techniques are used commonly to detect

antigen in infected tissue or cells, indirect immunohistochemical techniques are used to detect antibody in sera or other bodily fluids. Variations on the indirect IF test, such as amplification immunoassay systems utilizing various sandwich techniques (double indirect IF or anticomplement IF) or chemical amplification systems utilizing biotin–avidin complexes, are used in research settings [65]. Indirect IF methods are available in many laboratories for a variety of assays, although more automated techniques such as EIA are replacing IF techniques in many clinical settings.

In the indirect IF test, tissue or cells containing viral antigen are fixed on a glass slide, a serum dilution is added, and a fluorochrome-conjugated antibody indicator system is used to detect the resulting reaction. The conjugated detector antibody can be varied to specifically measure the presence of antibody classes, such as IgG, IgM, and IgA. IF techniques provide sensitive methods to detect antibody that can be related to immunity to viruses, such as VZV, to detect congenital infections in newborns based on IgM-specific antibody, and to detect epitope-specific antibody [65, 85]. In particular, the anticomplement-amplified indirect IF technique, utilizing a four-layer reaction including antigen, patient serum, complement, and fluorochrome-conjugated anti-C3 antibody, has been useful for the detection of the nuclear antigen of EBV, or EBNA [65]. The inclusion of standard positive and negative sera is needed in each test and independent reading by two observers is recommended to minimize errors in interpretations.

Advantages of indirect immunohistochemical methods include (1) the ability to use the system to detect antibody to many diverse viruses by varying only the initial step in the reaction, (2) the higher sensitivity and specificity compared with CF, (3) the simplicity and relative speed of an individual test, and (4) the reproducibility of the test by experienced personnel. Disadvantages of the test include the technical complexity, the lack of automation, the requirement for specialized cells and reagents, and the need for special equipment, such as a fluorescence microscope and darkroom.

7.4.4 Immunoblot

An immunoblot, also referred as a Western blot, is another widely used method for the detection of antibody to specific viral antigens. This technique relies on the incubation of patient serum with partially purified whole virus or recombinant viral proteins that have been separated by electrophoresis in a polyacrylamide gel and transferred onto nitrocellulose paper [86]. The assay is based on the same principal as EIA but has the advantage of identifying antibodies specific for several antigens of the same virus simultaneously. Quantitation of the specific reactions can be determined by a densitometer. Difficulties with immunoblots include the expense and time required for the test, the technical requirements for performing and interpreting the test, and the problems encountered with preparing reagents such as purified virus and labeled antihuman IgG. The time interval from virus acquisition and seroconversion by immunoblot may vary for different patients, as well as different viruses, indicating that diagnosis of infection by immunoblot analysis, while extremely sensitive, is not always definitive [87]. Furthermore, analysis by immunoblot will not differentiate maternal from fetal HIV infection in many cases. Despite these problems, the immunoblot assay is widely used today in laboratories around the world as the "definitive" confirmatory test for HIV, and it is also used as a confirmatory test in some circumstances for the serological diagnosis of hepatitis C virus infection [88]. It also has been used to differentiate type-specific serological responses to herpes simplex virus type 1 (HSV-1) and HSV-2 [89].

8 Interpretation of Laboratory Tests

Virus infection is usually identified by either detection of the virus or identification of a serological response to the virus. For some viral infections, both are required. However, the interpretation of the viral diagnostic assays must be made in the context of the assay sensitivity and specificity along with seasonal, clinical, and other epidemiologic factors.

Difficulties arise in interpretation of serological tests when only a single convalescent serum sample is obtained and a high antibody titer is found or if high titers are present in both acute and convalescent sera without a fourfold rise. These results can reflect either current infection or persistently high antibody levels from a previous infection. Significance may be attached to these findings if the disease is a rare one in which the presence of antibody is unique, if the test reflects a short-lasting antibody, or if specific immunoglobulin M (IgM) antibody can be demonstrated. A rapid drop in antibody titer in a subsequent specimen is also suggestive of a recent infection. Sequential testing of other family members also may be useful, since they may be in different stages of apparent or inapparent infection with the same virus. In an epidemic setting, comparison of the geometric mean antibody titer of sera collected early in illness from one group of patients with that from another group of patients convalescing from the same illness may permit rapid identification of the outbreak.

At times, a virus may be identified or an antibody rise demonstrated that is not, in fact, causally related to the illness. Sometimes dual infections with two viruses, or with a virus and a bacterium, occur, and the interpretation of the role of an individual viral pathogen in the disease process may be very difficult. On other occasions, no virus is isolated or the serological rise is not sufficient to demonstrate whether a specific virus is the real cause of the illness. A list of common causes for false-positive and false-negative results is given in Table 3.4.

Table 3.4 Viral diagnosis: some causes of false-positive and false-negative tests

False positive
Antigen detection
1. Persistent or reactivated virus from prior and unrelated infection
2. Two microbial agents are present, and the one isolated is not the cause of the disease
3. Mislabeled specimens
4. Cross-contamination within the laboratory
Serological rise
1. Cross-reacting antigens
2. Nonspecific inhibitors
3. Double infection with only one agent producing the illness
4. Rise to vaccination rather than natural infection
False negative
Antigen detection
1. Viral specimen taken too late or too early in illness
2. Wrong site of multiplication sampled, e.g., throat rather than rectal swab
3. Improper transport or storage of specimen
4. Low assay sensitivity
Serological rise
1. Specimens not taken at proper time, i.e., too late in illness or too close together to show antibody rise
2. Poor antibody response–low antigenicity of the virus or removal of antibody by immune-complex formation
3. Wrong virus or wrong virus strain used in the test
4. Nonspecific inhibitor obscures true antibody rise
5. Wrong test used for the timing of the serum specimens

9 Unresolved Problems

Many problems still must be resolved in the area of viral diagnosis. The development of newer molecular assays with improved sensitivity have supplanted many antigen detection methods with lower sensitivity, but the ease of performance of the antigen detection methods still makes these assays attractive to many laboratories. While molecular methodologies offer the potential for great sensitivity and specificity, they often require specialized equipment for their performance. Ultimately, the ideal diagnostic test will be rapid, easy to perform, inexpensive, sensitive, and specific and will not require the use of specialized equipment. However, even if such a test is developed, the significance of the identified virus to the observed disease process will remain in the hands of the epidemiologist, virologist, and clinician.

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Surveillance and Seroepidemiology

4

Ruth Jiles, Monina Klevens, and Elizabeth Hughes

1 Introduction

The term *surveillance* was employed for years in the restrictive sense to imply follow-up of exposed persons to determine whether disease developed within the limits of the incubation period. The dictionary definition of surveillance is "close observation, especially over a spy or criminal" [67]. Surveillance, in the context of health, has been defined as the systematic collection of data pertaining to the occurrence of specific diseases, the analysis and interpretation of these data, and the dissemination of consolidated and processed information to contributors, programs, and other interested persons. The Centers for Disease Control and Prevention (CDC) has described surveillance as the collection of "health related data essential to the planning, implementation, and evaluation of public health practice, closely integrated with the timely dissemination of these data to those responsible for preventing and controlling disease and injury" [89].

Surveillance systems are developed and implemented for the ultimate purpose of preventing or controlling diseases. Historically, the principles of surveillance were well described and documented by Langmuir [57] and other officials of the US Public Health Service, CDC [6, 66], and by Raška [76, 77] for the World Health Organization (WHO) [108].

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The techniques of surveillance and uses of surveillance data were applied first to infectious diseases and subsequently to occupational, environmental, and chronic diseases, as well as to injuries and emergency preparedness [6, 14]. *Healthy People*, the principal document of the US Department of Health and Human Services that outlines the health objectives for the United States, relies heavily on surveillance data to establish goals, baseline measures, and monitor progress towards achieving those goals [94]. The importance of surveillance and the need for improving surveillance techniques, both in developed and developing countries, have been well documented [68]. To improve data quality and timeliness of reporting, electronic transmission of surveillance data, mainly laboratory results, was initially implemented in the United States and France [39, 95]. Early challenges included standardization of case definitions and reporting methods among official sources. These issues were addressed in the US National Notifiable Disease Surveillance System through consensus and development of standard case definitions and reporting protocols.

This chapter discusses the background and elements of traditional surveillance, the concept and uses of serological and molecular epidemiology, and their application to the control of viral infections.

2 Surveillance

Traditionally, surveillance was based on the occurrence and reporting of a case of clinical disease or death. Currently, other life and infection-related events, such as births, hospitalization, risk behaviors and exposures, treatment, and healthcare system encounters, are also under surveillance.

2.1 Historical Background

A more detailed history of public health surveillance was published by Declich and Carter in 1994 [24] and summarized

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in an earlier version of this chapter [33]. Based on these accounts, various governmental actions to prevent spread of infections and diseases occurred during the seventeenth century. However, it is widely accepted that public health surveillance dates back to 1662, when John Graunt published the *Natural and Political Observations Made Upon the Bills of Mortality*. Graunt analyzed London's mortality data collected for the Bills of Mortality and was the first to count the number of deaths from specific causes (numerator), estimate the population (denominator), and use the information to determine death rates [41].

During the eighteenth century, some colonies in Rhode Island implemented various specific components of public health surveillance, requiring tavern keepers to report cases of "contagious" diseases such as smallpox, yellow fever, and cholera among their customers.

During the nineteenth century, several significant contributions were made in public health surveillance. Sir Edwin Chadwick, Secretary of the Poor Law Commission in England, used surveillance data to demonstrate that poverty and disease were associated and suggested improvement in the living conditions of the poor. In the United States, Lemuel Shattuck related living conditions to infant and maternal morbidity, mortality, and rates of death. He recommended collection of health data by such factors as age, gender, and occupation. William Farr, founder of a modern concept of surveillance, as the first Compiler of Abstracts for England and Wales, set up a system that not only tracked births and deaths, but also routinely recorded cause of death by occupation. Farr developed a nosology that is the basis for the International Classification of Diseases (ICD) (See http://www.cdc.gov/nchs/data/misc/classification_diseases2011.pdf).

Surveillance concepts and methodologies were developed in response to national needs for disease surveillance or in response to epidemics. Surveillance data were needed to define the magnitude of the problem and to inform policy/decision makers and others who were responsible for development, implementation, and/or evaluation of prevention and intervention programs and policies. Use of the term "surveillance" in the United States began in 1949 with the development of a modified program at the CDC called "Surveillance and Appraisal of Malaria." In 1951, the concept was applied to the residual smallpox cases in the United States.

On April 28, 1955, the Surgeon General of the United States directed the establishment of a "National Poliomyelitis Surveillance Program" in response to paralytic polio cases following the use of Salk vaccine (the "Cutter Incident"). Based on this program, established at the CDC, surveillance methods became an effective tool in following trends in the disease, in measuring the effectiveness of polio immunization programs, and in detecting suspected vaccine-associated cases. Likewise, other surveillance systems and units were developed in response to occurrence of other epidemics. WHO established regional, national, and international control programs with surveillance as an essential component of each type of control program. While European unification was anticipated, individual countries employed different approaches to surveillance; thus, there were concerns about comparability of data and coordination of activities [27]. The AIDS epidemic which began in the late 1970s and early 1980s forced many countries to establish new surveillance systems and improve existing ones.

While a set of legal and ethical principles of surveillance had been evolving gradually prior to the early 1980s, the pressing need for accurate information about HIV infection led to a broad array of new surveillance practices that have continued to test the balance between individual and community interests and responsibilities.

2.2 Types of Surveillance

Surveillance systems may be classified in a variety of ways: based on the method of reporting (passive or active), purpose of the system (Behavioral Risk Factor Surveillance System or BRFSS) [11], location (clinic based), or target population (Youth Risk Behavior Survey YRBS) [21].

The most common type of surveillance is passive. In passive surveillance the reporter, usually a physician or laboratory, regularly transmits a summary of all the cases of the specified disease or diseases observed to health authorities during the reporting period.

The US National Notifiable Disease Surveillance System (NNDSS) is an example of passive reporting [17]. States send case reports of nationally notifiable diseases to the CDC on a weekly basis. States determine which diseases are reportable within their jurisdiction and who (laboratory, physician, clinic, or some combination) initiates the report. State epidemiologists and CDC subject matter experts collaborate, through the Council of State and Territorial Epidemiologists (CSTE), to develop case definitions and define reportable data elements for reportable diseases. Generally, laboratories and/or physicians report cases to the local health departments, local health departments report cases to state health departments, and state health departments notify CDC of confirmed cases. Most laboratory reports and some physician reports are submitted electronically. However, reporting can be accomplished in print, by telephone, even using toll-free numbers or automatic recording devices available at all hours. Time and lack of resources greatly limit such a system to a small percentage of most reportable diseases, but as long as the system and requirements remain unchanged, the changes in incidence may reflect meaningful patterns of disease.

Active surveillance requires contacting the reporters at regular intervals and request for specific data on cases of

specified diseases. It permits more extensive data collection on epidemiologic features of certain diseases. CDC's Behavioral Risk Factor Surveillance System (BRFSS) is an active surveillance system [11]. States contact residents by telephone and administer a standardized questionnaire to eligible consenting adults aged 18 years or older to obtain information about a variety of chronic diseases and related risk behaviors. Relevant to viral infections, the BRFSS includes questions about receipt of influenza, shingles, and human papilloma virus vaccination. The BRFSS has also been used by CDC to provide state-specific estimates of HIV-related behaviors such as ever tested for HIV, where tested, and why tested. Some states conduct active surveillance for HIV/AIDS using other data sources.

For a few diseases, some special clinical feature of the disease may be used to estimate prevalence data. For example sudden severe acute lower respiratory illness may indicate hantavirus infection or jaundice may be evidence of hepatitis. In some instances, surveillance is based on laboratory data, as with the isolation of the agent or results of a serological test. In the case of viral hepatitis, the CDC/CSTE case definition includes both clinical features and laboratory test results. Most surveillance systems cover political jurisdictions (e.g., national, state, and county). However, some surveillance systems cover high-risk or specific population groups, requiring special reporting methods. Examples are military populations, hospitals, day-care centers, and homes for the elderly. Most surveillance systems incorporate a list of reportable diseases, but the focus of the reporting may be on specific disease entities, such as congenital defects as a reflection of the impact of rubella and cytomegalovirus infections [28].

An example of surveillance in a special population and location is CDC's school-based Youth Risk Behavior Survey (YRBS) [21]. In this survey, questions related to viral diseases, for example, HIV, are self-reported by participating high school students.

2.3 Sources of Surveillance Data

A wide variety of data sources are used for surveillance purposes. Some data sources were designed for the purpose of surveillance and are ipso facto "surveillance systems." Other data sources are used secondarily for surveillance. Sources of surveillance data vary from country to country, state to state, and across local jurisdictions. Availability of surveillance data is dependent on resources available to support the system, such as appropriate laboratory facilities and trained personnel. Table 4.1 lists some data sources that are commonly used for surveillance purposes. These sources are consistent with elements of surveillance summarized by WHO [108].

Table 4.1 Selected sources of data for surveillance

1. Viral statistics
2. Reportable disease systems
3. Surveys
4. Sentinel surveillance
5. Syndromic surveillance
6. Registries
7. Laboratory records
8. Pharmacy records
9. Administrative data

2.3.1 Vital Statistics

The oldest form of surveillance is mortality registration. In most countries, death registration is legally required. As a result, almost all deaths are included in the registries. Causes of death listed on the death certificate is dependent on the presence/absence of a physician or family member who is knowledgeable about the health of the deceased; severity of disease, complexity of the disease, associated illnesses, and whether or not an autopsy or diagnostic laboratory testing was performed. For many viral diseases, such as hantavirus, rotavirus, and influenza, only a small percentage of cases are fatal. However, the case fatality rate is high for other viral diseases, such as AIDS, rabies, Lassa fever, and certain hemorrhagic fevers. Thus, occurrence of viral infections is likely underestimated from the death certificate data. However, vital records are important to document severe complications of viral infections. In addition, surveillance from vital records can be useful for comparing viruses. For example, by 2007 in the United States, the number of deaths associated with HIV was lower than the number associated with hepatitis C [59].

Birth certificates are often used to monitor conditions such as congenital defects (due to rubella and cytomegalovirus infection), diseases transmitted from mother to child (e.g., hepatitis B virus infections), and other conditions of newborns that may impact immediate or long-term health status.

2.3.2 Reportable Disease Systems

The reporting of cases of specified infectious diseases also is legally required in most countries. The US National Notifiable Diseases Surveillance System includes over 70 diseases, of which approximately 30 are viral diseases including 6 arboviruses, 6 hepatitides, and 6 types of hemorrhagic fevers. Reportable disease systems form the backbone of surveillance for most state and local health departments and for many CDC programs. The advantages are that (1) reports are usually made by physicians and/or laboratories, (2) laboratory confirmation is generally available, and (3) there is typically an organized system of regional or national tabulation and reporting. The disadvantages are as follows: (1) the absence of some viral diseases from the required list; (2) the notorious underreporting of diseases despite legal

requirements, primarily because of lack of resources to support both reporting and education of physicians about the need to report; and (3) the variability of reporting efficiency from one jurisdiction to another. Lack of rapid, reliable, inexpensive diagnostic techniques also represents a discouraging obstacle to accurate identification and reporting of many viral diseases [17].

2.3.3 Surveys

Many types of surveys of infectious disease have been used in public health. Formal surveys support the collection of standardized information using standardized methods. Although survey data can be collected quickly over a wide geographic area, the cost of data collection may be prohibitive. The National Health and Nutrition Examination Survey (NHANES), conducted by the National Center for Health Statistics, a component of CDC, uses a complex sampling design to collect nationally representative data on the health and nutritional status of the US noninstitutionalized civilian population [16]. NHANES is a source of data for a number of infectious disease programs, mainly because the laboratory and physical examination components allow both confirmation of case status and collection of health information. For example, the Division of Viral Hepatitis at CDC uses NHANES data to estimate the prevalence of chronic hepatitis B and C for the US noninstitutionalized adult populations (see Sect. 3.3.2).

2.3.4 Sentinel Surveillance

Sentinel surveillance is used as a less costly alternative to population-based surveillance. Sentinel surveillance systems collect data from a sample of reporting sites. Generally a select group of reporting sources—hospitals, healthcare providers, agencies—are recruited to report all cases of one or more notifiable conditions. In the United States, sentinel sites report all cases of influenza-like illness to their state health department on a weekly basis. A network of sentinel providers in British Columbia, Canada, demonstrated the usefulness of sentinel surveillance in documenting the effectiveness of influenza vaccine [49].

2.3.5 Syndromic Surveillance

Syndromic surveillance uses clinical information about signs and symptoms of disease, before a diagnosis is made, as an early warning signal of a potential outbreak. Many syndromic surveillance systems use electronic data from hospital emergency room visits. The value of such systems relies on accurate assessment and coding of symptoms, as well as accurate data entry.

2.3.6 Registries

Registries are often established, usually at the state level, to collect information about persons diagnosed with a disease or condition. For example, cancer registries collect information about type of cancer, anatomic location, stage of disease at diagnosis, treatment, and outcomes. Childhood immunization registries maintain a record of vaccinations received by children within the jurisdiction of the registry [19].

2.3.7 Laboratory Records/Investigations

Laboratory identification of the causative agent of many viral infections has become a routine part of clinical care. Laboratory records are especially useful for public health surveillance. Appropriate laboratory facilities and experienced personnel are needed for the isolation and/or serological identification of the majority of viral infections. These may exist in national or regional public health laboratories, in specialized virus diagnostic institutes, or in university settings.

2.3.8 Pharmacy Records/Investigations

Pharmacy data may be used to identify those who are treated for specific diseases, monitor uptake of new medications, and evaluate effectiveness of specific treatments. For example, when treatment for HIV/AIDS first became available, prescriptions for zidovudine were used to identify potentially unreported cases [54].

2.3.9 Administrative Data

These data consist of electronic records prepared usually for billing or other administrative accounting and are sometimes available in a de-identified format and at no cost for public health use. Data from health plans such as Kaiser Permanente, Medicare, and Medicaid have proven to be useful to supplement routine surveillance data. Hospital discharge data, like insurance/health plan data, provide useful information on diagnosis, surgical procedures, other billed treatments, complications, length of stay, laboratory data, and associated costs. In addition to the wealth of data available in these sources, another attraction is that these data are already collected in electronic form, requiring fewer resources to analyze and summarize.

2.4 Surveillance Networks and Health Information Exchanges

2.4.1 Surveillance Networks

Surveillance networks grew out of the need for a more global approach to surveillance of infectious diseases. Surveillance of infectious diseases was often inadequate in the developing world due to a lack of resources and public health infrastructure. The need for early warning of outbreaks of emerging and reemerging diseases led the Federation of American Scientists to support the establishment of the first infectious disease network, the Program for

Monitoring Emerging Diseases (ProMED-mail) in 1993 [74]. ProMED-mail is an Internet-based system for reporting and disseminating information on infectious diseases outbreaks and acute exposures to toxins that impact human health. This open-source network receives reports from clinicians, public health officials and epidemiologists, laboratory scientists, medical missionaries, journalists, and laypersons. The editors also search the Web and press reports for information. A panel of experts screens, reviews, and investigates reports before they are posted to the website. ProMED-mail allows comparison of reports by geographic location. Thus, users of the system can identify similar outbreaks in both space and time.

Other surveillance networks include Canada's Global Public Health Intelligence Network (GPHIN) [75] and the World Health Organization's Global Outbreak Alert and Response Network (GOARN) [103, 104].

GPHIN, started in 1999, monitors internet websites, news wires, and other internet media to gather and provide information on disease outbreaks and other public health events. This network collects information about infectious diseases, outbreaks, contaminated food and water, natural disasters, bioterrorism events, and safety of products, drugs, and medical devices. GPHIN is part of GOARN [75].

GOARN, started in 2000 by WHO, links existing networks of government and academic centers of excellence, networks of laboratories, medical centers, scientific institutions, and other international organizations. The goal of GOARN is to provide countries with resources and expertise necessary to respond to infectious disease outbreaks. GOARN provides and coordinates technical support, investigates the event, assesses risk, streamlines processes to rapidly deploy field teams, and supports national preparedness [103, 104].

CDC surveillance systems may be viewed as networks connecting the Federal agency with local, state, and territorial health departments and international partners. A number of these surveillance networks focus on viral diseases. An example is the Influenza Surveillance Program [18]. The program which started in 1972 collaborates with local, state, and territorial health departments, clinical laboratories, healthcare providers, and emergency departments to collects and analyzes information on influenza in the United States. Information is collected from five categories of influenza surveillance: viral surveillance, including surveillance for novel influenza A viruses; outpatient illness surveillance; mortality surveillance; hospitalization surveillance; and geographic distribution. Information from these five categories of surveillance is used to track influenza-related illnesses and deaths and to provide a comprehensive overview of activities. CDC also collaborates closely with WHO in global surveillance, including surveillance for novel influenza viruses and in influenza vaccine strain selection.

WHO's Global Influenza Program provides technical support and guidance to Member States and maintains global surveillance for influenza. Virologic and epidemiologic data are collected from countries, areas, and territories through the influenza surveillance and monitoring system. Two platforms are provided for data collection and sharing: FluNet for virologic data and FluID for epidemiologic. These systems allow tracking of global trends, spread, and impact of influenza [106].

2.4.2 Health Information Exchanges

During the late 1900s and early 2000s, there were concerns about the ability of the United States to respond to possible acts of bioterrorism. Beginning with the anthrax attacks shortly after the September 11, 2001 destruction of the World Trade Center, epidemics of such emerging viral infections as West Nile virus, avian influenza, and SARS became a major issue in protecting the health of all Americans [58]. In response, the President of the United States signed Executive Order 12225 on April 27, 2004, which created the Office of the National Coordinator of Health Information Technology (ONCHIT) [58]. The primary objective of this order was to formalize the administration of the office and to advance the development and growth of health information technology as vital to improving the quality of healthcare while reducing its cost. The first step in the process to achieve this critical objective was to use medical records maintained by healthcare providers to build a national network of electronic health records on the majority of Americans, by the year 2014 [13].

Health information exchange (HIE) can be described as the "electronic movement of health-related information among organizations according to nationally recognized standards" developed by the Health Resources and Services Administration (HRSA) [92]. HIE encompasses two major concepts: the electronic sharing of health-related information among organizations and an organization that provides services to enable the electronic sharing of health-related information [15, 46]. The primary goal of HIE is to facilitate access to and retrieval of patient clinical data to provide more efficient, timely, effective, equitable, and safe healthcare. HIE secondary goals are to improve bidirectional communication, to enhance case reporting, and to focus on technology, interoperability, utilization standards, and harmonious collaboration between all patient-centered healthcare providers [13, 97]. The potential of HIEs to integrate the electronic transfer of vital health information among providers and public health agencies is critical in the effort to improve healthcare quality and increase safety for patients [46].

In 2007, CDC funded Health Information Exchanges to support situational awareness project [12]. The objectives of this project were to connect public health providers and organizations

with HIEs in order to improve public health with real-time awareness of the health and status of healthcare facilities within communities, bidirectional communication between healthcare entities, and enhanced case reporting [93]. An example of a successful merge of a HIE with a state partner is the Indiana Health Information Exchange (IHIE) [40, 48]. The IHIE displayed the interoperability of health information exchange by incorporating clinical messaging service ($DOCS_4DOCS$®) to improve communication between healthcare providers and public health agencies. With this system, clinical result messages can be forwarded to all physicians or targeted to clinical practices or specific geographical areas [90]. In 2009 and 2010, the $DOCS_4DOCS$® public health messaging system was utilized to alert nearly all physician practices and public health agencies about H1N1 influenza, a syphilis outbreak, an update on rabies treatment, and new vaccination requirements for school children [48].

Another example of a successful HIE is the Northwest Public Health Information Exchange (NW-PHIE) in Washington and Idaho states [90]. This HIE evaluated new influenza surveillance efforts and compared these with existing influenza surveillance data feeds through the Influenza-Like-Illness Network (ILINET). Results indicated that the NW-PHIE data were timelier, more stable, more extensive, and more broadly representative of the community. The NW-PHIE data accurately reflected trends in ILINET activity at the state and community levels [13].

HIEs benefit public health by improving the safety of patients and ensuring quality of healthcare. HIEs augment patient safety by serving as the connecting point for a standardized, organized process of data exchange across regional, state, and local jurisdictions; reducing duplication of services which may result in lower healthcare costs; reducing operating costs by automating many day-to-day organizational tasks; providing management of the data exchange process; and, most importantly, improving communication between providers and patients [46, 93].

3 Seroepidemiology

3.1 Introduction

Seroepidemiology is the systematic collection and testing of blood samples from a target population, or a sample of a population, to identify current and past experiences with infectious diseases by means of biological markers (i.e., antibody, antigen, or other tests). Findings from the tests are the outcomes that, when analyzed, are used to describe patterns and potentially identify factors associated with the outcomes. Seroepidemiologic studies are used worldwide to measure and characterize infectious diseases in the population. Sources of seroepidemiologic data are widely available and may be collected for other purposes (e.g., blood donor screening), but can be useful for public health purposes. In the context of viral infections, seroepidemiology serves at least three objectives:

1. To supplement surveillance data and inform immunization and public health planning programs
2. To generate hypotheses about the potential association between risk and occurrence of viral infectious diseases
3. To assess old and newly recognized viruses in different population groups

Just as epidemiology is concerned with the occurrence and distribution of clinical cases in different populations, serological epidemiology is concerned with the occurrence and distribution of various components of the blood that indicate past or current infection, that are biochemical markers for certain chronic infections, or that reveal the genetic attributes of strains in various population groups. The epidemiologic characteristics are detected in the laboratory rather than at the bedside; thus, laboratory support is fundamentally necessary to conduct seroepidemiologic surveys. Fortunately, laboratory testing is a part of routine clinical care and is readily available in most countries.

Largely based on the availability of resources to monitor any given viral infection, we might consider that seroepidemiologic data can be classified as:

1. The primary objective of the survey (i.e., a planned and designed survey or study)
2. A secondary use of specimens or data collected for other purposes (i.e., screening programs including blood donors, clinics for high-risk individuals, and other remnant sera)

As a primary objective, serological surveys are frequently integrated with other public health efforts. This section considers the history, methods, and uses of seroepidemiology as either a primary or secondary objective of data collection. As in the surveillance section, we draw heavily on the experience in the United States, but recognize excellent seroepidemiologic studies conducted in other countries.

3.2 Historical Background

The introduction of serological tests for the diagnosis of disease provided the basis for later serological surveys. As early as 1916, the Wassermann test was applied routinely to patients attending a prenatal clinic at Johns Hopkins Hospital by Williams [102] but this was more of a case-finding procedure than an attempt to delineate disease patterns. In 1930, the development of a neutralization test for poliomyelitis led Aycock and Kramer [4] to use the procedure to define the immunity pattern of a given population; this is a landmark in the history of serum surveys. In 1932, Soper et al. [85] mapped out the occurrence of yellow fever in Brazil by

antibody surveys under the auspices of the Rockefeller Foundation, and this technique has been widely used subsequently in studying arbovirus infections. Antibody surveys for influenza also date back to the mid-1930s. The discovery of swine influenza virus by Shope [83] in 1931 and of human influenza virus by Smith et al. [84] in 1933 was rapidly followed by population studies to measure antibody to these viruses among persons of different age groups [2, 9, 37].

The Yale Poliomyelitis Study Unit under Dr. John R. Paul employed serological survey techniques as early as 1935, and his analysis with Riordan of the poliomyelitis pattern in Alaskan Eskimos is a classic study [71]. He became one of the foremost users and promoters of the concept of serological epidemiology, and through his work and writing [69, 72], the utilization of this technique in public health practice and research studies has become a reality. The World Health Organization also took note of this development in 1960 and established three WHO Serum Reference Banks to practice and promote seroepidemiology in New Haven, Connecticut; Prague, Czechoslovakia; and Johannesburg, South Africa. An additional bank was established in 1971 in Tokyo, Japan. The activities and principles of these banks have been reviewed in two *WHO Technical Reports* [105, 107], in a book [72], and in several other publications [70, 73, 81]. Although WHO no longer formally supports these banks, the rationale for proper collection, cataloging, and storage of specimens for use by both primary and collaborating investigators has gained wide application in public health and academic research institutions. For example, WHO collected sera from household surveys in rural areas for the evaluation of the effectiveness of penicillin in mass eradication programs for yaws [42].

Seroepidemiologic techniques were critical to the discovery by Blumberg et al. [7] in 1965 of a particular antigen in the serum of an Australian aborigine; it was uncovered in the course of genetic studies of β-lipoprotein. Since the agent from which the antigen was derived could not be isolated or cultivated in the laboratory, serological surveys using immunodiffusion tests were carried out to detect its presence in the sera of different population groups and different disease entities. The results provided the sole initial evidence that this "Australia antigen" was associated causally with hepatitis B or "long-incubation hepatitis" [8]. Herpes viruses, HHV-6 and HHV-7, were not immediately recognized in association with previously defined disease entities, although the former has been unequivocally shown to be the major, if not the only, causal agent of exanthema subitum.

Many countries recognize the value of such biological resources and have created sizable repositories of serum and, increasingly, tissue or other cellular material containing nucleic acid suitable for molecular and genetic analysis. Seroprevalence studies of many different infections and from many countries have been published.

3.3 Methodology

3.3.1 Ethical Issues

Seroepidemiologic studies were used early in the HIV epidemic because there was a need to determine the unbiased frequency of infection in different populations. A series of serosurveys were conducted to cover different populations including persons attending STD clinics [100], childbearing women [43], and youth training programs [23]. To protect the identity of infected persons, these surveys were conducted anonymously and data were unlinked, such that notifying the person whose blood was tested was not feasible. The ethical issues discussed over time are described by Fairchild and Bayer [36]. The issues included participant consent, the participant's right to know and to control their information, and the responsibility of the health officials to inform the individuals of their test results. When treatment became available, serosurveys continued as long as voluntary counseling and testing was available to survey participants. As clinical data became a more complete source of similar surveillance information, the use of serosurveys became ethically indefensible [36].

Currently, in the United States, Federal Regulations (45 CFR 46.111) require that studies involving human research subjects satisfy the following rules: (a) risks to participants are minimized; (b) risks to participants are reasonable relative to anticipated benefits, if any, to participants, and the importance of the knowledge that may reasonably be expected to result; (c) selection of participants is equitable (i.e., could any special problems arise when research involves vulnerable populations, such as children, pregnant women, fetuses, prisoners, mentally disabled persons, economically or educationally disadvantaged persons); (d) informed consent will be sought from each prospective participant or the participant's legally authorized representative; (e) informed consent will be appropriately documented; (f) the research plan makes adequate provisions for ensuring the safety of participants; and (g) there are adequate provisions to protect the privacy of participants and to maintain the confidentiality of data. Institutions conducting epidemiologic studies maintain "Institutional Review Boards" to ensure that investigators address the above ethical requirements.

3.3.2 Statistical Considerations

When serosurveys are designed as primary data collection, representativeness of the population is usually desirable. In the United States, NHANES combines interviews, physical examinations, and laboratory specimens from a nationally representative sample of about 5,000 persons each year. These persons are located in different geographic areas called primary sampling units, of which 15 are visited each year. Health interviews are conducted in respondents' homes, whereas health measurements are collected in specially designed and equipped mobile centers.

NHANES uses a complex sample survey design [16]. Primary sampling units are generally single counties, where the sampling frame is all counties in the United States. The additional stages of selection in the probability design consist of clusters of households, where each person in a selected household is screened for demographic characteristics, and one or more persons per household are selected for the sample. As with any complex probability sample, the sample design information must be used when undertaking statistical analysis of the NHANES data. In particular, sample weights and the first stage of the cluster design need to be considered. Participants are compensated for participation and receive a report with their health results.

For rapid surveys or for surveys in developing countries, WHO has developed methods for cluster sampling that are more easily implemented and analyzed than multistage probability sample surveys [5]. Using this method, and assuming the survey is conducted to meet several health information needs, the first step is to plan carefully to ensure best use of resources. Sampling is conducted in several stages, starting with regions of a country, then districts within regions, then communities (e.g., villages, blocks, etc.) within the districts and households within the communities. Ideally, the community sampling framework should be a list of all the communities in the region, and there should be a measure of the population size of the community in order to sample with probability proportionate to size. This assessment is helpful because weighting at the time of analysis then becomes unnecessary. The total population divided by the number of communities to be selected determines the sampling interval, which will be used to select the communities after a random start. Also ideally, there is enumeration of the households within the selected communities such that a simple random sample can be selected. If that framework is not available, methods from the 30×7 Expanded Program on Immunization can be used since the selection of households is conducted in the field using a central start; interviews are conducted until information on seven children aged 12–23 months are completed [22, 45]. A slight modification to the above rapid method is "compact segment sampling," proposed by Milligan et al. [62]. In this method, the communities to be sampled are divided into segments of equal population size; then, one segment is randomly selected and all households in the segment are included in the sample.

An important analysis issue for sample surveys of any methodology is whether data have been collected consistently over time. When the same items have been collected using consistent methods, comparisons can be made over time, adjusting for the distribution of the age of the population.

3.3.3 Sources of Biological Material

When serostudies are designed for primary data collection, the collection of blood specimens can be targeted to a carefully and statistically selected sample of the population of interest. To achieve efficiencies from this type of study, serological surveys should be multipurpose in nature and can include measurement of antibodies, genetic material, and other clinical laboratory markers of health or illness. In the United States, the NHANES has produced a nationwide population-based health profile for more than two decades. As one of its many components, it has provided valuable seroprevalence data on a variety of viral infections, for example, measles, poliomyelitis, herpes simplex virus (HSV), hepatitis A virus (HAV) [53], and HBV [98].

Table 4.2 Sources of human biological materials for surveillance where planned serum surveys from targeted populations are the primary objective

Objective	Sources of biologic materials
Primary	Planned serum surveys from target populations
Secondary	Entrance and periodic examinations of different groups (military, industry, health clinics)
	Blood donors in Red Cross and similar programs (e.g., transplantation donors)
	Public health laboratories:
	Serological tests for HIV (e.g., premarital)
	Other immunologic and diagnostic tests
	Healthcare facilities:
	Entry tests for blood chemistries or syphilis
	Diagnostic tests for infectious diseases
	Blood banks and transplantation programs
	Prenatal; clinics (e.g., hemodialysis)
	Employee health services
	Counseling and testing sites, clinics for sexually transmitted diseases
	Drug treatment clinics

A list of several sources of material for survey analysis is shown in Table 4.2. Examples of sources of specimens for secondary use include blood collected for routine tests during physical examinations for the armed forces or industry or during an outpatient visit or admission to a hospital and from neonates screened for specific heritable disorders. Sera sent to a public health laboratory for serological tests for syphilis, viral diagnosis, or other diagnostic tests have also been employed. These collections of sera may not be representative of the age, sex, and geographic distribution of the entire population; the nature of the biases introduced must be recognized and evaluated. However, they are economical to obtain and sometimes may reveal important information about the presence or absence of a certain virus in the community or about the occurrence of a recent outbreak. An example of this type of serostudy was the description of the frequency of HIV and hepatitis B and C infection among injection drug users in several US cities [63].

Specimen Management. The collection and management of specimens depends on the type of specimen (e.g., viruses might be identified in stool samples, oral swabs). For blood, the specimen must be collected and separated under sterile

conditions. Aliquots of 0.5 ml each have been used by the WHO and CDC and are very useful for microtiter tests; several replicates of the entire collection may be prepared at the time of aliquoting so they can be shipped to other laboratories for testing. Sera are usually stored at −20 °C, often in a commercial warehouse. Temperatures of −70 °C are best but are more expensive to maintain. Lymphocytes can also be separated from anticoagulated blood, frozen at low temperatures in fetal calf serum and dimethyl sulfoxide (DMSO), and later thawed for examination of stable cell surface markers and other cell-associated products. Cellular material, even in extremely low concentration and a nonviable state, may be quite suitable for amplification and identification of fragments of nucleic acid. These techniques offer powerful tools for detecting genes characteristic of specific infectious agents and other biological material of interest.

3.3.4 Laboratory Tests

The general principles and techniques of laboratory testing for viral infections are presented comprehensively in Chaps. 2 and 3 of this text. The antibody tests most suitable to serological surveys of specific viruses are detailed in each corresponding chapter of this book. The criteria for a satisfactory test include simplicity, sensitivity, specificity, reliability, ability to detect long-lasting antibody, minimal interference from nonspecific inhibitors, the availability of satisfactory reagents, and the safety of the test for the laboratory technician [29, 107]. The microtiter procedure developed by Takatsy in 1950 in Hungary and popularized in this country by Sever [82] in 1962 has become the standard method in serological survey laboratories. It is adaptable to a wide variety of antibody determinations, it requires a minimal amount of serum (usually 0.1 ml) and other ingredients, and large numbers of sera can be efficiently tested. Several automated methods of dilution and of adding various reagents have been introduced to speed the testing even more [107].

The development of simple tests such as the enzyme-linked immunosorbent assays (ELISA) for antibody measurement in microtiter plates has provided a sensitive method for identification of antibody in serum samples from survey populations and can indicate both past and current infections [109]. The use of monoclonal antibodies in this and other antibody tests permits highly specific identification of individual strains of the virus, a special advantage in determining whether a new strain has been introduced in a community or if reinfection or reactivation has occurred in the individual. Commercial kits for many antibodies are now available, and new formulations are continually being devised for a variety of clinical, public health, and research applications. Detection of virus and genetic characterization of viruses can be accomplished using polymerase chain reaction (PCR) methods. Handling of specimens for PCR depends on two components: the source and virus, for example, varicella zoster virus can be identified in dried blood specimens, which can be stored at ambient temperature indefinitely. In general, PCR requires specialized kits that differ for qualitative or quantitative detection. Sequencing of select regions of amplified genetic material can be used to describe strains of virus circulating in a region or country; however, because of cost, these methods are frequently restricted to determine transmission during investigations of acute clusters of disease [1].

A full list of laboratory tests conducted using specimens collected as part of the NHANES 2009–2010 can be found here: http://www.cdc.gov/nchs/nhanes/nhanes2009-2010/lab_methods_09_10.htm.

3.4 Advantages and Limitations

The use of serological surveys is an important means of supplementing morbidity information, such as that obtained from routine case surveillance. Advantages are listed in Table 4.3. Because many viral infections may be clinically mild or inapparent, laboratory confirmation is necessary for accurate diagnosis of even overt cases. Data from serological surveys can reveal *total* burden of infection (apparent and inapparent), both currently and in the past. Selection of tests that reflect antibody of long duration permits measurement of the cumulative experience of the population tested with the disease in question; selection of a test based on short-lived antibody such as immunoglobulin M (IgM) allows identification of a recent infection or epidemic. Testing of two sera spaced in time permits measurement of the incidence of infection during the interval period.

An important advantage of carefully planned prospective serosurveys is that participants can become a cohort for other studies. For example, to determine the frequency with which hepatitis C-infected persons in the United States were aware of their infection, the National Center for Health Statistics conducted a follow-up survey of positive cases. A full interview with these participants yielded not only awareness, but also whether they sought medical care for their infection and their knowledge, attitudes, and practices related to hepatitis C [25].

Table 4.3 Advantages and limitations of seroepidemiologic studies by data collection objective

	Primary	Secondary
Advantages	Sample is representative of the target population	Less expensive
	Many possibilities of lab markers and behaviors	Saves time
		No participation bias from volunteers
Limitations	Expensive and labor-intensive	Convenience sample might not be representative of the population of interest
	Time consuming to design and implement	

An advantage of serostudies in providing information to guide vaccination programs is that serostudies measure immunity and are likely more reliable than self-reported vaccination coverage [26]. Another advantage is that aliquots of sera from a collection can be shipped, frozen, over long distances to a number of specialized reference laboratories for testing; therefore, the work can be divided among participating laboratories.

The disadvantages of seroepidemiology are the cost, time, and effort involved in the selection of the target population, the collection and analysis of data and blood, and the need for and cost of laboratory facilities equipped to carry out the tests. There must also be a satisfactory means of measuring antibody for the particular virus to be studied, and the method of carrying it out must be simple enough to allow performance on a large-scale basis. Finally, because serostudies designed for primary data collection are labor-intensive and prospective, there are significant delays in the availability of data. For example, the NHANES data require about 2 years between the end of data collection and the availability of datasets for public use.

3.5 Uses of Serological and Molecular Techniques

3.5.1 Prevalence

Prevalence from serosurveys, or seroprevalence, is defined as the number of persons whose sera contain a particular biomarker among the total number of persons examined at that point in time (frequently, 1 year). Unlike "case prevalence," which indicates the existence of disease at the time of the survey, the presence of antibody reflects the cumulative experience, past and present, with an infectious agent. The prevalence is a function both of prior and current infection and of the duration of the antibody tested. In contrast, antigen indicates presence of the virus at that time; in cases of latent or asymptomatic infection, it indicates prevalence of chronic infection.

Many antibodies such as the neutralization antibody for poliomyelitis or yellow fever virus; antibodies to the HIV core, polymerase, and envelope; and the hemagglutination-inhibition antibody for influenza, parainfluenza, rubella, measles, or arboviruses last for years, perhaps a lifetime. Thus, the cumulative experience of a population can be measured and infection acquired in childhood can be detected in persons of middle or perhaps even old age. Some waning of antibody titers (sometimes below the lowest detectable levels) may occur in older age groups after a childhood infection or vaccination. Similarly, the viral capsid antigen (VCA)-IgG antibody to Epstein–Barr virus measured by the indirect immunofluorescence test has been found to be of long duration; even complement-fixing antibodies to cytomegalovirus, herpes viruses, or dengue virus have been found to persist for years following infection.

Measurement of IgG and IgM antibody by tests such as the ELISA or immunofluorescent antibody tests provides a simple way in a single serum sample of reflecting current and past infection, respectively. Although IgM antibody usually denotes a primary infection, certain viruses such as herpes viruses may induce IgM on reactivation. It should also be reemphasized that unlike prevalence data for clinical infectious disease, serological prevalence data reflect total infection rates, representing both clinical and subclinical (or asymptomatic) infections.

Multipurpose antibody surveys have been carried out in a number of countries. In the United States, the successive NHANES surveys have provided large numbers of serum specimens for estimating cumulative exposure to various infections in representative samples of the population. For example, NHANES was critical in an understanding of hepatitis A immunity in the United States because a vaccination program was initiated in 1996 targeting select higher incidence areas. Stratifying seroprevalence by country of birth and race/ethnicity (Fig. 4.1) showed the higher levels of immunity among foreign-born and US-born Mexican Americans of any age. The impact of the initial vaccination strategy was evident in an analysis of seroprevalence stratified by geographic region and age group (Fig. 4.2) [53].

In a Barbados seroprevalence study [32], a 10 % household sample was randomly selected from a middle- and lower-socioeconomic-level community of 10,000 persons in Bridgetown. The results illustrate the type of information that can be derived from this type of study. Of 100 sera from children under age 10 tested, 30 % lacked protective levels of antitoxin against both diphtheria and tetanus, indicating the need for intensifying the immunization program against these diseases. The prevalence of protective levels against tetanus is a good indicator of the level of public health practice, since this antitoxin is acquired almost exclusively by immunization procedures and not through natural infection. The age distribution of antibodies to various viruses may provide useful information on the behavior of these infections in the community and of the need for immunization programs. On this basis, an active rubella immunization program was initiated in girls aged 12 years or younger. In subsequent years through 1978, a few sporadic cases were reported yearly, but no epidemic occurred [34]. Even sporadic serosurveys have demonstrated utility, for example, the researchers from the Barbados effort conducted a similar survey in St. Lucia [31]. They found important and unexpected differences compared to Barbados, including a higher prevalence of antibodies to dengue and rubella among young persons.

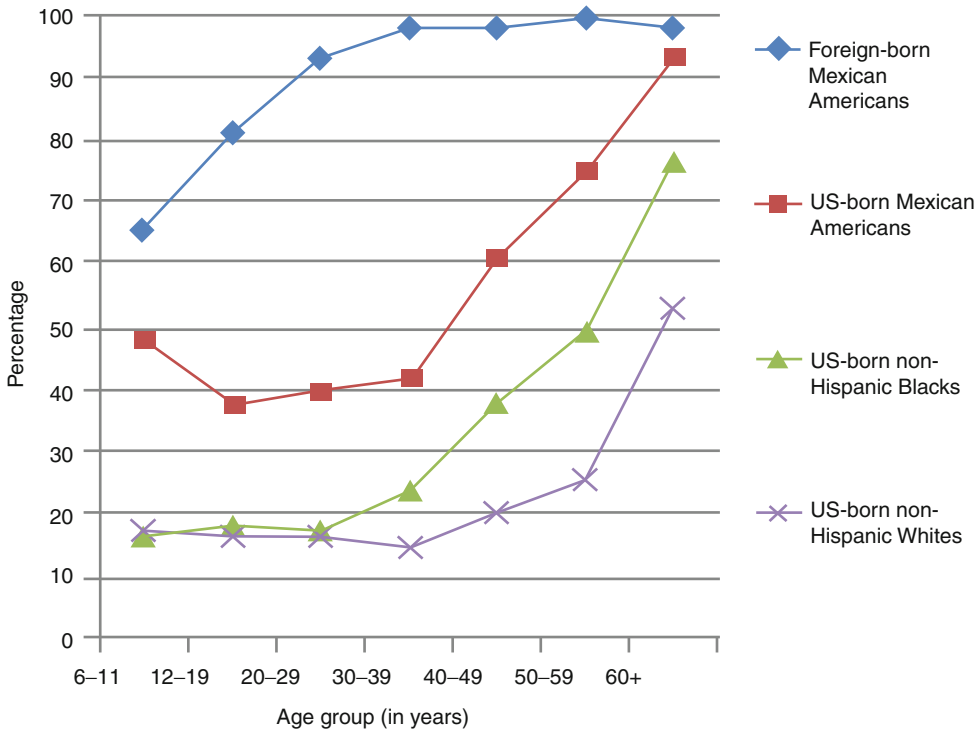

Fig. 4.1 Age-specific seroprevalence of antibodies to hepatitis A virus by country of birth and race/ethnicity, NHANES, 1999–2006

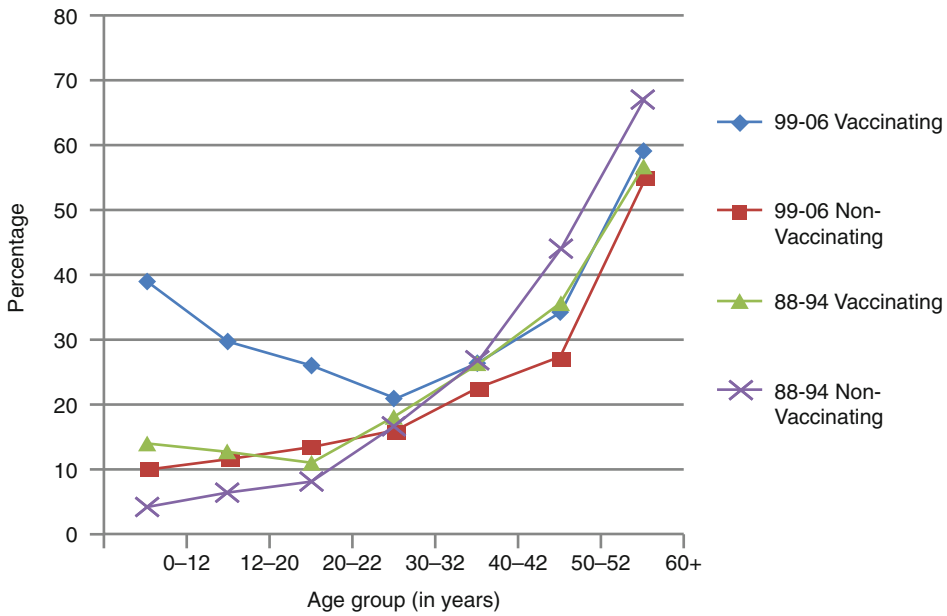

Fig. 4.2 Age-specific seroprevalence of antibodies to hepatitis A virus among US-born people by residence in vaccinating or non-vaccinating states based on 1999 recommendations, NHANES, 1988–1994 and 1999–2006

Initial tests for poliomyelitis antibody employing conventional microtiter neutralization procedures indicated that 27, 42, and 54 % of those tested at 1:5 or 1:8 serum dilution lacked antibody to poliomyelitis types 1, 2, and 3, respectively [32]. Subsequent tests on 304 sera using a 1:2 serum dilution and longer serum–virus incubation periods indicated that only 13.1 % lacked type 1 antibody, 6.5 % type 2 antibody, and 14.3 % type 3 antibody [34]. This emphasizes the need for sensitive methods for detecting low levels of antibody. Two mass poliomyelitis programs were carried out after the 1972 survey, one in 1974–1975 and another in 1977–1978. There have been no reported cases of poliomyelitis since 1972.

The 1972 Barbados serum collection was tested for human retroviruses after the agents were discovered. The HTLV-1 antibody was found in 4.25 % overall, rising in age from a 2.7 % prevalence for persons aged ≤10 years to 9.0 % among those aged 61–70 years [78]. Females had a higher prevalence rate (5.8 %) than males (2.3 %). The adult pattern raised the possibility of sexual transmission, and this was

strengthened by the finding of a prevalence rate of 14.1 % among persons with a positive VDRL test for syphilis as compared with 3.5 % of those who were VDRL negative. There was evidence of household clustering and of vertical transmission.

Advances in laboratory detection of viruses in oral fluids allow surveillance in high-risk groups with less-invasive techniques. For example, unlinked anonymous surveillance among injection drug users seeking services in England and Wales has been ongoing since 1990 [88]. The authors found that the prevalence of hepatitis C infection decreased from 70 % in 1992 to 47 % in 1998 before rising again to 53 % in 2006. Prevalence among women injecting drug users was higher than among men, and that prevalence was highest in certain geographic areas that could be targeted for prevention.

3.5.2 Incidence

Incidence is best calculated from cohort studies such that the appearance of the viral infection can be captured with a known denominator of a well-defined population of persons at risk. However, cohort studies are costly and subject to attrition over time.

Many prospective serological and clinical studies were used to investigate HIV infections and the development of AIDS and related clinical syndromes among high-risk populations such as gay men, injection drug users, persons with hemophilia, and their contacts. Some of the earliest studies were based on cohort methods using sera originally collected for evaluation of HBV vaccine, and others started afresh with a new cohort [50]. The findings in the New York [86] and San Francisco cohorts [10] were as follows: (1) the prevalence level of HIV antibody was 6.6 % in New York at entry into the study in 1978, and rose to 10.6 % by 1984; (2) in San Francisco the entering prevalence was 4.5 % in 1978, but rose dramatically to 73.1 % by 1985; and (3) the rate of viral infection (seroconversion) among those lacking antibody on entry was 5.5–10.6 % per year in New York and 11.2 % in San Francisco. Among gay and bisexual men entering the Multicenter AIDS Cohort Study (MACS) in Baltimore, Chicago, Los Angeles, and Pittsburgh in late 1984 and early 1985 [50], seroprevalence ranged from 23 to 49 %. In a pattern similar to those in other cohorts, the incidence rate of new infection in the MACS, documented at 3–5 % per year in early 1985, declined rapidly during the next 3 years to about 1 % [52]. In Africa and Thailand, measurements of seroprevalence and incidence among prostitutes and other high-risk groups have repeatedly demonstrated the alarming increases that have taken place over a very few years. Transfusion-associated HIV infection systematically documented through serosurveillance of hemophiliacs and other recipients [3, 54, 55], soon after known parenteral exposure, provided an important resource for comparative research on the natural history of HIV infections and the development of AIDS. Further details of serological studies of HIV infection can be found in Chap. 43.

The calculation of incidence is possible from such cohort studies because clinical, laboratory, and self-reported data are collected on participants at regular intervals. Therefore, negative results are available, such that a time period can be calculated between the first positive and the most recent previous negative result. Taken together, these and other cohort studies have yielded critical data for the empirically based projections about burden of infection and disease in select populations.

3.5.3 Outbreak Detection

The advances in genetic characterization and communication online have combined to expand the scope and breadth of serosurveillance into early detection of clusters of infectious diseases. In the United States, norovirus is the most common cause of foodborne disease outbreaks. Surveillance to detect early clusters of disease is conducted through routine notifiable diseases methods and by a laboratory network called CaliciNet [96]. It is a national surveillance network that includes a database for public health laboratories to submit gene sequences from human caliciviruses (noroviruses and sapoviruses) identified from outbreaks. The information is used to link norovirus outbreaks that may be caused by common sources (such as food), monitor trends, and identify emerging norovirus strains.

Measles in the United States provides an excellent example of the use of technology in outbreak detection. In 2000, the United States achieved measles elimination (defined as interruption of year-round endemic measles transmission) (http://www.cdc.gov/measles/global-elimination.html). However, importations of measles into the United States continue to occur, posing risks for measles outbreaks and sustained measles transmission. During 2011, a total of 222 measles cases (incidence rate: 0.7 per one million population) and 17 measles outbreaks (defined as three or more cases linked in time or place) were reported to CDC, compared with a median of 60 (range: 37–140) cases and four (range: 2–10) outbreaks reported annually during 2001–2010.

Measles has also reappeared in other developed countries, and a web-based, quality-controlled database with epidemiologic and nucleotide data for measles infection in the WHO/Europe region was developed: the Measles Nucleotide Surveillance (MeaNS) [60, 79]. The major objectives of the MeaNS initiative are to function as an epidemiologic surveillance tool and to monitor progress in measles control. Dynamic reports and graphical charts can be created on any user-selected fields in the MeaNS database (e.g., genotype or sequence variation in a geographical location or time period). Information about MeaNS is available at http://www.hpa-bioinformatics.org.uk/Measles/Public/Web_Front/main.php.

3.5.4 Diagnostic Serology

Sera sent to large hospitals or public health laboratories for various tests can be frozen and stored for later antibody testing against other antigens. The specimens must be adequate in amount and free of bacterial contamination. Specimens sent for viral antibody tests usually fulfill these criteria and are accompanied by minimal demographic and clinical data concerning the patient. There are many uses for this type of collection. All sera or exanthems coming from patients with central nervous system, gastrointestinal, or respiratory infections can be tested at the time of receipt or, later, against a battery of viral antigens in order to reveal the profile of agents likely to have caused the syndromes. An example of this was the evaluation of the importance of a new virus, called "the California encephalitis virus" in the causation of infections of the central nervous system by testing of all sera received in a state public health laboratory for this syndrome [44]. In Wisconsin, 5.7 % of 351 sera received in the state laboratory over the period 1961–1964 revealed evidence of the new California viral infection [91]; in Minnesota, 4.1 % of 1,617 retrospectively tested sera contained this antibody. A second and related application is the determination of the clinical spectrum associated with a newly discovered virus; this is accomplished by testing stored sera from patients with a variety of clinical syndromes and looking for evidence of infection with the new agent.

A third application for sera stored over time is the measurement of *secular trends* or antigenic shifts in viruses over time. This is especially useful in relation to influenza viruses. A fourth use, employing freshly received sera from high-risk populations, is the search for influenza antibody patterns that may reveal the beginning of an outbreak or a change in the antigen composition of currently circulating strains; this was used by Widelock et al. [101] at New York City public health laboratories. Comparison of the geometric mean antibody titer to influenza sera from persons in the acute phase of an unidentified respiratory illness with the titer in others convalescing from a similar illness may permit early identification of an outbreak without waiting for serial samples from the same persons.

Finally, investigators in South Australia made efficient use of samples taken in conjunction with blood donation. They searched for evidence of Ross River virus activity in different locations during the arbovirus season by measuring IgA-, IgG-, and IgM-specific antibody in samples from Red Cross blood donors. Differences in antibody prevalence by region indicated prior activity and helped identify endemic areas but revealed that acute infection had not occurred in that year at frequencies high enough to be detected [99].

3.5.5 Evaluation of Immunization Programs

The effectiveness of an immunization program is traditionally judged on the basis of clinical cases or epidemic behavior. A program is regarded as effective when cases decrease or epidemics do not occur. This information may be biased by the possibility that the decrease in clinical cases is related to poor reporting or that insufficient time has elapsed for another epidemic to have occurred. Currently, our knowledge of the utilization of vaccines depends on such sources as sales records of manufactures, public clinic and physicians' data, direct interviews, and school entry surveys [20]. Because of the inadequacy of these traditional surveillance techniques in determining the need for and the effectiveness of a given vaccine, serological surveys could play an even larger role in evaluation of immunization programs. Although the necessity for a venipuncture reduces the ease and acceptability of this method, public health professionals and physicians can help surmount such barriers by conveying the importance of seroepidemiology for such purposes. Newer techniques that obviate the requirement for blood sampling may further encourage the application of biological tools to the evaluation of vaccination effectiveness.

The uses of serological epidemiology in immunization programs are summarized in Table 4.4. Much of this information could be obtained in no other way. Serological surveillance has been of particular importance for the new epidemiologic settings created by substituting vaccine immunity for natural immunity as for poliomyelitis, measles, rubella, and to a lesser extent mumps and influenza [30]. With vaccines against HBV universally recommended for infants in the United States and a vaccine against varicella zoster virus, the patterns of susceptibility to and the distributions of these infections have changed substantially.

Table 4.4 Uses of seroepidemiology in immunization programs

1. Cross-sectional surveys to determine the need for immunization programs in:
(a) Different age groups
6–20 months—measure duration of maternal antibody
School/college entry—identify omissions, failures, loss of protective antibody
(b) Different geographic areas
(c) Different socioeconomic groups
(d) High-risk occupational groups
2. Follow-up measurements in immunized persons to determine:
(a) Proportion developing local, humoral, cell-mediated immune responses
(b) Quality and extent of response
(c) Nature and degree of interaction between vaccine components
(d) Duration of response
(e) Level of protection against disease and asymptomatic infection
(f) Degree of spread of live vaccine strains to exposed and susceptible contacts
3. Periodic serological surveillance to identify groups who are not receiving vaccines or who have inadequate responses

Patterns of susceptibility and immunity to all of these viruses may now vary from place to place, from age group to age group, and in various socioeconomic settings, depending on the immunization program instituted by health departments and the activities of physicians and clinics rather than on the inherent epidemiologic characteristics of the natural infection and disease. The methods of immunization practice, the frequency of repeated immunization programs, and the quality and duration of vaccine immunity will increasingly constitute the major determinants of the patterns of these diseases.

Over the years, serological surveys in American cities, such as Syracuse [56], Cleveland [38], and Houston [61], have uncovered serious deficiencies in the antibody patterns for viral diseases for which vaccines are available. The US military has used this approach to similar advantage. A national serosurvey of 1,547 Army recruits entering in 1989 at ages 15–24 years documented 15–21 % seronegativity for measles, mumps, and rubella; 7 % for varicella zoster virus; and 2.3, 0.6, and 14.6 % for poliovirus types 1, 2, and 3, respectively [51]. Likewise, a pre-vaccination survey of 1,568 US Navy and Marine entrants identified important differences in susceptibility to measles; susceptibility was greatest among males, younger recruits and whites [87]. Serosurveillance has also been used effectively in the developing world for such purposes as a general assessment of success in the WHO Expanded Program on Immunization [35] and a specific comparison of HIV-infected and uninfected Zairian infants for responses to polio vaccine [80]. The importance of surveillance programs and serological surveys to evaluate immunization programs has long been recognized [30, 47]. Thanks to testing of antibodies to hepatitis B that allow distinguishing immunization (where antibodies to hepatitis B surface antigen are positive and antibodies to hepatitis B core are negative), from past or from chronic infection, seroprevalence in NHANES has guided the US vaccination program for hepatitis B. Wasley et al. compared findings from the 1999–2006 NHANES with previous surveys and found that a history of infection with HBV had decreased from 1.9 to 0.6 % among children. Prevalence of antibodies consistent with immunization increased from 20.5 % in 1999–2002 to 25.2 % in 2003–2006 [98].

Hepatitis B also was examined in a study of data from 10 European countries. Residual sera were combined with sera from samples available at national serum banks [65]. Testing assays and algorithms were standardized to allow cross-country comparisons. The authors also collected information on country-specific vaccination policies. Six of the ten countries reported low levels (<3 %) of antibodies to hepatitis B core antigen. Of the eight countries testing for HBV surface antigen (HBsAg), Romania had the highest prevalence (5.6 %); the remaining seven countries had prevalence <1 %. Countries can apply findings from such comparisons to modify and adjust their own vaccination policies.

3.5.6 Biomarkers

Revolutionary advances in immunology and molecular biology have opened the possibilities for intensive analysis of humoral and cellular material—what some are calling broadly "molecular epidemiology"—analysis that will clarify the role of viral infection in the pathogenesis of autoimmune, degenerative, neoplastic, and even "genetic" diseases and will facilitate targeted drug and vaccine development. An example of one such possibility for molecular study is a European network for hepatitis B virus. The HepSEQ (http://www.hepseqresearch.org) is a pioneering online resource for the management, characterization, and tracking of hepatitis B infection in the area. An important use will be to detect mutations of the hepatitis B virus such as those consistent with antiviral resistance [64]. This information will be helpful in identifying transmission patterns that will guide prevention efforts.

4 Summary

Systematic collection of cases of infection or disease and of biomarkers of infection or immunity is an essential tool in the prevention and control of viral diseases. Surveillance and seroepidemiology have provided critical epidemiologic information to support public health policy at the local, national, and international levels. Continued advances in laboratory methods and communication systems will expand the applications of surveillance and refine their precision and efficiency.

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Viral Dynamics and Mathematical Models

5

Nimalan Arinaminpathy, Charlotte Jessica E. Metcalf, and Bryan T. Grenfell

1 Introduction

The application of mathematics to explore the dynamics and control of viral infections has a long history, which predates its application in many other fields of biology, as well as the germ theory itself [1]. A particularly fruitful area for development has been the dynamics of acute immunizing infections, with their relatively simple natural history and rich notification time series [2–4]. The last three decades have seen a considerable upsurge in the use of mathematical and computational models to explore the dynamics and control of a wide range of viral infections. This phase arose initially from developments in ecological population dynamics [4]; it was then greatly accelerated, both by the explosion in computational power and by the emergence of human immunodeficiency virus (HIV) infection, severe acute respiratory syndrome (SARS), and other potential pandemic threats [5].

In this chapter, we review basic concepts in infection dynamics and control, via a synthesis of epidemic models and data. We begin with acute immunizing infections, focusing on measles as a case study of the impact of herd immunity and other determinants of epidemic dynamics. We then extend the discussion to dynamical modeling applications to a range of other acute and chronic viruses, with a variety of more complex life histories. After a brief discussion of models for the within-host dynamics of viruses, we conclude with suggestions for gaps in knowledge and future research needs.

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2 Dynamics of Acute Immunizing Infections

2.1 Observed Epidemic Patterns

Acute immunizing infections typically generate recurrent epidemics in large communities. We illustrate these patterns with the best documented case – the dynamics of measles in large cities in England and Wales [2, 3]. Figure 5.1a shows the time series of raw weekly notifications for measles in London from 1944 (shortly after measles cases became notifiable) to 1994.

These data indicate three fairly distinctive dynamical eras:

- 1944–1950: Principally annual epidemics.
- 1950s–1960s: Regular, mainly biennial epidemic cycles, with intervening small annual peaks – incidence rates are markedly *seasonal* (Sect. 2.4).
- 1970s onwards: The vaccine era brought declining incidence, with increasing irregular, lower-amplitude epidemic cycles. By the end of the series shown in this figure, cases became very sporadic, with increasing levels of mis-notification of clinically identified cases [7].

As well as these fluctuations in individual large cities, there are also rich dynamical patterns in the *spatial spread* of epidemics among large and small communities (Sect. 4). Regional and temporal demographic variations, especially in *birth rate*, can also markedly influence dynamics (Sect. 2.4).

2.2 Epidemic Dynamics: The SEIR Model

The striking epidemic patterns of acute immunizing infections are particularly well documented for widely notifiable infections such as measles. The process of explaining these

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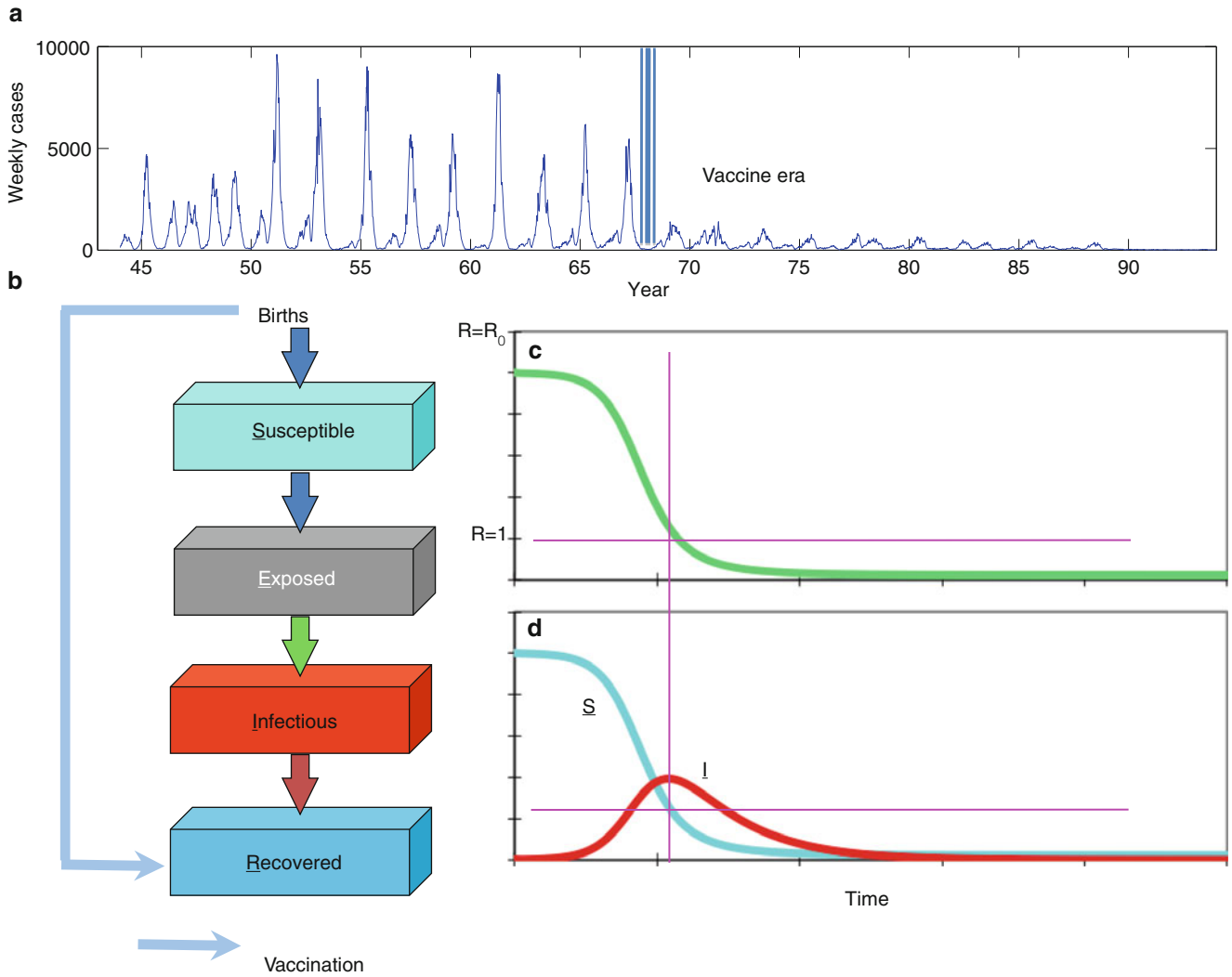

Fig. 5.1 (**a**) Observed spatiotemporal dynamics of measles in England and Wales, showing weekly aggregate time series of notifications for London. (**b**) Basic flows of individuals captured by the SEIR model. (**c**) Schematic time series of numbers of susceptible individuals, arising from flows in panel **b**, and in particular following the introduction of one infectious individual into a wholly susceptible population; the effect of birth rate on susceptibility is ignored. (**d**) Corresponding dynamics for the proportion of the population susceptible (line marked "*S*") and the proportion infected (line marked "*I*") (**b**, **d**: Taken from Fig. 2, Grenfell [6]. Figure found on p. 38)

cycles, as well as that of addressing associated public health issues, has spawned an extensive analytical literature, spanning public health epidemiology, theoretical biology, and population dynamics [4, 5, 8–10]. The key conceptual tool has been a family of compartmental dynamical models, based on the SIR (susceptible-infected-recovered) paradigm [2–5, 11, 12]. As described below, the SIR family successfully captures many key features of the epidemiological dynamics and control of viral infections.

The basic SIR formulation embodies the dynamics of immunizing infections. Because viruses reproduce rapidly in the host, we ignore within-host kinetics in the simplest SIR models (but see Sect. 8); depending on model details, this leads to a compartmentalization of the host population between (as yet uninfected) susceptible individuals, infected, recovered, and other classes. An initial taxonomic split here is between the SIR model (which crudely assumes that all infected individuals can pass on infection) and the SEIR (susceptible, exposed, infected, recovered) model, which adds an "exposed" (infected but not yet infectious) class [4]. SIR and SEIR models have qualitatively very similar dynamics [13]; however, we describe the latter with its more realistic depiction of viral incubation. The basic SEIR model, illustrated in Fig. 5.1b, reflects the following set of biological assumptions; we use measles as the classic acute exemplar here [14]. After a few months of maternally derived passive immunity, infants enter the virus-naïve "susceptible" (S) class; susceptibles can then become infected by close contact with infectious individuals (generally via

respiratory aerosol for measles). Infection moves individuals into the "exposed" (E) class, where they incubate but do not transmit the infection for around a week; this leads to the infectious (I) class, where virus is shed, again for approximately a week, after which individuals enter the recovered state (R). In the basic SEIR model, recovered individuals are assumed to be immune for life, both to clinical disease and to retransmitting the infection. However, subclinical infection (and hence boosting of immunity [15, 16]) is in principle possible. The lifelong sterilizing immunity induced by such an infection also makes this an excellent potential vaccine candidate [4]. For the SEIR model, vaccination is at its simplest assumed to be delivered to a proportion p of infants at the end of the maternal immunity period (i.e., near the effective "birth" of susceptibles). For individuals who seroconvert, immunity is then often assumed to be lifelong, moving susceptibles into the recovered class (Fig. 5.1c). There is also a considerable literature exploring more operationally realistic age distributions and immunogenic characteristics of vaccines in specific cases [5, 17].

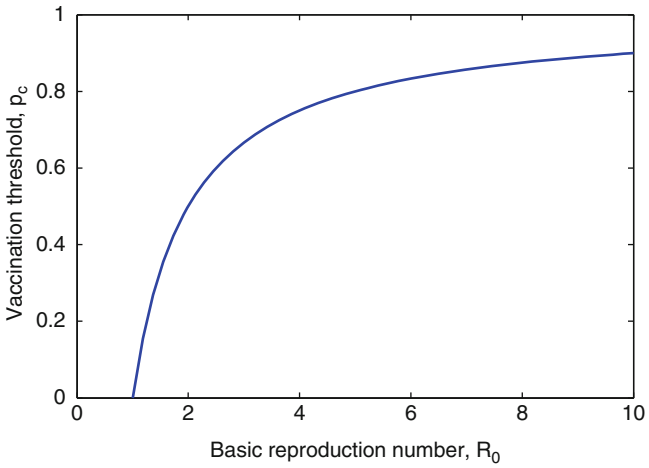

Fig. 5.2 The critical vaccination threshold (p_c) and its dependence on the basic reproduction number, R_0. In the region above the *blue line*, vaccination succeeds in local elimination of infection. Even in the region below the line, however, vaccination can substantially reduce overall transmission

2.3 Herd Immunity and the Impact of Vaccination

We can lay bare much of the dynamical behavior of immunizing epidemics by considering the case of the "simple" epidemic (Fig. 5.1c, d), in which the outbreak occurs sufficiently rapidly to ignore processes of host birth and death. The talismanic quantity here is the basic reproduction number (or ratio) of infection, R_0 [4, 5]. At its simplest, R_0 is defined as the total number of secondary cases caused by an infectious individual when introduced into a well-mixed fully susceptible population. To illustrate the ensuing dynamics, consider an epidemic of infection with $R_0>0$. Since each case initially causes R_0 secondary infections, the epidemic increases more or less exponentially over the first few infectious generations (Fig. 5.1c). However, these dynamics rapidly deplete the susceptible pool. This brings us to the other key parameter of epidemic spread: the *effective* reproduction number, defined as $R=[S/N]R_0$. Here, S/N is the proportion of susceptibles in the population and R is the realized value of R_0 as the epidemic develops [4]. Since S declines over the course of the epidemic (Fig. 5.1c), so does R (Fig. 5.1d).

This progressive decline in secondary infection rates through the epidemic is a manifestation of the key epidemiological process of *herd immunity* [18] – increasing natural immunity of the population that indirectly protects the remaining susceptibles from infection. Eventually, the effective reproduction ratio declines through unity (Fig. 5.1c) as population immunity increases; this corresponds to the herd immunity threshold, above which the epidemic will always decline, even if reintroduced to a closed population. This threshold is thus also a key aim of vaccination campaigns [4, 5]. Remembering that $R=[S/N]R_0$, the associated susceptible proportion at the herd immunity threshold ($R=1$) becomes $s_c=1/R_0$ (Fig. 5.1c); thus, immunizing at a level above $p_c=1-s_c=1-1/R_0$ will eliminate local transmission. As described below, these calculations have been refined considerably to allow for heterogeneities in transmission with age, space, and other characteristics (Sects. 3, 4, and 5). Nonetheless, our simple expression for p_c is an extremely useful metaphor for epidemic control: (1) because of indirect protection, not everyone needs to be vaccinated to eliminate transmission (corresponding to $p_c<1$) and (2) the effort required to increase this level of immunization increases with transmission rates. The latter point is made clear by a simple plot of p_c against R_0 (Fig. 5.2); more transmissible immunizing infections such as measles are harder to control than less transmissible agents such as smallpox. However, this refers to "random" mass vaccination; more targeted strategies such as ring vaccination coupled to active surveillance can promote elimination even below p_c, given (as with smallpox elimination [19]) the right biological characteristics and logistics. Partial immunity and other characteristics of the population can complicate the picture still further (see Sect. 5.2 below). Nonetheless, the metaphor that more transmissibility necessitates a stronger vaccination effort remains.

2.4 Seasonal Transmission and Recurrent Epidemic Dynamics

2.4.1 Observed Epidemic Patterns in Developed Countries

In the simplest analysis of an immunizing infection (Sect. 2.2), sustained cycles of infection will disappear, and the infected proportion in the population will settle to a constant level

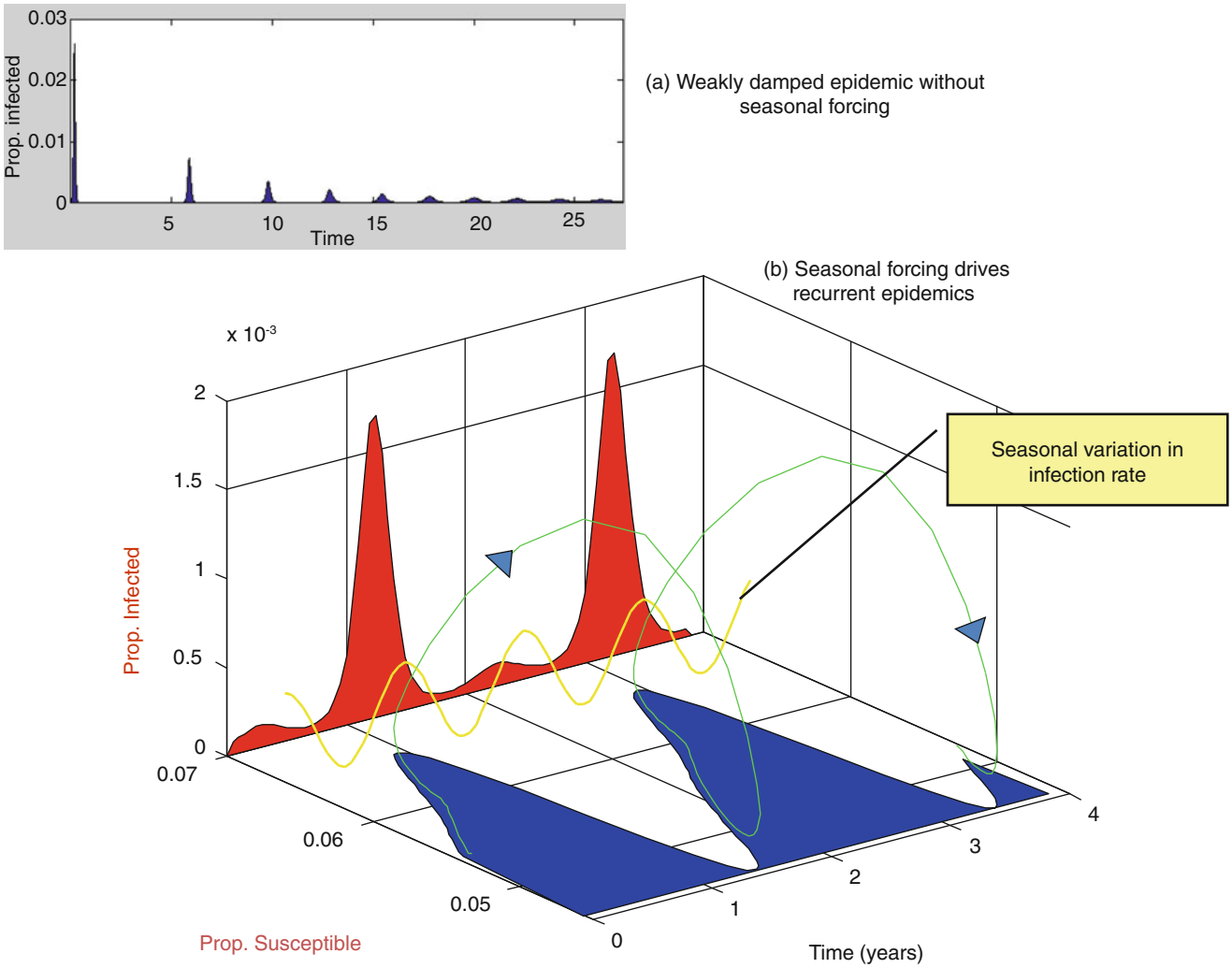

Fig. 5.3 (**a**) Simulated SEIR dynamics for measles with a birth rate, but in the absence of seasonal forcing of transmission rate; for full model specification and parameters, see Grenfell and Bolker [20]. (**b**) Same model, with sinusoidal forcing of the infection as shown (forcing amplitude set to 0.2; Grenfell and Bolker [20]). The green trajectory shows the joint dynamics of susceptible and infectious densities through time; area plots show the dynamics of infectious and susceptible individuals (Taken from Fig. 3 in above book chapter, found on p.39)

(Fig. 5.3a). The question of what maintains the recurrent epidemics [9] generally observed for immunizing childhood infections (e.g., Fig. 5.1) drove researchers to seek the key aspect of biological realism missing from the simplest SEIR model. For measles in England and Wales, seasonal variation in transmission driven by increased contact rates of children when schools were in session rapidly emerged as a possible candidate [12, 21] and has since received considerable empirical support [2, 3]. Stochastic fluctuations in incidence could in principle also contribute to the maintenance of cycles [22, 23]; however, for measles, the predominant driver is seasonality in transmission [2, 3]. Birth rate may modulate the periodicity of recurrent cycles driven by seasonality through its role in determining the rate of susceptible replenishment [24]. For example, for most of the postwar pre-vaccination era in London, seasonality generated sustained cycles by resonating with the biennial epidemic tendency of measles dynamics. However, during the postwar baby boom, births achieved sufficient levels to shift measles dynamics into annual cycles (Fig. 5.4).

2.4.2 Epidemic Dynamics in Developing Countries

It was realized early that seasonality could also result in more complex dynamics, including chaotic fluctuations, that is, very irregular dynamics with little long-term predictability. Contexts with both high and low birth rates (and both high and low transmission since birth rates and transmission act dynamically in very similar ways for immunizing infections [24]) can promote complex dynamics via coexisting attractors. Until recently, it was thought that chaotic measles dynamics were not likely to

be observed [3, 25], since observed seasonality in transmission was not strong enough to drive the associated violent epidemics. However, recent analyses of measles in Niger revealed very strong seasonality (driven by movement in and out of cities linked to rainfall); this, combined with high transmission rates and the highest birth rate in the world, results in irregular outbreaks consistent with expectations of chaos (Fig. 5.5). Erratic boom and bust outbreaks are expected to continue even as routine vaccination improves; and this suggests that high investment in reactive vaccination and surveillance is important, and pulsed vaccination approaches such as supplementary immunization activities could also play a helpful role in synchronizing dynamics [26].

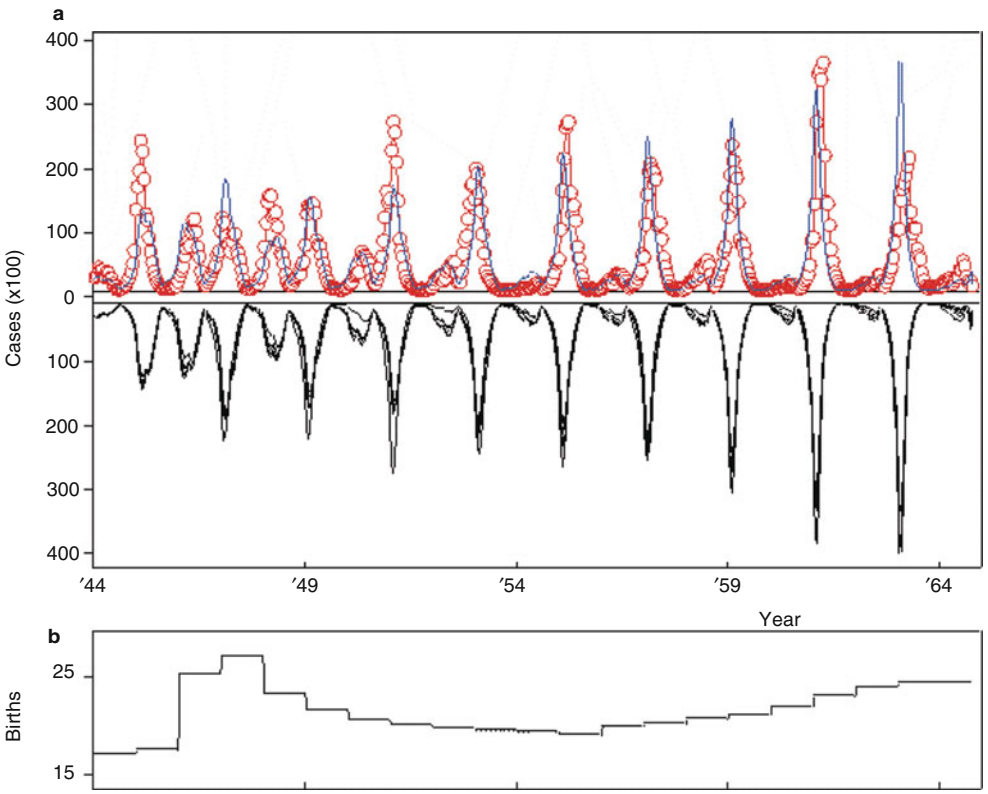

Fig. 5.4 (**a**) Observed pre-vaccinations measles dynamics for London (*red circles*), corrected for under-reporting, along with predictions of an autoregressive time series version of the SEIR model, starting at the observed initial density of cases and susceptibles (for more details, see [126]) (**b**) Birth rates accompanying the dynamics in panel (**a**) (Taken from Fig. 4 in above book chapter, found on p. 41)

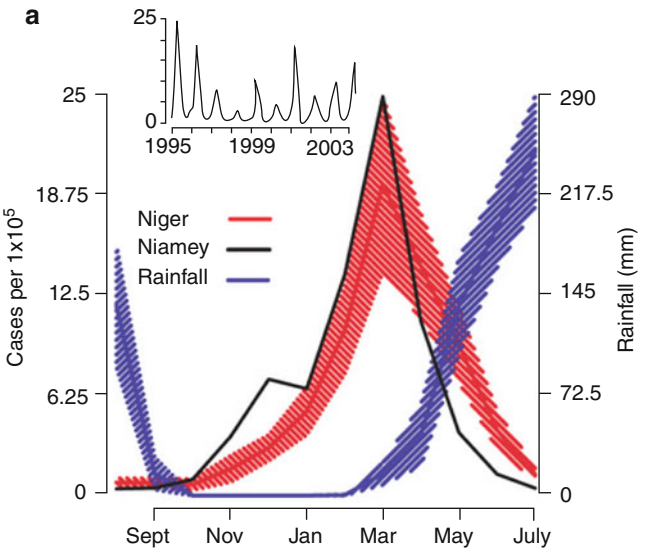

Fig. 5.5 Time series dynamics of measles outbreaks from Niger. (**a**) Mean number of reported measles cases per 10,000 nationwide in Niger from 1995 to 2004, and the mean monthly rainfall over the same time period (*blue*). Shaded regions give ±2 standard deviations. *Black curve*, mean monthly cases of measles in Niamey from 1986 to 2005. *Inset* monthly measles time series from 1995 to 2004. (**b**) Weekly measles case reports from seven departments of Niger, 2001–2005. *Red asterisk* Niamey. Each department is an aggregate of 3–8 arrondissements. (**c**) Case reports per month for the city of Niamey from 1986 to 2005. The *box* indicates the time frame shown in (**b**). *Black dots* months with 0 reported cases (Taken from Fig. 1, Ferrari et al. [26]; http://www.nature.com/nature/journal/v451/n7179/images/nature06509-f1.2.jpg)

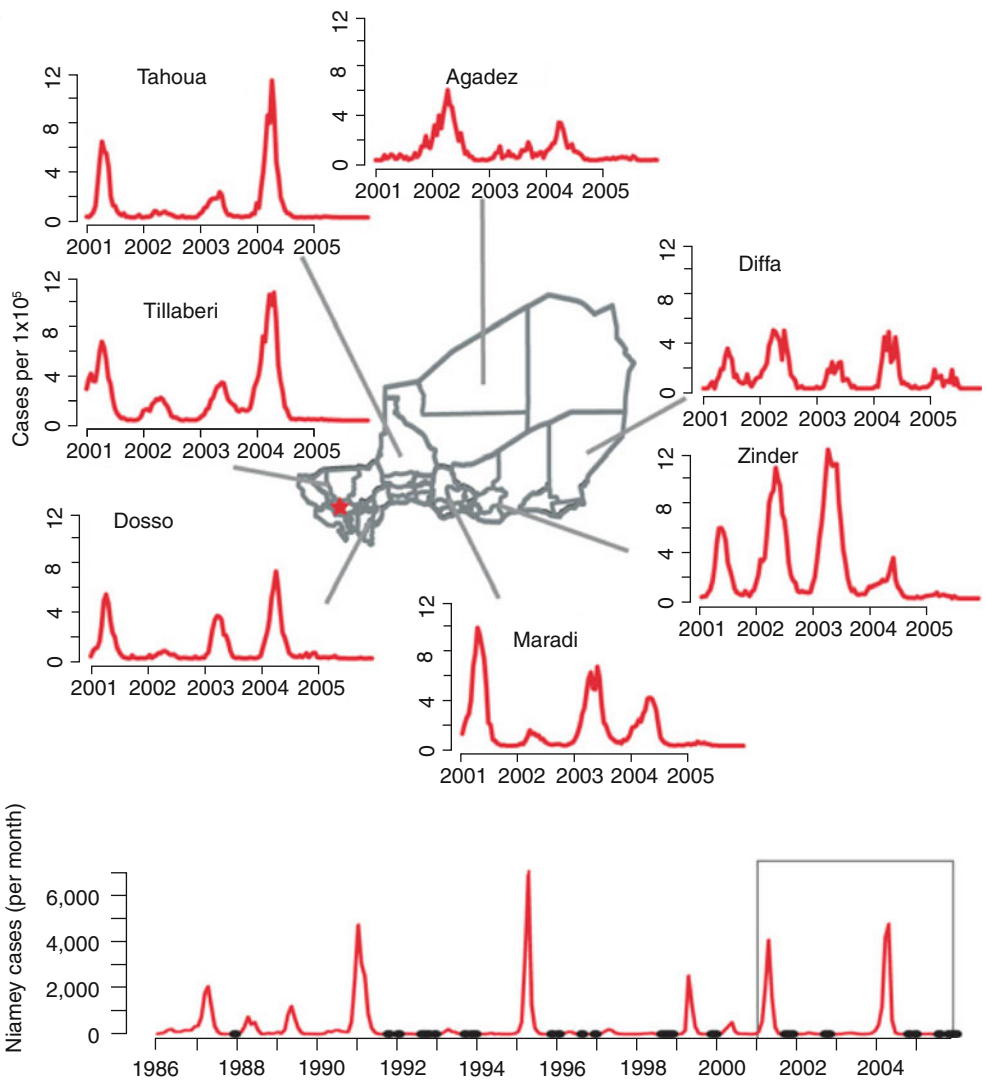

Fig. 5.5 (continued)

3 Age Structure, Demography, and Serological Profiles

The mean age at which individuals become infected by immunizing viral infection is generally lower for infections with higher rates of transmission or R_0 (defined above). Intuitively, this occurs because the faster an immunizing virus is spreading through a population, the younger the age at which individuals are likely to be exposed to it. Host demography modulates this relationship, and higher birth rate countries with an R_0 equivalent to that in lower birth rate countries will tend to have a lower average age of infection [27]. Maternal immunity, or protection of children after birth by transfer of maternal antibodies, will also have an impact, increasing the average age of infection for children whose mothers have been exposed to infection. Since maternal antibodies rarely persist for much more than a year, this effect will be greatest for infections with very low average ages of infection [4]. Beyond these broad descriptors, however, there is the added complication of possible relationships between age and probability of exposure to infection. Such relationships may arise for a variety of reasons. For example, only individuals beyond a certain age may work in areas where the disease is transmitted, or the disease may be only sexually transmitted. As a result, the force of infection, or probability that a susceptible individual will be infected, will show a distinct relationship with age.

For directly transmitted infections, like measles, mumps, rubella, and influenza, the age profile of the force of infection is determined by the rate at which individuals of different ages interact. There are two approaches to estimating this variation. The first is to use age profiles of seropositivity to infer the pattern of the force of infection and, from this, extrapolate to the pattern of contacts over age [28–31]. The link between these age profiles and the force of infection over age can be made since once an individual has been

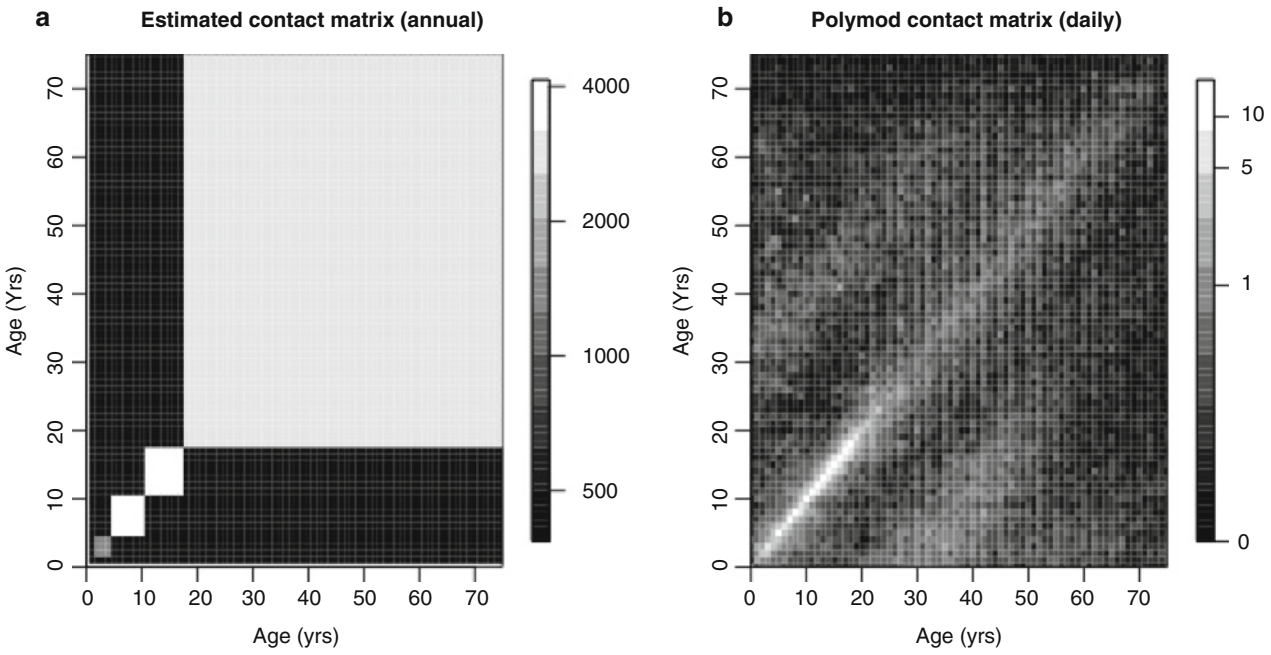

Fig. 5.6 (**a**) Contact matrix (annual) estimated from serology profiles and incidence data using maximum likelihood approaches (Ref. [30]), here showing a semi-assortative mixing matrix; (**b**) POLYMOD contact matrix (daily) obtained from diary studies showing records across all of Europe (Ref. [34])

infected, she/he can never be infected again. Therefore, to be immune at any age, the individual must have contracted the infection prior to that age. On a population level, cross-sectional seropositivity thus provides an indication of the total risk of infection for individuals up to a given age. (Longitudinal age-serological profiles can be even more powerful in quantifying epidemic risk [32]).

The age profile of infection can be framed mathematically [33], and parameters describing contacts between individuals of different ages inferred [31]. The resulting estimated matrix of contacts over age is known as the Who-Acquires-Infection-from-Whom or WAIFW matrix. Alternatively, patterns of contacts between individuals can be directly measured [34], for example, using diary studies (Fig. 5.6); and by combining this with the age profile of infection within the population, the force of infection over age can be inferred [35].

The infection dynamics themselves may also influence the relationship between age and force of infection. If outbreaks are separated by long intervals during which little exposure occurs, individuals may remain susceptible for many years and thus contract the infection at a later age than might be predicted from the age profile of contacts alone [36]. More subtly, detailed network analyses show that even within a single influenza outbreak, the burden of disease can cascade from children (where contacts are highest) to the less connected adults as immunity accumulates within the children, with implications for optimal vaccination distribution [37].

Understanding the processes underlying the average age of infection has a practical importance for any infection where the burden of disease shows an age profile. Rubella is a classic example. Infection during early childhood tends to be mild, but infection during pregnancy may result in birth of a child with congenital rubella syndrome, consisting of a range of birth defects (see Ref. [38]). A realistically complex age structure of mixing, as detailed in empirical studies, may thus be of crucial importance in establishing the burden of disease [39] but also how the burden of disease is likely to change as a result of vaccination.

4 Spatial Dynamics

So far, we have assumed that deterministic, spatially homogeneous dynamics govern infectious disease outbreaks. In fact, epidemics often spread across a heterogeneous landscape of human cities, towns, or rural communities, and this spread depends partly on the links between those locations. This leads us to move from the *deterministic* SIR model described above to *stochastic* models, which account for the random nature of individual infection dynamics and demography – for instance, individuals may or may not become infected with a given average probability, so that by chance, particularly in small populations, no new infections may occur, and the chain of transmission may be broken. During the troughs between epidemic outbreaks in smaller communities, incidence may fall to such low numbers that local extinction is likely.

Based on this observation, Bartlett [9, 40] used analyses of epidemiological data and stochastic models to develop the notion of critical community size (CCS), or population size below which stochastic extinction is expected, which was further developed by Black [41] in studies of measles persistence in insular populations. Bartlett demonstrated the existence of a CCS of around 300,000–500,000 for measles in England and Wales. For measles to persist in locations with a population size smaller than this CCS, immigration of infected immigrants from elsewhere in the metapopulation is necessary. The result is that the spread of measles across England and Wales in the pre-vaccine era resembled traveling waves spreading out from London [6], with a substantial epidemic lag in locations further away. The duration of the lag was also shaped by the size of the local populations. The duration of fade-outs following local extinction contains information on the degree to which a particular location is connected to the metapopulation as a whole [42]. Generally, this points to larger places being more connected. More detailed parametric analyses tend to confirm this (e.g., the gravity model [43]).

Similar processes may operate for immunizing or lethal infections in animal populations, with, for example, the spread of rabies through raccoon populations acting as an invasion wave structured by large landscape features such as rivers [44]. For more complex human infections, features such as imperfect immunity (see Sect. 5.2 below) will tend to shift the age class of hosts who disperse the infection. That shift may impact on the main mechanism of dispersal [45] and move the scenario away from invasion into a locally coupled landscape to a more demographically driven dynamic [46].

5 Comparative Dynamics

So far, we have explored the epidemiological dynamics of acute, immunizing viral infections. Though the resulting dynamics are both important and fascinating from a dynamical perspective, the natural (life) history of most viral infections departs, in one way or another, from this simple case. We review these complexities, and their epidemiological and control implications, in succeeding Sections.

5.1 From Acute to Chronic

A dramatically different life history from the transient infection paradigm represented by measles is observed with infections that are much more persistent (even lifelong). This difference may be expressed in terms of infectious period in individual hosts [4]. To illustrate how increasing infectious period alone modifies the violent epidemics of childhood infections, we retain the assumption of lifelong immunity of the SEIR model and vary the infectious period, from the roughly 1 week of measles to 10 years, corresponding to the approximate average pre-HAART treatment infectious period of HIV [4].

Figure 5.7 explores this comparison, simulating an infection which invades a partly susceptible population (full details are given in Ref. [6]; note that for clarity, the epidemic curves for 1- and 10-year infectious periods are raised above the curves for the more acute infections). Each simulation refers to a virus with a different infectious period. We assume that R_0 is identical for each of these infections. R_0 is roughly given by the product of mean *per capita* infection rate and infectious period; thus, to keep R_0 constant:

- A short infectious period implies a relatively high infection rate.
- A longer infectious period requires a lower infection rate.

This assumption imposes a simple evolutionary constraint on our set of model pathogens in Fig. 5.7, in that fitness (roughly equating to R_0) is kept constant as we increase the infectious period. Figure 5.7 indicates, first, that increasing the infectious period reduces, and eventually eliminates, the tendency for cyclical epidemics [48]; essentially, a longer infectious period "fills in the troughs" following major epidemics. As infectious period increases, we therefore see a transition from seasonally driven biennial epidemics (at the measles extreme of a 1-week infectious period) to low-amplitude annual epidemics (at 1 month). For longer infectious periods (1 year), we see slowly evolving epidemics with little seasonal activity and a modest post-epidemic overshoot. Finally, for a 10-year infectious period, we see a smooth slow epidemic, with an essentially "logistic" rise to a stable endemic plateau of incidence. Though crude, this exercise captures the essential dynamical transmission from the violent epidemics of acute infections to the much smoother and slower epidemic invasion of HIV [4]. Note that the seasonal variation in infection rate is assumed similar for the "measles" and "HIV" cases; however, the latter completely eliminates associated seasonal fluctuations in incidence due to the smoothing effect of prolonged infectious carriage.

Figure 5.7 also illustrates a second major dynamical impact arising from the trade-off of increased infectious period against lowered infection rate. "Fast" infections are much more prey to local stochastic extinction in the deep troughs between epidemics (Sect. 2.4) than the much more endemic incidence promoted by longer infectious period. On the other hand, acute immunizing infections can invade populations much more quickly than chronic infections for the same R_0 (Note that very imperfect immunity (corresponding to 'SIS' dynamics) could generate 'fast' invasion even for relatively chronic infections (Figure 5.7, inset), because of the increased supply of susceptible individuals (Sect. 5.2)). Note that, despite these great variations in dynamics, the assumption of a common R_0 means that the herd immunity

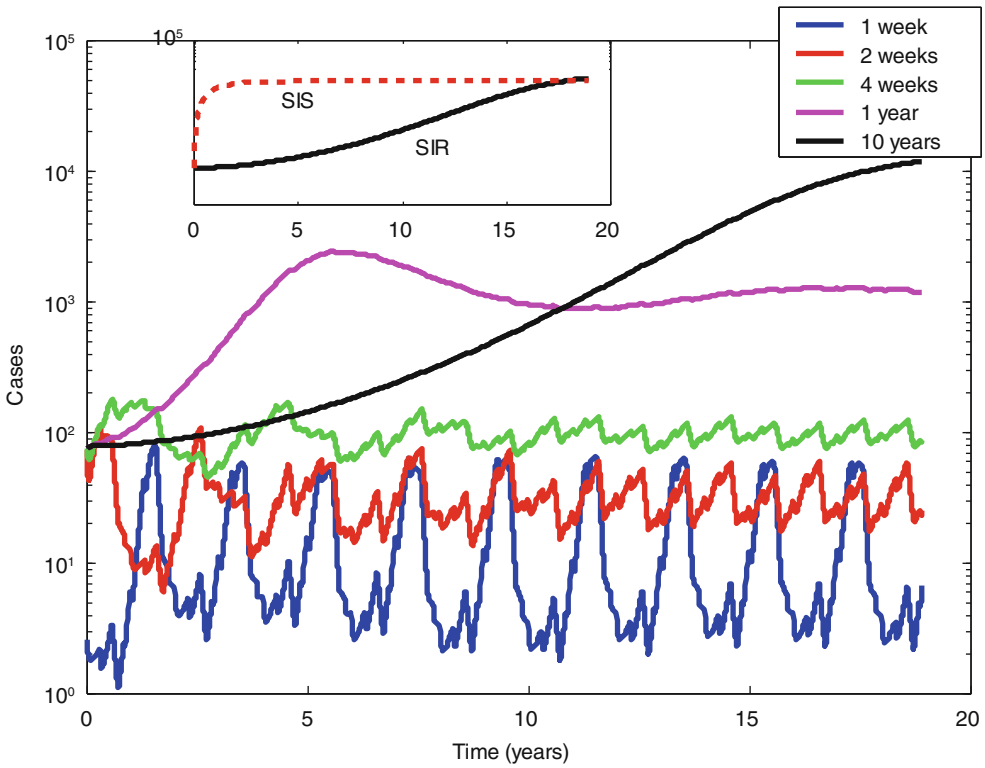

Fig. 5.7 Numerical solutions of seasonally forced SEIR models (see Fig. 5.3b), showing changes in the dynamics of infection caused by increasing the infectious period (see *color key*) while maintaining the basic reproduction number of infection at a constant level. To simulate crudely the initial dynamics of a "novel" infection, the system starts by introducing 6 % infectives into a 20 % susceptible population (a real novel epidemic might be much more violent if everyone is susceptible). *Inset*: Comparing the "slow" dynamics generated by a 10-year infectious period with a (sketched) solution of an SIS model with much faster dynamics (Taken from Figure in Box 2, Grenfell and Harwood [47])

threshold for elimination of infection is the same across this range of behaviors Sect. (2.3).

5.2 Departures from the SEIR Paradigm

We have seen that the SEIR framework (Fig. 5.1b) can be a powerful tool for capturing the dynamics of perfectly immunizing infections such as measles. With its various refinements to incorporate structure in the host population (such as age and spatial distribution), it yields a rich variety of dynamical behavior (Fig. 5.7). Given the range of immunological complexities and different viral life histories, however, it is clear that SEIR dynamics are only part of the story. In such cases, it is often possible to adapt Fig. 5.1b to more faithfully reflect such important biological complexities.

5.2.1 Waning Immunity

Many viruses are capable of reinfecting a host. This may be due to the waning of host immunity over time, as with respiratory syncytial virus (RSV) [49], or due to viral evolution for immune escape (e.g., with norovirus [50] and influenza) [51, 52]: see Sect. 6 for a more in-depth treatment of the latter. In either case, the overall dynamical effect is for recovered, immune individuals to eventually reenter the susceptible pool, as illustrated by Fig. 5.8a.

Where such waning of immunity is appreciable, the implications for vaccination can be profound. As the susceptible pool is replenished not only by births but also by previously immune individuals, the critical vaccination threshold (Sect. 2.3) is raised, making it more difficult to control the spread of infection. For this reason, vaccination against many imperfectly immunizing infections is aimed at direct protection of those receiving vaccine, rather than at raising indirect protection.

5.2.2 Partial Immunity

So far, the models we have considered assume that immune individuals have solid, transmission-blocking immunity, even if this should wane through time. In reality, however, it is common for immunity to be clinically protective (i.e., against symptomatic disease) but only partially protective from infection. This applies, for example, in the case of rotavirus, the leading cause of severe diarrhea in infants worldwide [53, 54]. Figure 5.8b illustrates a model structure developed to capture these dynamics [46].

5.2.3 Host Heterogeneity in Transmission

Recalling the qualitative differences between the dynamics of acute and chronic infections (Fig. 5.7), some viruses show a "mixed" character on the host population level: while some individuals clear infection relatively quickly, others may continue to harbor the virus as "carriers," either in a latent phase or in a chronic infectious state, for a longer period. An example is hepatitis B, in which 5–10 % of adult patients show chronic infection. As illustrated in Fig. 5.8c, this can be represented mathematically by identifying two infectious classes: one acute and the other chronic.

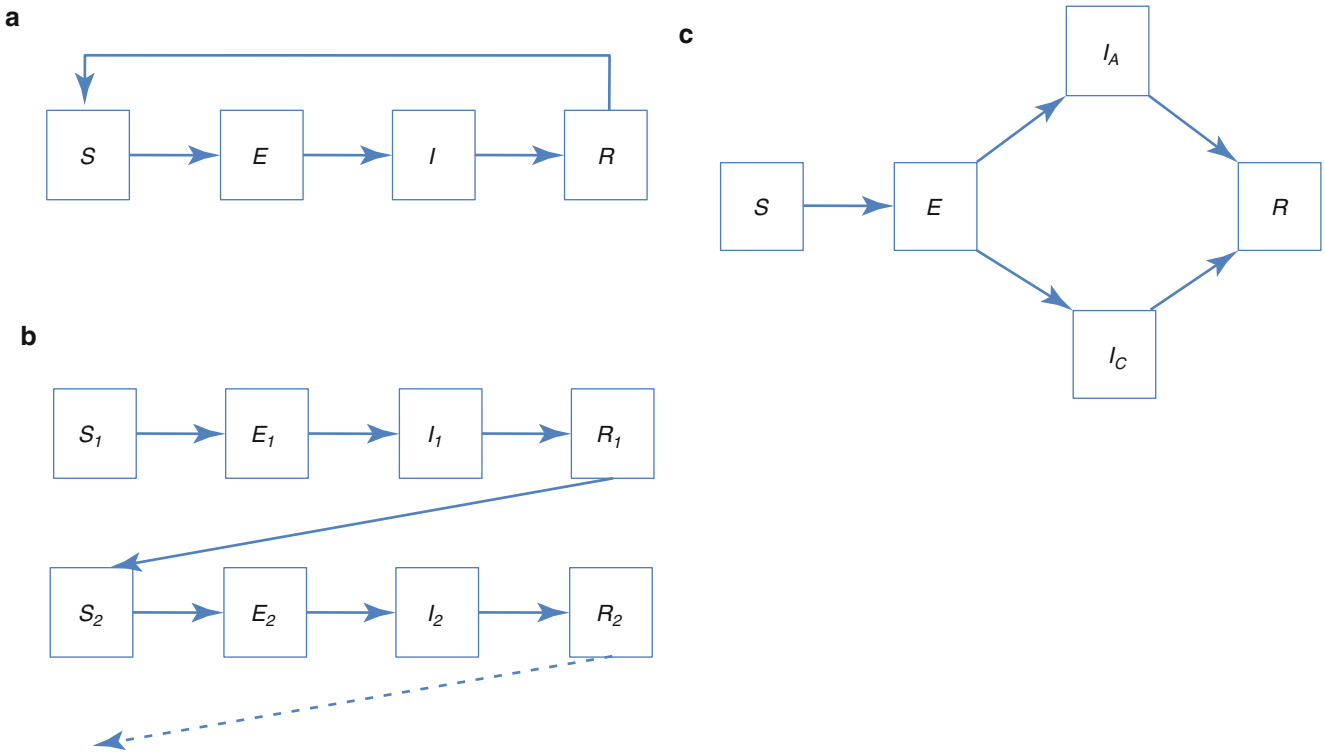

Fig. 5.8 Examples for extensions of the basic SEIR model (Fig. 5.1b). (**a**) Waning immunity can be represented by allowing individuals to return from recovered (R) to susceptible (S) status at a given rate. (**b**) If acquired immunity affords clinical but not sterilizing (transmission-blocking) protection, then additional SEIR stages may be incorporated to capture the corresponding effects on transmission dynamics. (**c**) An example of population heterogeneity, distinguishing "acute" cases (I_A) from "carriers" (I_C), the latter recovering at a slower rate than the former

Such factors can have implications for the dynamics and persistence of infections in a population [55]. In particular, where a disease shows epidemic cycles, facing the possibility of local extinction in the epidemic troughs, the presence of a small number of carrier hosts can facilitate viral persistence in the population, through these troughs. For example, in some cases infection with varicella zoster virus can be followed decades later by infectious shingles, an occurrence which could maintain the virus in small populations [56].

5.2.4 Complex Viral Strain Interactions

Strain structure, vector dynamics, and seasonality in transmission offer yet more complexities, as in the example of dengue [57]. Spread by *Aedes* mosquitoes, dengue has shown a significant emergence worldwide in the last 50 years.

Dengue dynamics are notable for the global circulation of four distinct serotypes (see Fig. 5.9a). Notably, prior immunity to a given serotype can elevate the risk of severe disease from subsequent infection by a different serotype [60–62]. It has been proposed that this arises from antibody-dependent enhancement (ADE) between different serotypes [63, 64], and modeling studies suggest that ADE could play an important role in shaping the epidemiological and strain dynamics of dengue, on the host population level [65–67].

However, there are also indications of a short period of cross-protective immunity (i.e., against all serotypes) for 2–9 months following infection [68], potentially mediating some degree of competition between serotypes. Several studies have addressed the dynamical implications of interactions between ADE and such immunity [69, 70]. Dengue dynamics are therefore complex and multifactorial: an understanding of these effects is important in understanding the potential for unexpected effects from transmission-reducing interventions [71].

6 Dynamics and Evolution of Immune Escape

Had viruses been discovered by the time that Darwin proposed "descent with modification" in the mid-nineteenth century, they may quickly have been recognized as fine examples of evolution in action. Indeed, today there is wide acknowledgment of the inextricable role that evolution plays in viral dynamics [58] and in the control of many different viral infections. RNA viruses in particular, lacking the replication fidelity of a DNA genome, are capable of considerable mutation rates [72]. In the presence of host immunity, natural selection

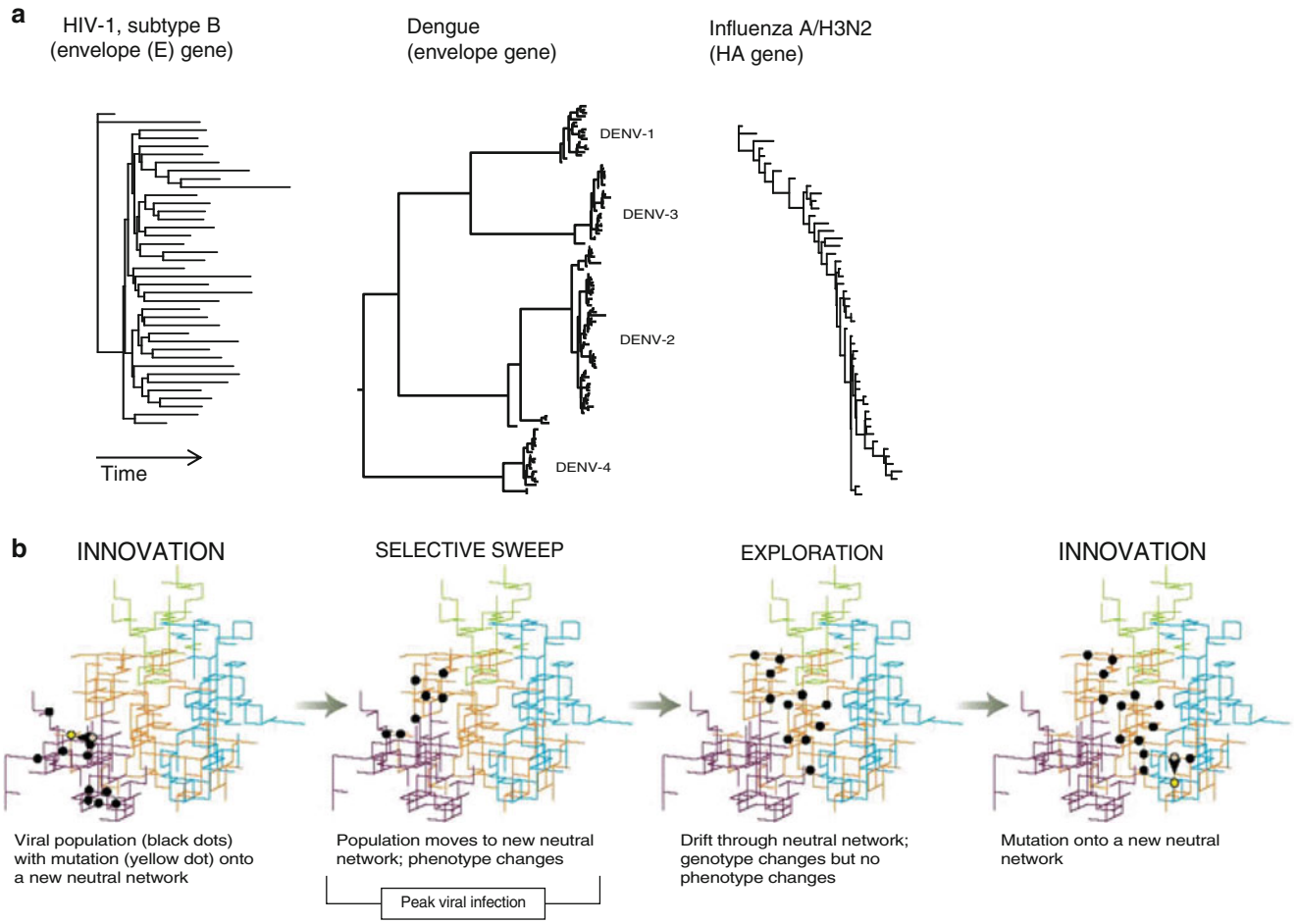

Fig. 5.9 (**a**) A diversity of viral diversity: examples of major human infections showing different patterns of evolution (Taken from (Ref. [58]), and references therein; Adapted from Fig. 1, Grenfell et al. [58]) (**b**) Schematic illustration of the "epochal evolution" model, which seeks to explain notable features in the phylogeny of human influenza A (panel **a**, rightmost phylogeny) (Taken from figure in van Nimwegen [59])

will favor those mutants most capable of evading immunity while still being capable of spreading between hosts.

As a result of these high mutation rates, population-level evolutionary patterns that can arise are quite diverse. Figure 5.9a illustrates how some infections, such as HIV, show significant population-level diversity [73], while others, notably seasonal influenza A, exhibit a markedly different pattern, with a "trunk-like" phylogeny on the population level [74].

The case study of influenza demonstrates not only interesting evolutionary patterns but also the following important features:

(i) The challenges that evolution can pose for infection control
(ii) The complex interplay, across a range of physical scales, between the evolution of a pathogen and its success in spreading in a host population
(iii) The many outstanding questions that remain, in understanding how viruses adapt to continue reinfecting their hosts

In what follows, we explore both within-host and population-level aspects of influenza evolution, with an emphasis on seasonal (interpandemic) influenza. We then set out the prevailing paradigms that seek to explain how these patterns arise, through complex interactions between viral evolution and epidemiology.

6.1 Immune Escape and Herd Immunity

Classic theoretical principles [4] provide a useful framework in which to think about viral evolution, where "evolutionary fitness" is determined ultimately by the capacity for transmission in a given host population. Recall from Sect. 2.3 that in the presence of prior immunity in the population, the effective reproduction number, R, serves as a compact measure of transmission potential. Vaccination aims to lower R through reducing the number susceptible in the population; however, evolving pathogens introduce the complexity of countering this effect, by evading immunity and thus acting

to restore R. Note the combination of immune escape and transmissibility encapsulated in R. In particular, on the scale of the host population and with a given level of existing immunity, the escape mutant with the greatest evolutionary fitness is that which maximizes R [5].

6.2 Molecular Aspects of Viral Evolution

As discussed in Chap. 21, the influenza surface protein hemagglutinin (HA) is a key immune target, with anti-HA antibodies capable of offering sterilizing immunity, that is, the potential to block host infection altogether. Indeed, raising such immunity is a central function of current influenza vaccines. However, HA is also the most variable viral component, continually under selective pressure to escape antibody binding [51]. For these reasons, while influenza evolution is by no means limited to HA, this viral component has attracted the most attention.

An important mechanism of immune escape is through conformational changes of HA epitopes to abrogate antibody binding, without compromising the ability of the virus to attach to host cells [75]. More recently, however, results from mouse experiments suggest that escape mutants can also acquire increased viral avidity for host cells [76]. Another potential immune escape strategy [77] is glycosylation, or the attachment of oligosaccharides to the HA molecule, to occlude epitopes from antibody binding. Such strategies are not without potential functional costs for replication [78], and yet there are indications that human influenza A/H3N2 has been accumulating glycosylation since its introduction into the human population in 1968 [79, 80].

6.3 Population-Level Manifestations of HA Evolution

On a genetic level, the evolutionary pattern for influenza HA (Fig. 5.9a), showing serial replacement of strains through time, is often characterized as "drift." The limited standing diversity, or "trunk-like" phylogeny, is especially paradoxical in light of the relatively high mutation rate of influenza A, where instead several lineages might have been expected to emerge and coexist in the population.

Moreover, how do such *genetic* patterns translate to *antigenic* properties, that is, viral interaction with anti-HA antibodies? In 2004, new techniques allowed a characterization of the antigenic evolution of the influenza subtype H3N2 [74] during the years since its introduction into humans in 1968. The resulting pattern is characterized by "punctuations" in antigenic evolution, occurring roughly every 2–8 years, in contrast with the more gradual pattern of genetic change shown in Fig. 5.9a. These punctuations have great importance for public health, often necessitating major reformulation of current, HA-based vaccines [81].

Two prevailing paradigms explaining these important phenomena, on both the genetic and antigenic levels, are the *epochal evolution* model [82] and that of *strain-transcending immunity* [83]. Both may be considered "phylodynamic" models [58], in the sense of aiming to capture the complex interactions between viral immune escape, viral population genetics, and epidemiology.

The picture of "strain-transcending immunity" [83] invokes a temporary mode of immunity, which immediately follows recovery from infection and protects the individual against infection by all influenza strains. Such broad immunity is postulated to arise, for example, either from T cells or from innate immunity [83]. Over the course of months, this broad immunity gives way to more long-lived, more narrowly acting immunity that is specific to the immunizing strain. On the population level, a key dynamical effect of such immunity structure is to impose population-level constraints on viral diversity, sufficiently strong to maintain a single dominant lineage through time.

The epochal evolution model [82] provides an alternative view. It builds on the proposal by Kimura in 1968 [84] that many amino acid substitutions in nature do not alter evolutionary fitness and are thus *phenotypically neutral*. If influenza HA evolution can be thought of as tracing a series of paths through a space of genotypes, the epochal evolution model proposes that, in phenotypic terms, such a space has a modular structure (Fig. 5.9b) in which each "module" is a group of genotypes sharing the same antigenic phenotype and a transition between modules corresponds to the observed antigenic "jumps." In particular, HA evolution diffuses through the local genotype space, accumulating neutral substitutions and thus genotypic diversity, over several years. Ultimately, a single strain accumulates the substitutions required to transition to a new antigenic phenotype. At this point, the emergent strain – owing to its antigenic novelty – causes a peak in infection and undergoes a selective sweep in the host population, to the exclusion of other strains. Such an event thus acts to periodically control antigenic diversity, maintaining the "trunk-like" shape of the influenza phylogeny.

Alongside these two prevailing paradigms of influenza evolution, yet another model [85] proposes that observed strains are drawn from a limited set of antigenic types, with their selection dependent on niches in host population immunity. While each of these models succeeds in capturing important features of influenza dynamics, they also highlight important gaps in our understanding of viral evolution and how to manage it.

7 Coevolution and the Evolution of Virulence

The degree to which viruses harm hosts varies considerably. Both within and between virus species, some variants may barely affect hosts at all, while others have significant health

impacts, up to and including host mortality [86]. An evolutionary perspective indicates that variation in virulence across clones should be shaped by the costs and benefits of virulence for parasite transmission [87]. The spread and persistence of virulence mutations may be favored if they covary with transmission. However, since host mortality can interrupt transmission, a range of values of virulence may lead to equivalent levels of overall fitness for the parasite [88]. While it is broadly agreed that the theoretical framework and empirical evidence on the existence of virulence-transmission trade-offs need to be further developed and extended [87], there are some systems where the general predictions appear to be borne out (e.g., [89, 90]). Perhaps most famously, the introduction of the myxoma virus into Australia was at first devastating to rabbit populations, causing near 100 % mortality. However, over subsequent years, the virulence of the infection decreased. Various lines of evidence suggest that this occurred in response to selection for increased transmission via decreased virulence – swift rabbit mortality was not an efficient mode of transmission for the virus.

8 Within-Host Dynamics

While we have so far largely discussed virus dynamics on the level of the host population, there are equally important processes to be considered on the within-host level. The "kinetics" of viral infection arise from a complex array of factors including viral replication and variability, the dynamics of the immune response, and pathogenicity to the host [91]. Over the past two decades, mathematical models have played an important role in studying these dynamics, often motivated by the need to understand the actual and potential impact of interventions such as treatment or immunization [91–93].

A major example is HIV infection (see Chap. 43); its clinical course is characterized by an initial acute phase of viral replication, lasting on the order of weeks, followed by an asymptomatic latent phase that can last decades before ultimately progressing to AIDS [94]. Major advances in the 1990s showed that despite the long clinical timescale involved, in vivo HIV replication is in fact a rapid process, with the viral life cycle being on the order of days [95, 96]. Subsequent studies modeling the dynamics of drug resistance underlined the need for early and aggressive drug therapy [97]. Conversely, more recent work capturing the dynamics of the immune response has illustrated how immunity raised by CD8+ T-cell vaccination elicits a response that is too weak and too late to achieve sterilizing immunity [98].

Hepatitis C virus (HCV) represents another major public health challenge and, like HIV, is a rapidly mutating virus capable of continually evading immunity to establish chronic infection [99]. HCV infection is currently treated with broad-spectrum combination antiviral therapy including interferon-alpha, ribavirin, and protease inhibitors, with upwards of 50 % of treated patients being responders; however, there is a need for a more rationally optimized approach. Models capturing viral dynamics in treated and untreated patients have contributed to an understanding of the action of these therapies [100–102], estimates of parameters such as rates of viral growth and of viral RNA clearance [102, 103], and correlates of long-term response to therapy [104].

Modeling approaches have been used to study the kinetics of acute infections too, notably in the context of influenza infection [105]. A key interest, for example, has been the relative importance of target cell depletion, innate immunity, and adaptive immunity in shaping the dynamics of viral infection [106–108].

Overall, while there remain significant gaps in our understanding of these and other major viral infections [103, 109], this work demonstrates the clinically relevant insights that can be derived from a careful study of within-host dynamics [91, 110].

9 Summary and Future Directions

This chapter has broadly outlined the multitude of factors shaping viral dynamics, as well as the role of mathematical approaches in elucidating these factors and their interactions. This growing body of work sets the stage for future directions.

The link between epidemiological modeling and policy is long-standing. Bernoulli's work provides perhaps the earliest case study [1]; recent high-profile examples include the use of models to guide responses to foot and mouth disease in the UK [111], smallpox [112], and influenza [113]. The key criterion of the effective deployment of models for policy is that they are embedded in testable hypotheses, embodying the best possible science. Continuing progress in this area thus calls for better understanding of the basic principles.

Especially in the context of viral evolution, whether in relation to immune escape or virulence, a complete biological framework calls for a linkage of processes across disparate scales, from the macroscopic structure of the host population to the molecular basis of viral replication and transmission. A key task at the heart of this challenge is arguably to quantify transmission potential in terms of immune escape, that is, to measure empirically how changes at the molecular level impact R (see Sect. 6.1). Indeed, recent equine influenza experiments provide some important steps in this direction [114].

New and emerging ways of tracking disease may also help to shed new light on the global spread of viruses and on how they may be better controlled. To name three examples, while disease surveillance continues to operate largely through public health channels, there is increasing interest in the use of alternative sources, including automated

tracking of Internet and social media trends [115, 116]. Second, genetic sequencing and analysis complements existing epidemiological approaches. The advent of high-throughput whole-genome sequencing is expected to shed new light on within-host viral diversity [117], an important aspect in our understanding of viral evolution. Third, any viral infection leaves its mark on the host immune system. Serological studies thus offer another valuable source of information in monitoring diseases [32] and already play an important role in many national surveillance programs (see Chap. 4). While in some cases there remain challenges in the clinical interpretation of quantitative serological data (e.g., Ref. [118]), its future use in estimating, for example, the prevalence of subclinical, but infectious, cases could be of significant value to public health efforts [119–121].

Finally, it is important to consider the role of the hosts themselves. With the host population providing the medium through which viruses spread, important factors in viral dynamics include heterogeneities among individuals (e.g., host genetic variation [122] and "superspreaders" of infection [123]) and patterns of human connectivity and mobility. Various approaches are beginning to unravel some of these patterns, both in the developed [34, 124] and in the developing world [125]. Future developments in such techniques will provide valuable new data for understanding the human role in viral dynamics.

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Part II

Viruses Causing Acute Syndromes

Adenoviruses

6

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1 Introduction

Adenoviruses are among the most widely distributed viruses in nature, infecting vertebrates as diverse as mammals, birds, fish, and reptiles [1]. Those adenoviruses that infect humans (HAdVs) are ubiquitous among the general population and have a worldwide distribution. Most HAdV infections occur in early childhood. They are most frequently associated with febrile upper respiratory tract illnesses with pharyngitis, pharyngoconjunctivitis, or coryza but can occasionally cause pneumonia. Other manifestations of HAdV infections include gastroenteritis, keratoconjunctivitis, and rarely cardiac, genitourinary, and neurologic diseases. Most HAdV diseases are self-limited and do not require therapy. However, severe and sometimes fatal infections can occur in immunocompromised hosts, neonates, and occasionally healthy children and adults. Although there have been no clinical trials performed to document efficacy, severe HAdV infections are most frequently treated with cidofovir, an antiviral agent licensed for the treatment of cytomegalovirus disease.

HAdVs can cause persistent infections, with the virus being shed for months after acute infection. Additionally, HAdVs are heterogeneous and subject to recombination among serotypes. Consequently, new types and variants of established serotypes appear over time.

Certain HAdV serotypes have caused epidemics of acute respiratory disease (ARD) in military training camps. Live enteric HAdV vaccines have been used safely and effectively to prevent outbreaks of ARD in new military recruits since the early 1970s. After the manufacture of the HAdV vaccines was discontinued in 1996, outbreaks of ARD reemerged in both military recruits and some civilians, prompting redevelopment of the vaccines.

Adenoviruses are also under intensive investigation as vectors to deliver foreign genes. Although the immunogenicity of adenovirus vectors and the high prevalence of adenovirus-specific memory immune responses have been obstacles for long-term gene therapy, recombinant adenovirus vectors have potential for use to immunize against other pathogens and as tumor vaccines.

2 Historical Background

In 1953, Rowe et al. [2] described an agent that caused spontaneous degeneration of surgically removed human tonsils and adenoids in tissue culture. In 1954, similar agents were isolated from military personnel with febrile respiratory disease [3], and in 1956 the term "adenovirus" was first adopted [4]. Subsequent studies revealed that these newly discovered agents were a major cause of epidemics of acute respiratory disease (ARD) in military training camps [5].

There is evidence that HAdV infections were present long before the 1950s. Epidemics of ARD were described in military training camps as far back as the Civil War. The adenoviral syndrome epidemic keratoconjunctivitis (EKC) was first described in German workers in the late 1800s [6]. Outbreaks of EKC were commonly observed during World War II in US shipyards, where the disease was referred to as "shipyard eye" [7]. Transmission likely took place in medical facilities where workers sought treatment for foreign bodies or chemical irritation of the eye.

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Epidemics of a febrile illness with conjunctivitis related to swimming activities were first described in the 1920s [8]. When diagnostic tools for HAdVs were developed in the 1950s, all swimming-pool-associated outbreaks of pharyngoconjunctival fever were traced to them [9]. HAdVs also became recognized as important causes of febrile respiratory illnesses in infants and young children [10]. Continued outbreaks of ARD in military training camps led to a large number of epidemiologic studies of HAdV infections in the military [11, 12]. These investigations culminated in the development of HAdV vaccines that were introduced in 1971 for use in military recruits to help prevent ARD [13, 14].

3 Virus Characteristics

3.1 Virion Structure and Antigenic Composition

Adenoviruses exhibit a distinct morphology and chemical composition. The virion consists of a 70–90 nm diameter nonenveloped icosahedral capsid that packages a single double-stranded linear DNA genome of approximately 35 kb in length [15]. The capsid is composed of 252 capsomeres, of which 240 are homotrimeric hexons and 12 are pentameric pentons located at the vertices of the icosahedron and from which project a single, variable-length homotrimeric fiber (Fig. 6.1) [16]. The species F enteric HAdVs uniquely possess two different lengths of fibers that occur alternately on the vertexes. Four minor proteins (IIIa, VI, VIII, and IX) that stabilize the structure are also elements in the capsid [17]. Six other proteins are located in the virus core, of which five (V, VIII, Mu, IVa2, and the terminal protein) are associated with the viral genome and the remaining component protease facilitates virion assembly. Type-specific antigenic determinants that give rise to neutralizing antibodies are located primarily on the surface of the hexon capsomere, the fiber, and, to a lesser extent, the penton protein.

Type-specific determinants on the hexon map to loops L1 and L2 of the hexon monomer and are encoded by seven hypervariable regions of the hexon gene, HVR1-6 (L1) and HVR7 (L2) [18]. A genus-specific antigen is located on the interior surface of the hexon. In addition, the fiber is a potent hemagglutinin that also contains type-specific antigenic determinants recognized by hemagglutination inhibition (HI).

3.2 Adenovirus Classification

Adenoviruses are classified within the family *Adenoviridae* and further into five genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*, and *Ichtadenovirus*. HAdVs, located within the genus *Mastadenovirus*, are divided into seven species, A–G, based on several of the following characteristics: phylogenetic distance, genome organization, DNA homology (G+C%), number of virus-associated RNA genes, ability to recombine, cross-neutralization, oncogenicity in rodents, and differential ability to agglutinate human and animal erythrocytes [19]. Species B can be further divided into two subspecies, B1 (3, 7, 16, 21, 50) and B2 (11, 14, 34, 35), based on homology of the viral genomes, tissue tropism, and associated disease patterns [20, 21].

There are 51 recognized HAdV serotypes defined classically by serum neutralization (SN) (Table 6.1), and several new "types," including one that belongs to the new species G (HAdV-52), have been recently described by molecular analyses [22]. For further discussion of the use of molecular and immunotyping methods for classifying HAdVs, see Sects. 12 and 12.1, below.

Some HAdVs show considerable intraserotypic genetic variability, best documented by genome restriction fragment analysis [23, 24]. For example, more than 20 DNA variants or genome types of HAdV-7 have been identified among strains isolated worldwide, and regional shifts or replacement of predominant genome types have been documented [25]. Designation of HAdV genome types is based on *Bam*HI as the "type" defining restriction enzyme, with different genome types denoted with a character, e.g., "p" for the prototype strain and then "a" through "k" for *Bam*HI variants. Genome types that are further distinguished by restriction pattern with additional enzymes are given an Arabic numeral (e.g., Ad7p, p1, a, a1-6).

Many HAdV species and serotypes exhibit distinct tissue tropisms and clinical manifestations, reflecting preferential infection of the respiratory, gastrointestinal, and urinary tracts and eyes. Typically, HAdV species B (particularly serotypes 3, 7, 14, and 21), C, and E cause respiratory tract infections, whereas the enteric species F serotypes 40 and 41 and species A (particularly serotype 31) are more commonly associated with infantile gastroenteritis. Species D serotypes 8, 19, and 37 are responsible for most outbreaks of EKC. Naturally occurring intermediate strains, which arise from intermolecular recombination [26] and possess the hemagglutinin (fiber) of one serotype and serum-neutralizing determinants (hexon) of another serotype, are well documented and may exhibit modified tropism and host pathology [20]. For example, species B HAdV-11p, identified in the 1950s in association with acute hemorrhagic cystitis, was later identified in outbreaks of respiratory disease as a novel genome type, HAdV-11a [27, 28]. Subsequent molecular analyses revealed that HAdV-11a (also referred to as HAdV-11/H14 or HAdV-55) is a recombinant virus that acquired the HAdV-14 fiber gene and possibly enhanced tropism for the respiratory tract [28–30].

3.3 Adenovirus Replication

Adenoviruses exhibit a highly restricted host range. Nonhuman primate species are poor hosts for HAdVs, as

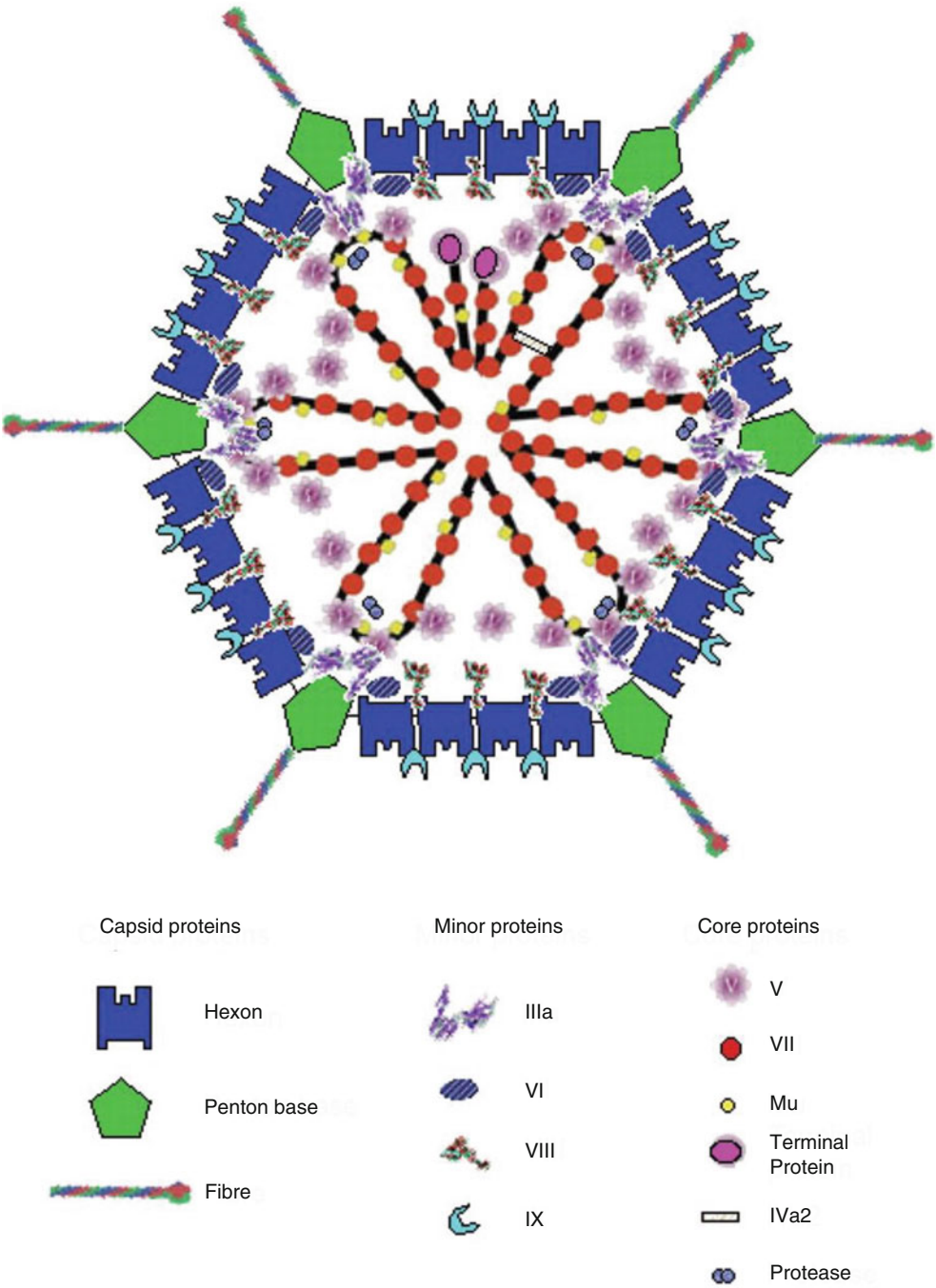

Fig. 6.1 Schematic diagram of the adenovirus virion and associated structural proteins (Reprinted with permission from Russell [16])

shown by the inefficient replication of HAdVs in simian cells unless coinfected with the helper papovavirus, SV-40 [31]. Although most HAdVs do not induce clinically apparent disease in common laboratory animals, hamsters, cotton rats, rabbits, and mice have been used to model respiratory and ocular infections with some HAdVs [32]. All HAdVs can transform cultured rodent cells to a malignant state, and species A, and to a lesser extent species C viruses, can induce tumors in newborn hamsters. However, there is no clear evidence that HAdVs can cause cancer in humans. For further discussion, see Sects. 12 and 12.4, below.

The adenovirus infectious cycle consists of an early and late phase separated by the onset of viral genome replication [15]. The early phase begins with virus entry into the host cell that proceeds by a two-step process: (1) binding of the fiber knob domain to the cell receptor followed by (2) interaction between a specialized motif on the penton base (Arg-Gly-Asp (RGD) peptide sequence) and cellular integrin, as a secondary receptor, triggering virus internalization [33]. Two well-documented HAdV receptors are the membrane cofactor protein (MCP or CD46), shown to bind most species B HAdVs, and the coxsackievirus-adenovirus receptor (CAR),

Table 6.1 Properties of human adenovirus serotypes

Species[a]	Serotypes	Hemagglutination groups[b]	Oncogenicity in newborn hamsters	Fiber length (nm)	DNA			Most common disease syndromes[d]
					% homology[c]	% G+C	Number of *SmaI* fragments	
A	12, 18, 31	IV	High	28–31	48–69/8–20	47–49	4–5	Gastroenteritis
B	3, 7, 11, 14, 16, 21, 34, 35, 50	I	Weak	9–11	89–94/9–20	50–52	8–10	URI, LRI, cystitis
C	1, 2, 5, 6	III	Negative	23–31	99–100/10–16	57–59	10–12	URI, LRI, endemic viruses
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51	II	Negative	12–13	94–99/4–17	57–60	14–18	EKC, asymptomatic rectal carriage
E	4	III	Negative	17	4–23	58	19	URI, LRI
F	40, 41	III	Negative	~29	62–69/15–22	52	10–12	Gastroenteritis

Adapted from Schnurr [278], Hierholzer [20], and De Jong et al. [21]

[a]Species G and types identified solely by molecular methods are not included in the table. Please see Sects. 12 and 12.1

[b]*I* complete agglutination of monkey erythrocytes, *II* complete agglutination of rat erythrocytes, *III* partial agglutination of rat erythrocytes, *IV* little or no agglutination

[c]Range of percent genome homology among serotypes of the same species (first listed) and between serotypes of different species (second listed)

[d]*URI* upper respiratory illness, *LRI* lower respiratory illness, *EKC* epidemic keratoconjunctivitis

that binds HAdV serotypes from all other species [34]. Integrins, CD80/CD86, sialic acid, and other cellular glycoproteins have also been implicated as receptors for some HAdVs. Following virus entry and passage to the cell nuclease, selective transcription and translation of the early viral genes occur that facilitate viral genome replication. There are five immediate early transcription units: early region 1A (E1A), E1B, and E2–E4. E1A encodes proteins that activate transcription and induce the host cell to enter the S phase of the cell cycle, and E1B proteins prolong cell survival and block apoptosis. E2 proteins provide machinery for viral genome replication and ensuing transcription of late genes. E3 proteins are nonessential for virus replication but help subvert the host's defense mechanisms to prolong survival of the infected cell in vivo (see Sects. 7 and 8, below). E4 proteins facilitate virus messenger RNA metabolism, genome replication, and shutoff of host protein synthesis. The late phase consists of transcription of the late transcriptional units (L1–L5) that leads to production of the viral structural components, assembly of the virus particles in the nucleus, and eventual release from the host cell. Replication-deficient mutants with deletions of the E1 transactivating region have been developed for use as vectors (see Sect. 11, below).

4 Methodology Involved in Epidemiologic Analysis

4.1 Sources of Data

HAdV disease is not reportable in the United States outside of the military. Nevertheless, numerous studies conducted in military training camps, pediatric institutions, day-care centers, primary schools, groups of families, the broader community, and other settings have allowed a detailed description of the epidemiology of HAdV infections. Global data assembled by the World Health Organization on type-specific HAdV isolates in the early 1980s [35] and more recent data from the United States on the prevalence and risk factors for severe HAdV disease from 2004 to 2006 are also available [36].

4.2 Laboratory Tests

Because the disease syndromes caused by HAdVs are not easily distinguished clinically in the absence of epidemics, laboratory testing is crucial for diagnosis. Laboratory diagnosis can be accomplished by virus isolation, polymerase chain reaction (PCR) assay, antigen detection, or serology. Viral detection is greatly enhanced by proper collection of specimens from affected sites in the early phase of illness. The type of specimen collected is dictated by the clinical presentation of the patient and the type of test to be administered. Throat and nasopharyngeal specimens are suitable for detection of HAdVs in the upper respiratory tract. Fecal specimens or rectal swabs are the specimens of choice for HAdV-associated gastroenteritis and may complement diagnosis of respiratory tract infections as virus shedding from the gastrointestinal tract may persist for prolonged periods after disappearing from the upper respiratory tract. Conjunctival swabs are suitable for HAdVs causing ocular infections. Appropriately timed blood samples collected as soon as possible after the onset of symptoms and 2–4 weeks later are needed to establish diagnosis by serology.

4.2.1 Viral Culture

Isolation of HAdVs in cell culture provides the most conclusive evidence of viral infection. Recovery of the virus is usually not very difficult in the acute phase of illness. With the exception of the enteric HAdVs, most HAdV serotypes can be easily isolated in continuous human cell lines of epithelial origin such as A549, HEp-2, and HeLa. Fibroblast cell lines like MRC-5 and WI38 are generally less sensitive but can grow HAdVs after short adaptation. The enteric HAdVs can be isolated in Graham 293 cells, a human embryonic kidney cell line that was stably transformed with HAdV-5 E1 transactivating region [37]. Centrifugation of specimens directly onto culture cells using the shell vial technique has greatly reduced time for detection [38]. Adenoviruses assemble in the nucleus of infected cells, and the nuclear morphologic changes can be diagnostic on histologic examination. Typical cytopathic effect (CPE) appears as a rounding and clumping of cells in "grape-like" clusters within 1–2 weeks after inoculation. Adenovirus CPE does not immediately result in cell death, however. Rounding and detachment are caused by the penton base-induced cell detachment domain (RGD) located on the penton capsomere that is produced in excessive amounts [39].

4.2.2 PCR Assay

In recent years, the application of molecular methods such as PCR has significantly improved the sensitivity of HAdV detection and shortened the time for reporting test results. Numerous PCR methods have been developed for detection and identification of HAdVs. Conventional PCR assays targeting conserved regions in the hexon, fiber, or VA RNA genes using broadly reactive primer sets are capable of detecting all recognized HAdV types [40–42]. Commercial multiplex PCR assays that can simultaneously detect multiple respiratory pathogens including HAdV are now available that greatly enhance the ability of laboratory diagnosis and epidemiology investigation [43, 44]. Quantitative real-time PCR assays for pan-HAdV detection have become popular [45, 46], particularly in the clinical diagnostic setting. Quantitation of viral load by real-time PCR has aided our understanding of HAdV pathogenesis and has proven to be an essential tool for early diagnosis and monitoring of antiviral therapy in immunocompromised patients, especially in the posttransplant setting [47–49].

4.2.3 Antigen Detection, Histology, and Electron Microscopy

Antigen detection methods using monoclonal antibodies to the hexon group antigen permit rapid detection of HAdVs in clinical specimens. Immunofluorescence (IF) assays are widely used in clinical settings as they permit rapid, specific, and relatively low-cost diagnosis of HAdV infections [50]. Commercial latex and enzyme immunoassays for detection and identification of enteric HAdVs in stool specimens are also available. However, antigen detection techniques tend to be less sensitive than culture and PCR, are not amenable to high throughput, and may be insufficiently informative for epidemiologic studies.

Histologic examination of biopsy or autopsy tissues can help identify HAdV disease. HAdV-infected tissues often reveal cells with enlarged nuclei containing amphophilic or basophilic inclusions with indistinct nuclear membranes known as "smudge" cells [51]. Confirmation of intranuclear adenovirus antigen by immunohistochemical testing can localize infection in affected organs, providing a more definitive diagnosis (Fig. 6.2) [52].

Electron microscopy (EM) has been useful to visualize HAdVs that are not easily grown in cell culture, such as the enteric HAdVs. Because of their unique morphology, HAdVs can be rapidly identified by EM, but the method is insensitive and not widely available.

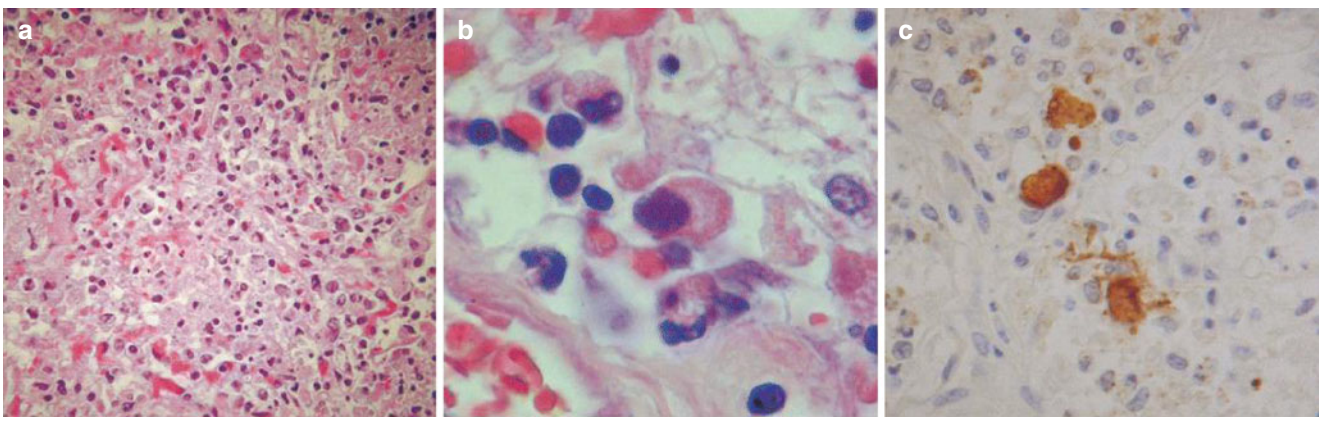

Fig. 6.2 Histologic section of the lung from a fatal case of adenovirus pneumonia. (**a**) Low-power photomicrograph of lung tissue demonstrating the presence of necrotizing exudates containing fibrin, inflammatory cells, and karyorrhectic nuclear debris (×200). (**b**) Same photomicrograph of lung tissue at higher magnification, demonstrating a degenerating, inclusion-bearing nucleus (smudge cell; ×1,000). (**c**) Immunohistochemical stain showing multiple nuclei containing immunoreactive adenovirus antigen (×400) (Reprinted with permission from Barker et al. [52])

4.2.4 Serology

As noted above, detection of HAdVs in respiratory or gastroenteric specimens can be difficult to interpret, especially when sensitive molecular methods are used. In such cases, serology can be helpful in distinguishing past from present infection. Detection of HAdV-specific antibodies in single serum specimens indicates prior infection with the virus. Seroconversion or a ≥4-fold rise in antibody titer between acute- and convalescent-phase sera is considered evidence of recent infection or possible reactivation of latent virus. Commonly used serological tests include enzyme immunoassay (EIA), serum neutralization SN, and hemagglutination inhibition HI. EIAs are easy to perform, but do not distinguish between serotypes. Although more laborious to perform, SN and HI assays provide type-specific information and have been useful for epidemiologic and vaccine efficacy studies [53].

4.2.5 Virus Typing

In the clinical setting, once a diagnosis of a HAdV infection has been made, further identification of the virus is often unnecessary. However, for research and epidemiologic purposes, species- and type-specific information may be useful to strengthen association with disease and document emergence of new, potentially more virulent strains. For example, detection of species B or E viruses in specimens from persons with acute respiratory illness or species F viruses with gastroenteritis would be more meaningful than detection of species C viruses, due to the latter's tendency to shed persistently from the upper respiratory and gastrointestinal tracts. Detailed molecular analyses documented genetic signatures unique to the recently reemergent species B HAdV-14 [54] and revealed patterns of virus transmission during outbreaks [55].

Identification of a HAdV isolate is classically performed by immunotyping (SN and HI), but these procedures are laborious and time-consuming, and reference antisera are not widely available. In the early 1980s, molecular typing by genome restriction fragment analysis as described above was used as an adjunct to immunotyping [24, 56], but this procedure also depends on culture-grown virus for implementation. Molecular methods have resolved many of these problems and are now used routinely for identification of HAdVs. PCR assays using species- or type-specific primers targeting the hexon, fiber, and VA RNA genes have been used for direct identification of HAdVs in clinical specimens [41, 57–59]. PCR and amplicon sequencing of hexon HVR1-6 [60] and HVR7 [61] have been shown to correlate with SN. The addition of fiber gene sequencing permits identification of some intermediate HAdV strains [62]. The availability of reference hexon and fiber gene sequences in the public domain makes preliminary type identification of HAdV strains possible. Full genome sequencing combined with bioinformatic analysis has allowed for more comprehensive characterization of HAdVs, which has been useful for exploring pathoepidemiology and evolutionary relationships [63].

5 Descriptive Epidemiology

HAdVs most commonly cause disease in infants and young children and are especially prevalent in day-care centers and households with young children. Longitudinal studies performed in families and pediatric facilities documented that species C HAdV infections occur early in life and are often endemic [64, 65]. Transmission among families was extensively investigated by the Virus Watch studies in the 1960s, during which biweekly collection of both fecal and respiratory specimens was performed [65, 66]. These studies identified a large number of asymptomatic infections with species C viruses and demonstrated a high frequency of fecal shedding of virus. HAdVs were found intermittently in fecal specimens for extended time periods, up to 1–2 years. Species F serotypes 40 and 41 cause gastroenteritis in infants and young children. In contrast, a large proportion of species A and D adenoviral infections are asymptomatic [67].

Species B (particularly serotypes 3, 7, 14, and 21) and E (serotype 4) infections tend to occur later and cause symptomatic respiratory disease, including epidemics of acute respiratory disease (ARD) in military recruits [12] and pharyngoconjunctival fever in summer camps [68]. In a study of 1,653 clinical isolates collected from 22 hospital laboratories in the United States between 2004 and 2006, species B HAdV-3 was the most commonly identified (35 %), followed by HAdV-2 (24 %) and HAdV-1 (18 %) [36]. These data are consistent with the fact that HAdV-3 is less prevalent than HAdV-1 and HAdV-2 but is more likely to cause a severe disease that requires hospitalization and/or testing.

5.1 Synopsis of Descriptive Epidemiology

5.1.1 Incidence and Prevalence

The incidence of infection is highest in infants and young children, during which time infections with the endemic species C HAdVs most commonly occur. In one family study, over 90 % of children under age 2 had SN antibodies to HAdV-1 and HAdV-2 [66]. Young children also most commonly shed virus in stool for prolonged periods after the resolution of symptoms. Most transmission occurs among families with young children, and the incidence is higher in lower socioeconomic groups. Rates of HAdV infections are higher in day-care centers and pediatric facilities.

5.1.2 Epidemic Behavior

The species E HAdV-4 and species B serotypes 7, 14, and 21 occur mostly in epidemics; species B HAdV-3 is sometimes endemic and sometimes epidemic.

Table 6.2 Most common adenoviral disease syndromes associated with specific patient populations

Patient population	Clinical syndromes
Infants and young children	Pharyngitis, coryza, tonsillitis, otitis media
	Pneumonia
	Gastroenteritis, mesenteric adenitis
Children	Pharyngitis, coryza, conjunctivitis
	Pharyngoconjunctival fever (PCF)
	Pneumonia
	Gastroenteritis
Adults	Acute respiratory disease (ARD)
	Epidemic keratoconjunctivitis (EKC)
Immunocompromised hosts	Pneumonia
	Gastroenteritis, hepatitis
	Hemorrhagic cystitis, interstitial nephritis
	Meningoencephalitis

5.1.3 Geographic Distribution

The species C HAdVs are endemic in most areas of the world [35]. Certain other types such as the species D HAdV-8 are endemic in underdeveloped countries such as parts of the Middle and Far East and Africa but primarily occur in epidemics in developed countries.

5.1.4 Seasonality

HAdV infections occur throughout the year but are more commonly found in late winter, spring, and early summer in temperate regions [65].

5.1.5 Age

Most children have been exposed to several types of endemic HAdVs by the time they enter school. Infections with the less common species E HAdV-4 and species B serotypes can occur later in life.

5.2 Epidemiologic and Clinical Aspects of Specific Syndromes

The clinical manifestations of HAdV disease vary according to the HAdV serotype and the age and immune status of the host (Table 6.2) [32]. HAdVs are responsible for 5–10 % of febrile illnesses in infants and young children [66].

5.2.1 Respiratory Illness

Infants and young children have the highest attack rates of endemic HAdV infections. These infections commonly present as mild upper respiratory tract illnesses and are most frequently caused by species C serotypes 1, 2, 5, and , to a lesser extent, 6 [69, 70]. Pharyngitis is frequently associated with conjunctivitis, laryngitis, or bronchitis. Fever is common and may be associated with malaise, headache, myalgia, and occasionally abdominal pain [71]. HAdVs are among the most common causes of tonsillitis in young children. Less frequently, HAdVs have been associated with otitis media (in children under age one), a pertussis-like syndrome, bronchiolitis, coryza without fever, or an exanthem.

The species B serotypes 3, 7, 14, and 21 have been associated with a more severe disease compared to the species C viruses [72, 73]. In particular, HAdV-7 has been observed to be one of the most pathogenic serotypes, causing a number of fatal lower respiratory tract infections [74–76]. Pneumonia is more severe in infants than older children and can be associated with extrapulmonary complications such as meningoencephalitis, hepatitis, myocarditis, and nephritis [77–80]. Other rare complications include disseminated intravascular coagulation and a toxic shock-like syndrome [73, 81]. Notably, there is a high incidence of pulmonary sequelae following HAdV pneumonia in children, especially bronchiectasis [82, 83].

5.2.2 Pharyngoconjunctival Fever

This HAdV syndrome is characterized by pharyngitis, conjunctivitis, and spiking fevers. Either one or both eyes can be involved, and lymphadenopathy is often observed. Diarrhea and coryza can sometimes occur, and tonsillar exudates can be observed. Pharyngoconjunctival fever is most commonly caused by species B HAdV-3 and HAdV-7 and species E HAdV-4 [68, 84]. The disease can be seen sporadically and be transmitted among families. However, this syndrome is best known as an epidemic disease in summer camps. Studies suggest that infection is transmitted by direct contact with water from contaminated swimming pools and small lakes; virus is likely introduced into the eye or upper respiratory tract [68]. Outbreaks of pharyngoconjunctival fever have been specifically associated with inadequate chlorination of swimming pools [85].

5.2.3 Acute Respiratory Disease (ARD)

ARD epidemics can occur under the special conditions of fatigue and crowding present in military training camps [13, 86]. Symptoms typically include fever, sore throat, and cough, sometimes with coryza, headache, or chest pain. Malaise is characteristic and lasts for approximately 10 days. Pneumonitis can also occur, resulting in rare fatalities [87, 88]. ARD epidemics have been associated with species E HAdV-4 and species B serotypes 3, 7, 11a, 14, and 21. Outbreaks in military training camps usually peak at about 3–6 weeks after the onset of training, resulting in morbidity rates as high as 6–17/100 per week.

Introduction of live, oral HAdV-4 and HAdV-7 vaccines for US military recruits in the early 1970s significantly reduced the incidence of ARD [14]. After the manufacture of HAdV vaccines stopped in 1996, however, there was a prompt rebound in high rates of HAdV infections [89, 90].

Rare new fatalities were reported in military recruits due to probable HAdV pneumonia with or without encephalitis [91]. In a study of 584 clinical isolates from military recruits collected between 2004 and 2006, 93 % were HAdV-4 [36]. HAdV transmission among new military recruits was studied using active surveillance for illness and enrollment and end-of-study viral throat cultures and serology [86]. Febrile respiratory infections due to HAdV-4 were identified in 25 % (67/271) of new recruits, and the percentage of recruits seropositive of HAdV-4 increased dramatically from 34 to 97 % during the first 4 weeks of training. The authors proposed that introduction of recruits with asymptomatic viral shedding into new training groups was likely a primary cause of continual transmission of HAdV-4 in the camp. In 2006, species B adenoviruses became more prevalent, including serotypes 3, 7, 14, and 21 [92].

Species B HAdV-14, which was first identified in the Netherlands in 1955 and caused sporadic outbreaks in Europe, emerged in US military bases in 2005. Since the initial reports, HAdV-14 has caused several outbreaks of ARD in both military recruits and civilians [88, 93, 94]. In a report of 140 cases, 38 % were hospitalized, 17 % were admitted to intensive care units, and 5 % died [95]. An analysis of 99 isolates from military and civilian cases in the United States revealed that all isolates were identical and suggested that they arose from recombination between HAdV-11 and HAdV-14 strains [54].

Due to the increasing incidence of ARD in military recruits, live, oral HAdV-4 and HAdV-7 vaccines were redeveloped by a new manufacturer and licensed in the United States in 2011 [96, 97].

5.2.4 Epidemic Keratoconjunctivitis (EKC)

In contrast to the benign course of pharyngoconjunctivitis, certain species D serotypes (8, 19, and 37) can cause a more serious keratoconjunctivitis [98, 99]. This disease is characterized by conjunctivitis, followed by the development of corneal infiltrates that cause pain, edema of the eyelids, photophobia, and lacrimation. One or both eyes may be involved, and preauricular lymphadenopathy is common. Although this disease does not usually result in permanent corneal damage, it can cause severe pain and blurry vision lasting up to 4 weeks.

A number of outbreaks of EKC have occurred in medical facilities, in particular ophthalmology offices. Infections can be spread from contaminated instruments, surfaces, or hands [100, 101]. Adenoviruses are relatively resistant to disinfectants, so heat sterilization of ophthalmology instruments (in particular, tonometers) is preferred. Hand washing with soap and water does not efficiently remove adenoviruses, so gloving is important. In one investigation of a HAdV-8 epidemic, almost 50 % of patients diagnosed with EKC carried virus on their hands, and the virus remained viable on inanimate surfaces for up to 35 days [102].

5.2.5 Gastroenteritis

Species F serotypes 40 and 41 are responsible for about 5–10 % of acute diarrheal illnesses in infants and young children. In one study of over 400 cases of acute infantile gastroenteritis, enteric adenoviruses were the sole recognizable cause of diarrhea in 7.2 % of cases [103]. Outbreaks have occurred in day-care centers and pediatric healthcare facilities. In a review of an outbreak in several day-care centers, 38 % of 247 young children tested had positive stool specimens for HAdV-40 or HAdV-41, although only half were symptomatic [104]. These fastidious enteric adenoviruses require special cell lines for growth but can be readily detected by enteric adenovirus antigen assays. The species A HAdV-31 is a less common cause of infantile diarrhea [105]. Most other serotypes have not been clearly associated with diarrhea, but are commonly shed in stool for months after infection. However, non-enteric species C HAdVs have been associated with mesenteric adenitis, which may mimic appendicitis and occasionally cause intussusception in infants and young children [106, 107].

5.2.6 Uncommon Syndromes

HAdVs can cause acute hemorrhagic cystitis in children (species B serotypes 11 and 21) [108]. Rarely, HAdVs have caused urethritis in adults (species D serotypes 19 and 37), and sexual transmission has been postulated [109, 110].

Meningitis and encephalitis have been reported occasionally in association with HAdV infections [111]. Neurologic involvement usually occurs in association with severe pneumonia, especially with HAdV-7.

HAdVs have been associated with acute myocarditis in children [112]. In one study, 38 myocardial tissue samples from 34 children with acute myocarditis were tested by PCR for a panel of viruses [113]. HAdV was detected more commonly (15 samples) than enterovirus (8 samples) and was not detected in control samples.

HAdVs have been occasionally associated with polyarthritis syndromes [114, 115]. Reye's syndrome has also been reported in association with HAdV infections in infants [116, 117].

5.2.7 Immunocompromised Hosts

HAdVs can cause severe, life-threatening infections in immunosuppressed persons, especially in the pediatric population [118–122]. Disease can be caused by primary infection or reactivation of latent infection. In transplant recipients, HAdV infections can also rarely be transmitted from the donated organ or bone marrow. Severe neonatal HAdV infections occur primarily in premature infants, and vertical transmission may play a role [123].

Pediatric hematopoietic stem cell transplant (HSCT) recipients have the highest incidence of HAdV disease and mortality [124, 125]. In one study of 204 pediatric HSCT recipients, 15.1 % had HAdV infections and 8.8 % developed

severe disease [126]. In contrast, in one study of 1,050 adult HSCT recipients, 4.8 % had HAdV infections and only 0.9 % had invasive disease [127].

The clinical spectrum of HAdV infections in HSCT recipients can range from asymptomatic shedding to fatal disseminated disease. A wide range of clinical syndromes has been reported, including pneumonia, colitis, hepatitis, hemorrhagic cystitis, tubulointerstitial nephritis, encephalitis, and disseminated disease. Disease is most frequently associated with the common species C serotypes 1, 2, and 5. Interestingly, there is also a preponderance of urinary tract infections due to species B serotypes 11, 34, and 35, which are infrequently isolated in the general population [127–129]. One hypothesis is that these types frequently cause latent infections of the genitourinary tract that can reactivate following severe immunosuppression.

In contrast to the HSCT population, HAdV disease typically involves the donor organ in solid organ transplant recipients. For instance, hepatitis is the most common manifestation of invasive adenoviral disease in pediatric liver transplant recipients. In one large study of 484 liver transplant recipients, 14 (3 %) developed HAdV hepatitis [130]. HAdV-5 was the most common isolate, and 43 % of patients died. In another study, examination of pre- and posttransplant sera from recipients and donors suggested that virus was transmitted from the donated organ [131].

In renal transplant recipients, HAdV can cause acute hemorrhagic cystitis, sometimes complicated by interstitial nephritis [132]. The incidence has not been well defined but is low, and this disease is exclusively associated with species B serotypes 11, 34, and 34. The prognosis is generally good, although infection occasionally results in fatal disseminated disease [133]. In one study of 339 adult renal transplant recipients, 17 (5 %) developed HAdV-related hemorrhagic cystitis [134]. Symptoms included dysuria, hematuria, fever, and bilateral testicular pain and lasted a mean duration of 2 weeks (range, 0.5–9.6 weeks). Reversible allograft dysfunction occurred in ten patients. One patient developed disseminated HAdV disease and died from bacterial sepsis.

In a study of pediatric cardiac transplant recipients, HAdVs have been implicated as a cause of graft loss and coronary vasculopathy [135]. HAdVs was detected by PCR in myocardial biopsies from 24 of 149 patients (16 %) and was associated with reduced graft survival.

Severe, frequently fatal neonatal infections have been described [123, 136]. In addition to the usual routes of transmission, neonates can acquire HAdV infection from exposure to cervical secretions at birth [137]. Rare cases of intrauterine infection have also been described [138]. Primary HAdV infections can also cause severe disease in children with immunodeficiency syndromes such as severe combined immunodeficiency disease (SCID) [139].

HAdVs are an uncommon cause of morbidity and mortality in HIV-infected individuals. However, a number of serotypes have been isolated from AIDS patients, including species B serotypes 11, 34, and 35 in urine and rare species D serotypes and intermediate strains in stool samples [140, 141]. Occasional fatal cases of adenoviral infection in AIDS patients have been reported, especially in the pediatric population [142, 143].

6 Mechanisms and Routes of Transmission

Transmission of HAdVs primarily occurs by the fecal-oral and respiratory routes. Fecal-oral transmission is common in households with young children [144]. Moreover, prolonged fecal shedding can sustain transmission in households and day-care centers. HAdVs can be transmitted via inhalation of droplets dispersed in the air by coughing and via direct contact with infected secretions or contaminated surfaces. The respiratory route is an important means of transmission in epidemics of ARD in military training camps. In one study, 50 % of air samples from recruit barracks were positive for HAdV DNA [86]. This study also detected viral DNA from 14 to 39 % of surfaces, suggesting that environmental contamination of living quarters is a possible contributing factor. Direct contact with contaminated pool water plays a major role in causing outbreaks of pharyngoconjunctival fever in summer camps [68].

Nosocomial transmission has been well documented [145, 146] and can be challenging to prevent because HAdVs are resistant to a number of common disinfectants and can survive on surfaces for weeks [100, 147]. In one investigation of a large community outbreak of EKC, HAdV-8 was isolated from multiple ophthalmic solutions from one physician's office [148]. In another report, HAdV-8 conjunctivitis occurred in 7 premature infants who had undergone ophthalmological examination in a neonatal intensive care unit; infection was also transmitted to 9 staff and 12 family members [149]. There were 11 deaths from HAdV-7 infections among 61 residents of a long-term care pediatric facility during an 8-week period; 23 staff members had febrile respiratory illnesses during this period, suggesting sustained transmission between patients and staff [150].

Occasionally, neonatal infections have been caused by vertical transmission of HAdV, especially with species B HAdV-11 and HAdV-35 [136, 151]. Intrauterine infections have rarely been described, and ascending viral infection from the cervix has been proposed as one mechanism [137].

7 Pathogenesis

HAdVs are lytic DNA viral pathogens that usually cause self-limited localized disease but can disseminate in immunocompromised hosts and rarely in healthy children and

adults, causing significant morbidity and mortality. HAdVs cause pathology during the process of viral replication and lysis of susceptible cells. Additionally, investigations of replication-defective adenoviruses in animal models and clinical studies have revealed that HAdVs can cause significant inflammation in the absence of viral replication. Thus, the clinical manifestations of HAdV disease appear to result from both the direct effects of the infection and the host inflammatory responses.

7.1 Tissue Tropism

Although certain HAdV serotypes are associated with distinct clinical manifestations, the basis for these differences is not well understood. For example, the species D serotypes 8, 19, and 37 can cause a more severe eye disease than other types. The species E HAdV-4 and species B serotypes 3, 7, 14, and 21 have been associated with severe respiratory disease. In contrast, the species B serotypes 11, 34, and 35 primarily cause hemorrhagic cystitis. It is likely that differences in the fiber and penton capsid proteins that mediate cell receptor binding and cell entry contribute to serotype-specific differences in clinical manifestations. For instance, HAdV species display different cell receptor preferences that are mediated by the fiber. Most serotypes from species A, C, D, E, and F bind to CAR (the coxsackie adenovirus receptor), which is a member of the immunoglobulin superfamily expressed on multiple cell types [152]. Most species B viruses bind to a ubiquitously expressed membrane complement regulatory molecule CD46 [153], whereas the species B HAdV-3 and HAdV-7 bind to the related molecules CD80 and CD86 [154]. Members of the group D viruses can also utilize ubiquitous sialic acid receptors [155, 156]. Following viral attachment, penton base protein binds to cellular $\alpha v\beta 3$/$\alpha v\beta 5$ integrins [157], thus facilitating virus internalization via clathrin-coated vesicles into endosomes for further processing [158].

7.2 Pathology

In a natural infection of the human host, epithelial cells are the primary targets of adenoviral cytopathology [159]. The respiratory epithelial cells affected during adenoviral pneumonitis can develop acidophilic intranuclear inclusions. Infected cells with enlarged nuclei containing amphophilic or basophilic inclusion bodies surrounded by thin rims of cytoplasm referred to as "smudge cells" have also been described. There are no syncytia or multinucleated cells, as seen in herpes virus infection. In the rare case of fatal illness, the virus has been recovered from most body organs [160]. In such cases, extensive pathology is found in the lungs, with microscopic necrosis of tracheal and bronchial epithelium characterized as necrotizing bronchiolitis. Rosette formation, mononuclear cell infiltrates, and focal necrosis of mucous glands appear to be characteristic. Typical intranuclear virus particles have been observed in alveolar lining and bronchiolar cells by electron microscopy [72]. In infants who recover from adenovirus pneumonia, severe sequelae may follow, including bronchiectasis, radiolucent lung syndrome, persistent lobar collapse, and bronchiolitis obliterans [161].

HAdVs can also cause pathology in the absence of replicating virus. For example, replication-defective HAdVs can trigger innate immune responses, primarily by recognition of capsid proteins via pathogen recognition receptors (PRRs) with activation of dendritic cells and macrophages [162]. Capsid proteins can also activate the complement system via both classical and alternative pathways [163, 164]. This results in a proinflammatory response with the release of multiple cytokines. The viral capsid proteins also trigger humoral and cell-mediated immune responses that further exacerbate the developing proinflammatory response (see Sect. 8, below). For instance, antiviral antibodies can increase Fc receptor-dependent viral internalization in macrophages and amplify intracellular innate pathways [165]. The ability of this virus to induce an inflammatory response in the host in the absence of replication has implications for the clinical use of replication-deficient adenovirus vectors. For further discussion, see Sect. 11, below.

7.3 Immune Evasion Mechanisms

In natural infections, HAdVs have evolved several mechanisms to evade or downregulate the host immune response, which can help counteract the above inflammatory responses. The viral early region 3 (E3) encodes several small proteins (range 6.7–19 kDa) that play a prominent role in evading host innate and acquired immune defenses [166]. The E3 promoter also has NFkB binding sites that can be induced by cytokines such as TNF-α, and as a result, E3 protein expression is directly upregulated during an inflammatory response [167]. The E3-19K glycoprotein can block the transport of major histocompatibility complex (MHC) class I antigens to the cell surface, thereby inhibiting the recognition of virus-infected cells by cytotoxic T lymphocytes (CTLs). E3-19K evokes two distinct mechanisms to inhibit MHC class I molecule presentation of viral peptides to CTLs. First, E3-19K directly binds to class I antigens in the endoplasmic reticulum (ER) and inhibits their egress from the ER to the cell surface [168, 169]. Additionally, E3-19K can directly bind to TAP (transporter associated with antigen processing) and block class I/TAP association [170]. As a result of this interaction, the transfer of viral peptides processed in the cytosol to class I antigens is also inhibited.

Other E3 proteins enable the virus to evade apoptosis by inhibiting signaling from death receptors. CTLs express Fas

ligand on their cell surface and induce apoptosis upon interaction with the Fas ligand receptor CD95 on target cells. Similar apoptotic pathways are triggered by TNF-α and its ligand TRAIL (TNF-related apoptosis-inducing ligand). However, in the presence of the E3-10.4K and E3-14.5K proteins, these pathways are inhibited. Two units of E3-10.4K and one unit of E3 14.5K hetero-trimerize to form the RID complex (receptor internalization and degradation), which stimulates the degradation of receptors for Fas ligand and TRAIL via endocytosis and lysosomal degradation [171–173]. Thus, RID inhibits apoptosis through the TNF receptor 1 (TNF-Rl), Fas, and TRAIL-R1 pathways. The E3-14.7K protein is also a potent inhibitor of apoptosis mediated by TNF-α [174].

HAdVs also can counteract host antiviral immunity by interfering with the antiviral activity of type I interferons (IFNs). About 1 % of the viral genome is transcribed into noncoding virus-associated RNA (VA RNA). The synthesis of VA RNA begins during the early phase of the infectious cycle and accelerates during the late phase. VA RNA interferes with IFN-dependent phosphorylation of cellular proteins that inhibit viral peptide chain initiation [175, 176]. As a result, the viral polypeptide chain synthesis proceeds uninhibited. E1A proteins also inhibit the function of type I IFNs by blocking signal transduction pathways via IFN-stimulated gene factor (ISGF3). Conserved regions of E1A bind directly to STAT 1, the transcription factor that mediates IFN-stimulated transcription [177].

7.4 Viral Persistence

Although most HAdV illnesses are acute and self-limited, infection may be prolonged, and asymptomatic infections are common. Viral shedding from the gastrointestinal tract may persist for weeks to months, thus increasing the risk for horizontal transmission. By devoting about a third of the genome towards gene products that counteract host defense mechanisms, HAdVs have evolved strategies (discussed above) to help facilitate persistence after primary infection. HAdVs can be isolated from at least 50 % of surgically removed tonsils [178], occasionally from the kidney [131] and also from lymphocytes [58], suggesting that infection may remain latent for a very long time, possibly for life. Mechanisms for maintaining persistent and latent HAdV infections are not well understood. For further discussion, see Sect. 12.2 below.

8 Host Immune Responses

Adenoviruses are highly immunogenic and elicit strong innate and adaptive immune responses. In natural infections, the immune responses to HAdVs are moderated by multiple mechanisms related to expression of E1 and E3 region proteins, as well as VA RNA (as described above). However, these immune evasion mechanisms are not activated by infection with replication-defective E1-deleted adenovirus vectors.

8.1 Innate Immune Responses

The innate response to adenovirus acts as a first line of defense and serves to control infection locally and recruit effector leukocytes, such as granulocytes and monocytes/macrophages, to the site of infection. The viral capsid proteins are primarily responsible for inducing innate immune responses and for activating and recruiting Kupffer cells, endothelial cells, neutrophils, splenic macrophages, and dendritic cells [162, 179, 180]. Binding of capsid proteins to complement, pattern recognition receptors (PRRs), erythrocytes, and other blood factors further enhances the ensuing immune response [181, 182]. For example, recognition of HAdVs via Toll-like receptors (TLRs) triggers activation of NFκB and signal transduction via the mitogen-activated protein kinases (MAPKs), resulting in the transcription of host chemokine and cytokine genes [183]. Overall, the inflammatory response generated from leukocyte recruitment provides additional local control of infection and facilitates antigen presentation by infiltrating cells such as macrophages and dendritic cells. A robust innate response is thus essential for the development of adaptive immunity.

Capsid proteins induce the production of proinflammatory cytokines and chemokines such as TNF-α, IL-6, IL-1B, IL-8, IL-12, RANTES, MIP-1, and MCP-1 [162, 184, 185]. While the primary role of these cytokines is to locally enhance the antiviral activity (via recruitment of appropriate effector cells) and enhance the immune cascade, overproduction may result in acute toxicity. In one study, 38 children hospitalized with HAdV infections were found to have high levels of IL-6, IL-8, and TNF-α without evidence of bacterial infection. Very high levels of IL-8 were found in all 16 fatal cases of HAdV infection, comparable to the levels seen in septic shock in adults [186]. Similarly, TNF-α was more frequently detected in the fatal group. Thus, an association between high proinflammatory cytokine levels and the severity of a natural HAdV infection was noted in infected children.

Virus-induced innate responses are also clinically relevant with regard to gene therapy using adenovirus vectors. The inflammatory response to replication-deficient adenovirus vectors significantly reduces gene transfer efficiency and vector persistence in both animal and clinical studies [180, 187, 188]. Moreover, one of the initial gene therapy clinical trials to treat ornithine transcarbamylase deficiency using a replication-deficient, E1-deleted HAdV-5 vector resulted in the unfortunate death of an 18-year-old patient.

In this case, mortality coincided with the induction of a cytokine storm [189]. There was a massive release of proinflammatory cytokines, including IL-6 and IL-10, causing a systemic inflammatory response syndrome. The patient developed disseminated intravascular coagulation and multiple organ system failure, leading to death 98 h following gene transfer. This case highlights the need to develop strategies to moderate HAdV vector-induced innate immune responses. In contrast, for the purposes of vaccine development or cancer immune therapy, the innate immune response to adenovirus capsid proteins can have a desirable adjuvant effect to enhance the host immune response or cause a bystander effect [179, 190]. For further discussion, see Sect. 11, below.

8.2 Adaptive Immune Responses

The development of an adaptive immune response is critical in the resolution of HAdV infections. Virus-specific antibodies (mainly secretory IgA) are present in the upper respiratory tract within 3 days of infection. Approximately 7 days postinfection, antibodies can be detected in serum, and nasal secretions contain both secretory IgA and IgG [191, 192]. Serotype-specific neutralizing antibodies (Nabs), predominantly directed against the hexon, fiber, and penton base, are generated following the first exposure to each HAdV serotype [193, 194]. Anti-fiber and anti-penton Nabs prevent cellular entry of the virion, while anti-hexon antibodies prevent virus uncoating and nuclear entry of viral DNA [195, 196]. Nabs inhibit HAdV transduction of cells by a variety of methods, including prevention of cell attachment and facilitation of virus aggregation and clearance [197]. In addition, anti-hexon Nabs that allow virus entry but prevent virus gene expression have been identified [198]. Nabs generated against hexon target hypervariable regions (HVRs), which account for 80–95 % of virus-specific Nabs, appear to be most critical for virus clearance. Studies have shown that the presence of Nabs prevents reinfection with the same serotype [66, 86], and Nabs have been demonstrated 10 years after documented infection [199]. However, the protective effect of Nabs is serotype specific and does not allow for broad protection against multiple HAdV serotypes.

The severe HAdV infections observed in patients with a suppressed cellular immune system reflect the importance of cell-mediated immunity in limiting HAdV replication and preventing dissemination of infection [118, 128, 200]. Adenovirus-specific memory CD4+ T cell responses can be detected in the vast majority of healthy adults [201, 202]. These CD4+ T cell responses are Th1 type, based on the cytokine secretion pattern [203] and, unlike Nabs, are cross-reactive against different serotypes. Several CD4+ T cell epitopes have been mapped from the hexon, including a highly conserved epitope located in the COOH terminus of hexon that is restricted by a HLA class II allele present in 75 % of the population [204]. Lower frequencies of adenovirus-specific CD8+ T cells can be detected in most healthy adults. Hexon is also recognized by CD8+ T cells, and multiple cross-reactive hexon epitopes have been identified [205–207]. In addition to the hexon, conserved regions of HAdV early proteins like the E2 DNA polymerase and DNA-binding protein (DBP) have been identified as CD8+ T cell targets and are recognized by both healthy and acutely infected adults [208]. Thus, cross-reactive virus-specific memory CD4+ and CD8+ T cell responses can be detected in most individuals.

Cross-reactive memory T cell responses to HAdVs pose a challenge to the development of adenovirus vector applications (see Sect. 11, below). However, virus-specific memory T cells from HSCT donors have potential use for immunotherapy of severe HAdV infections in HSCT recipients. Several reports have demonstrated an association between recovery of HAdV-specific T cells and resolution of acute HAdV infections following HSCT [209, 210]. Specifically, HSCT recipients recovering from HAdV disease can mount CD4+ and CD8+ T cell responses to hexon, as well as CD8+ T cell responses to the above early protein targets [208, 211]. Therefore, passive transfer of virus-specific donor T cells is being explored as an adjunctive therapy for severe HAdV infections in HSCT recipients [212, 213]. For further discussion, see Sect. 9 below.

9 Treatment

The majority of HAdV infections are self-limited and do not require specific therapy. There are no agents licensed specifically for treatment of HAdV disease.

9.1 Antiviral Agents

Of the available antiviral agents, only cidofovir has reproducible in vitro activity against adenoviruses [214, 215]. Cidofovir has been most commonly used to treat invasive infections in immunocompromised patients [126, 216, 217]. Treatment with cidofovir has been associated with clinical responses and declines in viral loads in some HSCT recipients with invasive HAdV disease [218, 219]. Early treatment and immune reconstitution appear to be important factors associated with improved outcomes [220]. Although some pediatric HSCT programs perform active HAdV surveillance using blood PCR assays and treat preemptively [221, 222], the nephrotoxicity of cidofovir is often dose limiting. Less nephrotoxic lipid-ester derivatives of cidofovir are being investigated for the treatment of serious HAdV infections

[223, 224]. Disseminated adenoviral disease was successfully treated with the cidofovir prodrug CMX001 in a pediatric stem cell transplant recipient [225].

9.2 Other Therapies

Pooled intravenous immune globulin has high titers of Nabs against common species C HAdV and is commonly used as adjunctive therapy for severe HAdV infections in immunocompromised patients [226, 227]. A benefit from immune globulin was demonstrated in an immunosuppressed mouse model where passive transfer of mouse adenovirus (MAV)-specific IgG caused a marked delay in mortality after MAV infection and survival correlated with levels of MAV-specific antibodies [228].

Additionally, adoptive transfer of virus-specific donor T cells has been investigated in a small number of pediatric HSCT recipients. In one study, virus-specific donor T cells were isolated and infused into nine children with systemic adenoviral infections [212]. Viral clearance was demonstrated in 5 of 6 evaluable patients. In another study, patients were treated with donor lymphocytes stimulated in vitro with HAdV [213]. Reductions in viral loads were documented in all three patients with active infection, and one patient with adenoviral pneumonia had a clinical response. Methods to optimize recovery and in vitro expansion of donor virus-specific T cells for immunotherapy trials are being investigated [211, 229].

10 Prevention

Prevention of transmission of adenoviral infections is challenging. Transmission is common in households with young children and day-care centers. HAdVs can be intermittently shed in stool for weeks to months and pose an ongoing source of infection. Nosocomial transmission can also occur, as discussed above. In an outpatient study, 126 (7 %) of 1,870 ophthalmology patients developed EKC due to HAdV-8 [101]. Inadequate disinfection of instruments and finger-to-eye transmission by healthcare workers were implicated in this outbreak.

Adenoviruses are nonenveloped viruses that can remain viable on environmental surfaces for weeks and are relatively resistant to commonly used disinfectants. In one study, only bleach (1:10 dilution) and 2.4 % glutaraldehyde were effective disinfectants against HAdV-8 under all test conditions [230]. The disinfectants 70 % ethanol and 65 % ethanol plus 0.63 % quaternary ammonium compound also demonstrated at least a 3-$\log_{10}$ reduction in titers, but their virucidal activity was significantly reduced in the presence of organic matter. Other common disinfectants, including 70 % isopropyl alcohol, 0.63 % quaternary ammonium compound alone, 4 % chlorhexidine gluconate, and 10 % povidone-iodine, were significantly less active. Additionally, one study found that HAdVs are not completely eliminated from hands after washing with soap and water [102]. These viruses are also relatively resistant to UV light [231] but can be inactivated by heat.

Infection control measures can reduce the risk of HAdV transmission in some settings. For instance, adequate chlorination of swimming pools can help prevent pharyngoconjunctival fever outbreaks [68, 85]. The spread of EKC in ophthalmology offices can be prevented by heat sterilization of instruments, the use of single-use ointments and solutions, and the use of gloves and strict hand hygiene practices. However, during a large community outbreak of EKC where over 100 nosocomial cases occurred at a large ophthalmology center, transmission continued to occur despite the institution of strict hand washing, gloving, and bleach disinfection of equipment [232]. Transmission was stopped only after patients were rigorously triaged and cohorted, highlighting the difficulties in preventing the nosocomial spread of HAdV infections.

In healthcare facilities, the Centers for Disease Control and Prevention recommends to use contact precautions (gloves and gown) for children with suspected or documented adenoviral conjunctivitis or gastroenteritis (if incontinent or in diapers). Both contact and droplet precautions (surgical mask) are recommended when caring for children with suspected or documented adenoviral respiratory tract infections. Although there are no specific recommendations regarding adults, isolation precautions should also be considered for adult patients with HAdV disease [146].

Immunization with live, oral, enteric-coated HAdV-4 and HAdV-7 vaccines was used safely and effectively for years in military training camps. After the sole manufacturer stopped production in 1996, outbreaks of ARD reappeared [90]. Efforts to control the spread of HAdVs and prevent epidemics in new military recruits were largely unsuccessful. In response, new live oral HAdV-4 and HAdV-7 vaccines were produced [96] and were relicensed in 2011 for military use [97].

11 Adenovirus Vectors

A large number of studies have investigated replication-defective, E1-deleted adenovirus vectors for gene therapy. Species C HAdV-5 has been the primary platform for the construction of vectors. Adenovirus vectors can infect a variety of cell types, including nondividing as well as dividing cells, and can be prepared readily in large quantities in tissue culture. These vectors can accommodate large inserts (up to 8 kb) and express higher levels of recombinant proteins than most other viral vectors.

A major obstacle to utilizing adenovirus-mediated gene therapy is the fact that adenovirus vectors are highly immunogenic, as discussed above. As a result, in vivo transgene expression is transient in both animal and clinical studies and is associated with substantial inflammatory responses [233–235]. Additionally, most individuals have serotype-specific Nabs to HAdV-5 and exhibit cross-reactive memory T cell responses to HAdVs, which further limit transgene expression from HAdV vectors [205, 236]. Therefore, a number of strategies to reduce immunogenicity in order to prolong transgene expression have been investigated, including (1) making additional vector deletions to reduce adenovirus protein expression [237], (2) preparing "gutless" or helper-dependent vectors [238, 239], and (3) inserting or upregulating proteins that inhibit immune responses such as Fas ligand or the adenovirus E3 region [240, 241]. Additionally, alterations in the fiber protein, which mediates virus attachment to cells, have been engineered to enhance binding to cell types that are negative for CAR, the major HAdV cell receptor [242].

Since 1993, about 400 clinical protocols have been implemented using adenovirus vectors. Most clinical trials have focused on the administration of E1-deleted HAdV vectors by direct injection into tumor masses. HAdV vectors expressing the tumor suppressor p53 have shown modest activity in patients with head and neck cancer and non-small cell lung cancer [243]. HAdV vectors expressing the herpes simplex virus thymidine kinase have been used as a suicide gene therapy in patients with glioblastoma or mesothelioma [244]. Additionally, HAdVs engineered with E1 mutations that preferentially allow viral replication and cell lysis in tumor cells, so-called "oncolytic" adenovirus vectors, have had modest activity in combination with chemotherapy in patients with head and neck cancer [245, 246].

Additionally, both live and E1-deleted replication-defective adenoviruses are being investigated as vaccine vectors for immunization against other infectious pathogens. A large phase II trial (the STEP study) of a replication-defective HAdV-5 vector expressing HIV-1 gag, pol, and nef proteins failed to prevent HIV-1 infection or to reduce early HIV replication after infection [247, 248]. However, priming with recombinant protein or DNA, followed by boosting with an adenovirus vector, may improve vaccine efficacy by evading preexisting memory immune responses to adenoviruses [249]. For instance, an influenza matrix protein 2 DNA prime, followed by a recombinant adenovirus boost, conferred broad protection against influenza A infection (including H5N1 influenza) in mice [250]. Other strategies under investigation to help evade preexisting immune responses include (1) administration via the intranasal route [251, 252], (2) use of vaccines based on animal adenoviruses or rare HAdV serotypes [253, 254], (3) insertion of immunostimulatory proteins [255], and (4) incorporation of novel antigens into adenovirus capsid proteins [256]. For instance, in one study, sublingual administration of a HAdV-5 vector expressing HIV Gag in HAdV-5-immune mice elicited a broad CTL response in both systemic and mucosal compartments [257].

12 Unresolved Issues

12.1 Typing Nomenclature

Typing of HAdVs has historically been accomplished by establishing their immunologic distinctiveness by quantitative neutralization with hyperimmune animal antisera. Designation and numeric assignment of a new serotype require demonstration of either no cross-reactions with other serotypes or a homologous-to-heterologous titer ratio of ≥16 in both directions. For a HAdV with a borderline SN titer ratio, distinctiveness is assumed if the hemagglutinin can be distinguished by HI or if substantial biophysical, biochemical, or phylogenetic differences exist. As noted above, naturally occurring intermediate strains that possess hemagglutinins of one or more serotypes and neutralizing antigens of another have been designated with the SN component first and the HI component following a slash (e.g., HAdV-11/H14) [20]. Other intermediate strains that are doubly neutralized by two prototype antisera have been described and molecularly characterized [258, 259].

Because of the lack of immunotypic clarity with some HAdV strains, the challenging requirements of virus cultivation and antisera preparation, and the limited access to reference prototype antisera, alternative typing schemes like genome restriction analysis have been proposed as noted above. With the increasing availability of cost-effective methodologies in DNA sequencing and computational analysis, full genomic sequencing has become the agreed upon standard for future classification of adenoviruses [63]. However, the criteria used for assigning new numbers based solely on genomic sequences have been unclear [260], and in the last few years, a plethora of new HAdV numeric "types" have appeared in the literature (types 52–65) [259, 261, 262], many of which were previously described intermediate strains. To facilitate better integration into the existing system, others have proposed retaining the preeminence of the hexon gene as the primary identifier and the fiber gene as the major determinant of tropism in designating a new virus type [263]. However, the degree and character of sequence divergence necessary to consider a candidate virus as a new numeric type has yet to be agreed upon.

12.2 Mechanisms of Latency

HAdVs were originally isolated from tonsil and adenoid explants. Using a sensitive real-time PCR assay, Garnett et al. [264] detected a high prevalence of adenoviral DNA in

human tonsil tissues from children, but infectious virus was isolated from only 13 of 94 donors (11 %). The average amount of viral DNA was 280 copies per cell, and all species C serotypes were identified. Notably, adenoviral DNA replication could be induced in most samples by lymphocyte stimulation in culture. In another study, analysis of lymphocyte populations from tonsil tissue indicated that adenoviral DNA was associated with T cells, not B cells [58].

There is also evidence for HAdV reservoirs in lymphocytes from other tissues and other cell types. HAdVs are frequently shed in feces, suggesting that the gastrointestinal tract may be a source of persistent infection. In one study, intestinal tissue from surgical specimens and the lymphocytes isolated from these specimens were tested using a nested adenoviral PCR assay [265]. Intestinal tissue from 21 of 58 specimens and intestinal lymphocytes from 21 of 24 evaluable specimens were positive for adenoviral DNA. Surprisingly, species E serotype 4 sequences were most commonly detected – this virus has rarely been isolated from stool samples in civilian epidemiologic studies or from clinical specimens in immunocompromised hosts. Species B and C sequences were also detected. Rare reports implicating HAdV transmission from donor organs (liver and kidney) suggest that these viruses can maintain reservoirs in other tissue types [131, 133]. Additionally, adenoviral DNA was commonly detected by PCR assay in brain tissue specimens in one study [266] (see Sect. 12.4, below).

What are mechanism(s) of latency in lymphocytes? Although HAdVs infect lymphocytes and monocytes very inefficiently in vitro, one group found that a subset of B and T cell lines supported short-term viral replication [267]. Another study found that adenovirus genomes were maintained at varying levels in different B and T lymphocyte cell lines in association with downregulation of CAR expression [268]. Additionally, viral DNA was maintained as a monomeric episome in the lymphoma cell line BJAB. There is also one report of a spontaneous persistent HAdV infection of an EBV-positive B cell lymphoma in a HSCT recipient [269]. These studies suggest that HAdVs can exist in a latent form as episomal DNA in lymphocytes and can be induced to replicate upon certain activation signals.

12.3 Role in Obesity

Several animal and clinical studies have identified an association between obesity and species D HAdV-36 infection. HAdV-36 increases adiposity in experimentally infected mice, chickens, and marmosets [270–272]. In one study of over 500 adult volunteers, HAdV-36 antibodies were associated with increased body weight and lower serum lipids [273]. In another study of 124 children, the prevalence of antibodies to HAdV-36 was significantly higher in obese children (15 of 19, 78 %) compared to nonobese children (15 of 67, 22 %) [274]. However, a study in military personnel did not confirm a correlation between HAdV-36-specific antibodies and obesity but rather identified differences in HAdV-36 seroprevalence related to race and sex [275].

12.4 Oncogenicity

All HAdVs can transform cultured rodent cells, a cell type in which infection is highly restricted. Species A HAdVs such as HAdV-12 and HAdV-18 and, to a lesser extent, species C viruses can induce tumors in newborn hamsters [276]. This transformation process is mediated by the adenovirus E1 transactivating region [277]. However, there is no definite evidence for oncogenicity in humans. In one study using a highly sensitive quantitative PCR assay to test over 500 pediatric tumor specimens, including leukemias, lymphomas, and solid tumors, there was no evidence of HAdV DNA in any tumor cell types except in brain tumors [266]. Although HAdV DNA was detected in 25 of 30 glioblastomas and 22 of 30 oligodendrogliomas, HAdV DNA was also detected in most control brain tissue samples. Predominantly, species B and D HAdV DNAs were identified in both malignant and nonmalignant brain tissue specimens. These data suggest that the brain may represent another reservoir of latent HAdV infection.

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Alphaviruses: Equine Encephalitis and Others

7

Scott C. Weaver and Ann M. Powers

1 Introduction

Arthropod-borne viruses (arboviruses) are an ecologically defined set of viruses that share a common mode of transmission involving arthropod vectors that transmit to vertebrate hosts. Most are biologically transmitted, requiring replication in both arthropod and vertebrate host with transmission between vertebrate animals by the bite of mosquitoes, ticks, sandflies, or midges [1]. The vast majority of arboviruses belong to one of five families: *Togaviridae, Flaviviridae, Bunyaviridae, Reoviridae*, and *Rhabdoviridae*. Information on their isolation, morphology, sensitivity to inactivation by chemicals, arthropod vectors, vertebrate hosts, laboratory propagation, serological reactions, geographic distribution, clinical manifestations, and epidemiology is found in the *International Catalogue of Arthropod-Borne Viruses*, compiled by the American Committee on Arthropod-Borne Viruses [2]. This exhaustive reference source has been used freely in preparing this chapter.

The biological transmission of arboviruses is characterized by their replication in the arthropod. The period from ingestion of an infectious blood meal until the virus replicates, reaches the salivary glands, and can be transmitted is called the extrinsic incubation period. After being fed upon by an infected arthropod that secretes saliva to block hemostatic responses and often to limit pain, the vertebrate host becomes infected and usually viremic. This part of the transmission cycle takes from 1 day to more than a week and constitutes the intrinsic incubation period. Biological transmission should be distinguished from mechanical transmission in which the virus contaminates the mouthparts of the arthropod and can be transmitted immediately to a new vertebrate host without replication in the invertebrate. Also, nonviremic transmission has been described for a few arboviruses whereby virus inoculated via the saliva is sufficient in amount to generate an immediate, nonreplicative viremia [3, 4].

Arboviruses are usually maintained in a reservoir cycle that consists of both arthropod and vertebrate hosts. Both are needed to maintain the virus in nature [5]. A subset of arboviruses, including members of the family *Togaviridae* [6, 7], is transmitted vertically through the egg of the arthropod. In these cases of transovarial transmission, the arthropod alone may be the reservoir of the virus and may maintain the virus in the absence of a vertebrate animal (for a limited duration assuming the rate of vertical transmission is <100 %).

2 Sources of Mortality Data

Mortality data are collected systematically but passively by national governments for the encephalitic alphaviral infections and other diseases such as epidemic polyarthritis. Data are published in the *Morbidity and Mortality Weekly Report* of the US Centers for Disease Control and Prevention, in the *Weekly Epidemiological Report* of the Pan American Health Organization, and the *Weekly Epidemiological Report* of the World Health Organization. The mortality data are underreported, but may serve as a comparative database, since underreporting may be uniform throughout much of the world.

The information flow to the World Health Organization is sometimes facilitated by informal networks of scientists and interested citizens. Nevertheless, the organization is constrained from action until official reports are received. This constraint can mean a delay in control of a disease of regional or world importance.

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3 Sources of Morbidity Data

The same sources supply morbidity data as supply mortality data. In the USA, of the arboviral diseases, only encephalitis is reportable. An observed trend of increased nonspecific encephalitic cases during years of arbovirus epidemics may be due to increased physician requests for more diagnostic tests, leading in turn to diagnosis in a higher percentage of cases in these years. If so, this implies underdiagnosis in other years [8].

4 Serological Surveys

Alphaviruses are distributed focally nearly throughout the world [9]. The distribution of any given arbovirus is ecologically limited by the range of the vector and vertebrate hosts. Serological surveys are ideally suited for arboviruses to determine the point prevalence in different geographic areas. Often distribution of the antibody will give clues to the ecological conditions necessary for maintenance of the virus. Surveys of different age groups will show if the virus is more prevalent as the population ages, typical of an endemically transmitted agent. Another prevalence pattern in which antibody is present only in persons born before a certain year may indicate an epidemic in that year. Alternatively, a relatively constant percentage of antibody in each age group may indicate a recent introduction of the virus causing a virgin soil epidemic.

Broadly based serological surveys of large populations can provide extensive information about virus distribution in different geographic areas, rural versus urban populations, different age and sex groups, and different occupational types. However, arbovirus serosurveys have limitations. Cross-reactions occur among viruses of the same serogroup using certain tests. This is especially true of the hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA). The neutralization test is more specific and should be used where feasible. Surveys with HI or ELISA must be interpreted with caution unless one is certain that only one virus is extant in the region, or unless the results have been confirmed by neutralization test with a portion of the negative and positive sera.

The serosurvey usually will not indicate when the infection responsible for the antibody occurred. If the antibody is suspected to be of recent origin, the IgM antibody-capture ELISA is useful for detection of infections originating within recent months.

5 Laboratory Methods

The laboratory is an all-important resource in the study of the epidemiology of arbovirus diseases. Diagnosis can rarely be made with certainty by clinical examination. Isolation of virus from arthropods, wild or domestic animals, and people is essential to determine the natural history of infection with these agents.

Positive findings on survey sera are greatly bolstered by virus isolations from the vector, nonhuman vertebrates, or humans. Virus isolation procedures for serum specimens require inoculation of a small amount of serum into laboratory animals or cell cultures or both. Mice are observed daily, or more than once a day, for evidence of illness. A subpassage may be made to attempt to enhance the virulence of the virus, and when one is assured that a virus has been isolated, a stock pool of virus is established and hyperimmune mouse serum or ascitic fluid is prepared if needed. In fatal cases, 10–40 % suspensions of the tissue—brain, liver, lung, or spleen (purified by centrifugation)—may be inoculated as for serum.

Cell culture systems (vertebrate or insect cells) can also be used for virus isolation. For certain alphaviruses and certain cell cultures, the cell culture systems, are as sensitive as laboratory animals (considering an equal volume of total inoculum). Intracerebral inoculation of infant mice will serve to isolate a much wider total range of arboviruses than will any single cell culture system. Conversely, in the investigation of a specific virus, a cell culture system that has been predetermined to be suited to the virus can be easier, cheaper, involve fewer regulatory hurdles, and atleast as reliable than techniques that require laboratory animals.

In the study of material derived from patients, it is highly desirable to have a pair of serum specimens to work with. The first should be taken early in the course of illness and can serve as material both for virus isolation and for the determination of baseline serum antibody levels before the patient has developed antibodies to the infecting virus. A second serum should be obtained at least 3 weeks after onset of illness or as late as several months after onset. A seroconversion (four-fold or greater rise in antibody titer) demonstrable between the early and later specimens is a strong evidence of a recent virus infection. Demonstration of IgM in ELISA permits a presumptive diagnosis with a single serum as long as two etiologic agents that produce similar disease are not circulating during the same time period [10, 11].

Details of the techniques of CF, HI, and virus neutralization relating specifically to alphaviruses are available in current references [11]. Fluorescent antibody (FA) techniques; antigen detection ELISA, often coupled with monoclonal antibody; and RT-PCR are widely used for antibody, antigen, and RNA detection, respectively. Additional specialized assays for the serotype determination and genetic characterization of specific alphavirus groups are detailed below.

6 Biological Characteristics of the Viruses That Affect the Epidemiologic Pattern

A prerequisite for the survival of arboviruses in nature is a period of viremia in a vertebrate host at a level sufficient to infect the arthropod vector. Even with alphaviruses that generate high-titer viremias and highly susceptible vectors, a viremia titer in excess of 10,000 infectious doses of virus per milliliter of blood is frequently required to sustain transmission. Such high levels of viremia are generally found in the natural host and often are attained in the more commonly used laboratory animals such as mice, hamsters, guinea pigs, chicks, and monkeys. For some alphaviruses, however, the natural hosts or natural vectors, or both, are either unavailable or not known, so that the postulate of a level of viremia in the vertebrate adequate to infect a susceptible vector remains undemonstrated.

7 Epidemiology

The epidemiology of arbovirus infections in humans is influenced by three major determinants: (1) the behavior of the arthropod vector, including the ecological setting in which its larval habitats occur, its pattern and range of mobility, its biting habits and species preferences for feeding, its longevity, and the factors affecting the infection, replication, dissemination, and secretion into the saliva of virus within the arthropod host; (2) the frequency, nature, and duration of exposure of humans to the infected arthropod vectors, as influenced by the presence, level, and specificity of humoral antibody and by use in the population of insecticides, insect repellents, and protective clothing; and (3) the requirements for the presence of a necessary and/or amplifying vertebrate host for the virus, such as horses, birds, or rodents, and of the availability of humans as alternative hosts.

8 Epidemic Behavior

Outbreaks of alphavirus infections in the USA involving humans occur periodically and unpredictably, as evident from the activity from 1955 through 1987. Only 182 cases of eastern equine encephalitis (EEE) were reported during this time, of which 12 cases and 6 deaths were in 1968; during the same period, 1,030 cases of western equine encephalitis (WEE) were reported, of which 172 occurred in 1965, with four deaths, and 133 in 1975, with six deaths (http://www.cdc.gov/ncidod/dvbid/arbor/arbocase.htm). In contrast, from 1988 to 2010, 159 EEE cases were reported, while only five WEE cases occurred. Venezuelan equine encephalitis (VEE) produced its first outbreak in Texas, USA, in 1971, with no epidemic activity in the USA since that date.

9 Geographic Distribution

Alphavirus infections are nearly worldwide in distribution and may occur whenever the appropriate mosquito vectors occur in sufficient numbers in proximity to humans and a suitable amplifying host. Table 7.1 includes the geographic distribution of alphaviruses.

10 Temporal Distribution

In the USA, mosquito-borne alphaviruses produce human infections primarily in late summer and fall. Tropical alphavirus infections tend to occur during rainy seasons when mosquito vectors are most abundant. However, chikungunya virus (CHIKV), which occurs both in enzootic, sylvatic primate-amplified and urban, human-amplified cycles, can cause human disease throughout the year.

11 Age and Sex

Infections with alphaviruses can occur at any age. The age distribution depends on the degree of exposure to the particular transmitting mosquito relating to age, sex, and occupational, vocational, and recreational habits of the individual or group of individuals. For most alphaviruses, there is not a strong association between these groups and markedly higher rates of exposure. However, once humans have been exposed and infected, the severity of the disease may

Table 7.1 Members of the genus *Alphavirus*

Antigenic complex	Species	Antigenic subtype	Antigenic variety	Clinical syndrome	Distribution
Barmah Forest	*Barmah Forest virus* (BFV)			Febrile illness, rash, arthritis	Australia
Eastern equine encephalitis (EEE)	*Eastern equine encephalitis* (EEEV)	I		Febrile illness, encephalitis	North, Central and South America
	Madariaga virus	II–IV			
Middelburg	*Middelburg virus* (MIDV)			None recognized	Africa
Ndumu	*Ndumu virus* (NDUV)			None recognized	Africa

(continued)

Table 7.1 (continued)

Antigenic complex	Species	Antigenic subtype	Antigenic variety	Clinical syndrome	Distribution
Semliki Forest	Semliki Forest virus (SFV)			Febrile illness	Africa
	Chikungunya virus (CHIKV)			Febrile illness, rash, arthritis	Africa, Asia
	O'nyong-nyong virus (ONNV)			Febrile illness, rash, arthritis	Africa
	Getah virus (GETV)			None recognized	Asia
	Bebaru virus (BEBV)			None recognized	Malaysia
	Ross River virus (RRV)	Sagiyama		Febrile illness, rash, arthritis	Australia, Oceania
	Mayaro virus (MAYV)			Febrile illness, rash, arthritis	South America, Trinidad
	Una virus (UNAV)			None recognized	South America
Venezuelan equine encephalitis (VEE)	Venezuelan equine encephalitis virus (VEEV)	I	AB	Febrile illness, encephalitis	North, Central, South America
			C	Febrile illness, encephalitis	South America
			D	Febrile illness, encephalitis	South America, Panama
			E	Febrile illness, encephalitis	Central America, Mexico
	Mosso das Pedras virus (MDPV)		F	None recognized	Brazil
	Everglades virus (EVEV)	VEE-II		Febrile illness, encephalitis	Florida (USA)
	Mucambo virus (MUCV)	VEE-III	A	Febrile illness, myalgia	South America, Trinidad
			C (strain 71D1252)	Unknown	Peru
			D	Febrile illness	Peru
	Tonate virus (TONV)	VEE-IIIB		Febrile illness, encephalitis	Brazil, Colorado (USA)
	Pixuna virus (PIXV)	VEE-IV		Febrile illness, myalgia	Brazil
	Cabassou virus (CABV)	VEE-V		None recognized	French Guiana
	Rio Negro virus (RNV)	VEE-VI		Febrile illness, myalgia	Argentina
Western equine encephalitis (WEE)	Sindbis virus (SINV)			Febrile illness, rash, arthritis	Africa, Europe, Asia, Australia
		Babanki		Febrile illness, rash, arthritis	Africa
		Ockelbo		Febrile illness, rash, arthritis	Europe
		Kyzylagach		None recognized	Azerbaijan, China
	Whataroa virus (WHAV)			None recognized	New Zealand
	Aura virus (AURAV)			None recognized	South America
	Western equine encephalitis virus (WEEV)	Several		Febrile illness, encephalitis	Western North, South America
	Highlands J virus (HJV)			None recognized	Eastern North America
	Fort Morgan virus (FMV)	Buggy Creek		None recognized	Western North America
Trocara	Trocara virus (TROV)				South America
Salmon pancreas disease (SPD)	Salmon pancreas disease virus (SPDV)			Pancreatic disease (salmon), sleeping disease (trout)	Atlantic Ocean and tributaries worldwide
Southern elephant seal	Southern elephant seal virus (SESV)			None recognized	Australia
Eilat	Eilat[a]			Incapable of infecting vertebrates	Middle East

[a]Recommended for species designation [12] but not yet approved by the International Committee on the Taxonomy of Viruses

also be influenced by age. For example, WEEV and VEEV tend to produce the most severe clinical infections in young persons.

12 Mechanism and Route of Transmission

By definition, arboviruses must be transmitted by arthropod vectors. There is often a high-level virus–vector specificity associated with biological transmission, usually manifested at the initial infection of the mosquito alimentary tract. This biological transmission may be supplemented under some circumstances by mechanical transmission.

The duration of the necessary period of virus replication within the arthropod host before it becomes infectious varies from virus to virus and vector to vector and is also directly temperature dependent. For some alphaviruses, the extrinsic incubation period can be as little as 2 days under average summer temperature conditions [13, 14]. However, mosquitoes generally must develop their eggs and oviposit before a subsequent blood meal, resulting in transmission, occurs; this gonotrophic cycle length may limit the minimum time until transmission if it is longer than the time for virus to reach and replicate in the salivary glands after an infectious blood meal. Once infected, vectors often remain infected and able to transmit for life, which can be many weeks or even months.

Transmission of alphaviruses transovarially in mosquitoes, often referred to as "vertical transmission," has been demonstrated for CHIKV [6] Sindbis [15] and Ross River virus [7].

13 Pathogenesis and Immunity

Most alphavirus infections are transmitted by the bite of a mosquito vector, so that the skin represents the sole portal of entry. With early wide dissemination throughout the host, multiplication follows in generally poorly determined target cells and tissues. Viremia in the host then provides the seedbed for infection of succeeding cohorts of biting arthropods. The incubation period is usually 3–15 days.

The site of replication of most arboviruses remains undetermined but is presumed to be in the vascular epithelium and the reticuloendothelial cells on the lymph nodes, liver, spleen, and elsewhere. Liberation of virus from these organs constitutes the "systemic phase of viremia," resulting after 3–7 days of symptoms such as fever, chills, and aching. A number of arbovirus infections have two phases—this early phase and then a second phase with or without a few days of freedom from symptoms. The second phase may be attended by encephalitis, joint involvement, rash (sometimes hemorrhagic), and involvement of the liver or kidneys. In most arbovirus infections, only the first phase occurs, and the disease is "nonspecific." In other instances, the early phase may be missed, and only the severe manifestations occur. The early phase is often accompanied by leukopenia and the second phase often by leukocytosis [16]. Tissue injury may be the direct effect of viral multiplication in susceptible cells, as is the case with lymphodepletion following VEEV infection.

Humoral antibodies regularly appear early in the course of alphavirus infection and constitute the major basis of immunity. Such immunity may be lifelong. The role of cell-mediated immunity in alphavirus infections has been poorly studied but can protect mice deficient in antibody production from lethal VEEV infection [17]. It is possible that it may be important in controlling virus persistence and in determining the immunopathological lesions suspected in certain manifestations of infection.

14 Patterns of Host Response

14.1 Clinical Features

Inapparent and subclinical human responses predominate in most alphavirus infections, with the exception of CHIKV and VEEV. For example, infection with EEEV and WEEV results principally in mild and inapparent infections; the reasons for these differences are unknown.

14.2 Diagnosis

Cases of alphavirus infections in the USA are not likely to be diagnosed unless there is a high degree of clinical suspicion. Outbreaks of encephalitis in horses during the summer, caused by EEEV, WEEV, or VEEV, serve to focus attention on febrile illness in humans associated with symptoms or signs indicating involvement of the CNS. Recognition of the arbovirus infection acquired by the traveler outside the USA also depends on the alertness of the examining physician. Rapid jet transport now permits exposed overseas travelers to reach home and fall ill even within the short incubation period of such infections. This trend was exemplified by the 35 CHIKV-infected patients diagnosed after their return from epidemic regions of the Indian Ocean and Asia during 2006 [18]. The physician must maintain a high degree of suspicion when seeing CNS infections or influenza-like illnesses occurring in travelers recently returned from areas endemic for alphaviruses. Testing for alphavirus infection is not widely available, so special testing must often be done through arrangements with regional, national, or international public health agencies and/or reference centers. It

should be noted that arbovirus infections constitute only a small fraction of the encephalitis cases seen in the USA with the majority of cases of undetermined etiology.

The laboratory diagnosis depends on the isolation of the virus from the blood, a fourfold antibody rise in titer between acute and convalescent sera, or the presence of specific IgM taken during the acute phase. Often, the suspicion of an alphavirus infection in individual cases, especially in cases of neurologic disease that occurs several days after the onset of illness, arises too late for virus isolation from the blood. Under these circumstances, the presence of a high antibody titer in a single serum may be significant if the infection is an uncommon one in that region and particularly if antibody surveys reveal a low antibody prevalence or if prior surveys have demonstrated the absence of antibody in that community. The appropriate procedure in suspected cases is to (1) notify the health department and seek background epidemiologic and clinical data and (2) send acute and convalescent serum samples to the nearest public health laboratory (usually a state laboratory), with a request for antibody tests for alphaviruses. Some state laboratories may not have all necessary testing available, at which point a request for transshipment of sera to the CDC for additional testing might be included.

14.3 Control and Prevention

Major control methods include (1) control of the arthropod vector, which may be by elimination of aquatic larval sites or their treatment with insecticides or by direct control of adult female mosquitoes that are responsible for transmission, through insecticide applications such as malathion; (2) avoidance of exposure to mosquito vector bites by screening of houses, by using protective clothing, by staying indoors during times of peak host seeking by vector mosquitoes, and by applying insect repellents when outside in high-risk areas. Control of mosquito vectors through biological approaches ranging from larvicidal bacteria, predatory fish, and the introduction of genes deleterious to the vector population [19] or of *Wolbachia* bacteria that render adult female mosquito vectors refractory to transmission [20] is receiving much attention as an alternative control strategy.

15 Characteristics of Selected Arboviruses

15.1 Alphaviruses of Importance in the USA

Of the alphaviruses found in North America, only eastern (EEE), western (WEE), and Venezuelan equine encephalitis (VEE) viruses are important human pathogens. The former two viruses are enzootic within the USA, Canada, and Mexico, while VEE is enzootic and endemic only to Mexico but has spread into the USA on at least one occasion.

15.2 Eastern Equine Encephalitis Virus

15.2.1 Natural History/Transmission

In North America, most human and equine EEEV infections occur near enzootic foci of transmission, which occur in hardwood swamps populated by the principal mosquito vector, *Culiseta melanura*, and passerine birds that serve as amplification hosts (Fig. 7.1). These habitats occur along the Atlantic and Gulf coasts but also at inland locations in the upper Midwest (Fig. 7.2).

However, southeastern USA foci in locations where this mosquito is not abundant suggest the involvement as enzootic vectors of ornithophilic *Culex* spp. mosquitoes including *Culex erraticus* [21]. Because *Cs. melanura* feeds primarily on avian hosts, bridge vectors, probably including *Aedes vexans, Coquillettidia perturbans*, *Ae. canadensis*, and *Cx. salinarius,* have been assumed to transmit from birds to humans and horses [21, 22] (Fig. 7.3).

However, some studies suggest that *Cs. melanura* may bite mammals at sufficient frequency to implicate them in human and other mammal infections [23, 24]. Passerine birds that inhabit swamps are considered the principal enzootic amplification hosts in North America. However, recent serological and experimental infection data from the southeastern USA suggest that amphibians and reptiles could be involved in enzootic circulation [25, 26]. Although a wide variety of domesticated mammals and birds suffer severe and often fatal disease after EEEV infection, none is believed to be efficient at amplification via mosquito transmission because viremia levels are generally low [27–29]. Thus, unlike VEE outbreaks that can spread far from their origin due to efficient equine amplification, cases of EEE rarely occur far from enzootic foci, which tend to occur in relatively remote locations.

Presumably because they receive greater exposure to mosquito feeding, equids suffer a higher incidence of EEE than humans in North America. EEE epizootics in horses and pheasants often precede human cases, and the former are thus useful sentinels for detecting epizootic circulation [30]. Human and equine outbreaks are associated with excessive rainfall [31, 32] and usually peak in early fall in temperate regions of North America.

EEEV strains closely related to North American strains isolates have also caused equine outbreaks in the Caribbean [33] and northern Mexico [34]. Because there is no clear evidence of enzootic circulation in these locations, these outbreaks may represent introductions followed by epizootics of limited duration. Similarly, it remains unknown if the detection of epizootic

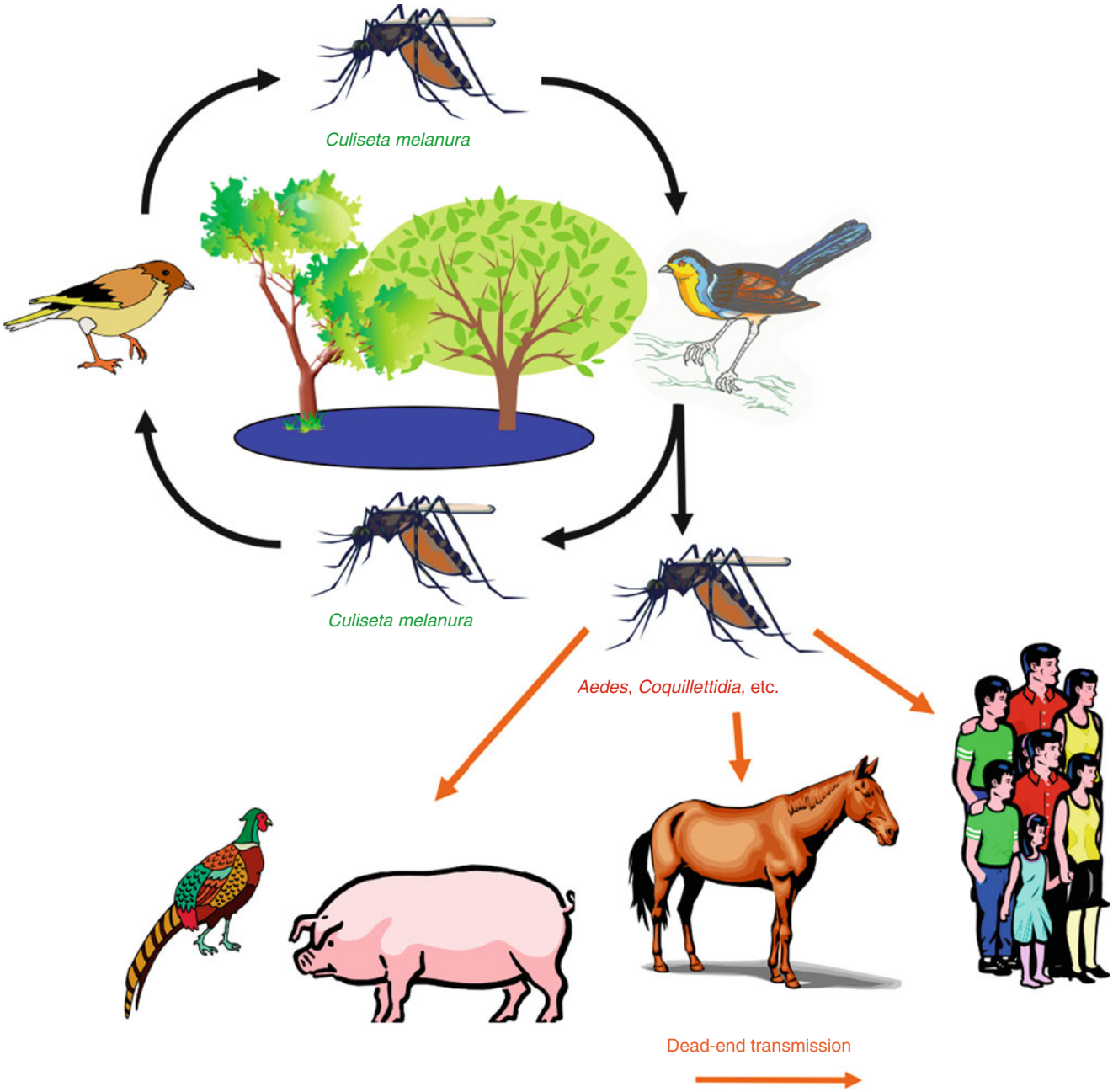

Fig. 7.1 Enzootic transmission cycle of eastern equine encephalitis virus in North America and epidemic or epizootic spillover to humans and domestic animals, respectively

EEEV circulation as far north as northern Maine in 2009 [35] represents stable enzootic circulation or a temporary introduction. In Central and South America, strains recently assigned to the species *Madaringa virus* (MADV have been isolated repeatedly from mosquitoes, principally members of the subgenus *Culex* (*Melanoconion*), as well as from equids suffering from neurologic disease. The most important reservoir hosts in South America remain unclear but may involve both small mammals and possibly birds [36]. Prior to 2010, despite clear evidence of their exposure during epizootics and by living in areas of enzootic transmission, only three cases of human EEE were reported in Latin America [37–39]. Genetic and antigenic distinctions between EEEV strains that circulate in North versus South American strains (MADV), as well as differences in sensitivity to and induction of interferons, suggest fundamental differences in human virulence [40, 41]. However, a 2010 EEE outbreak in Panama, involving both horses and humans, may reflect a change in human virulence there [10].

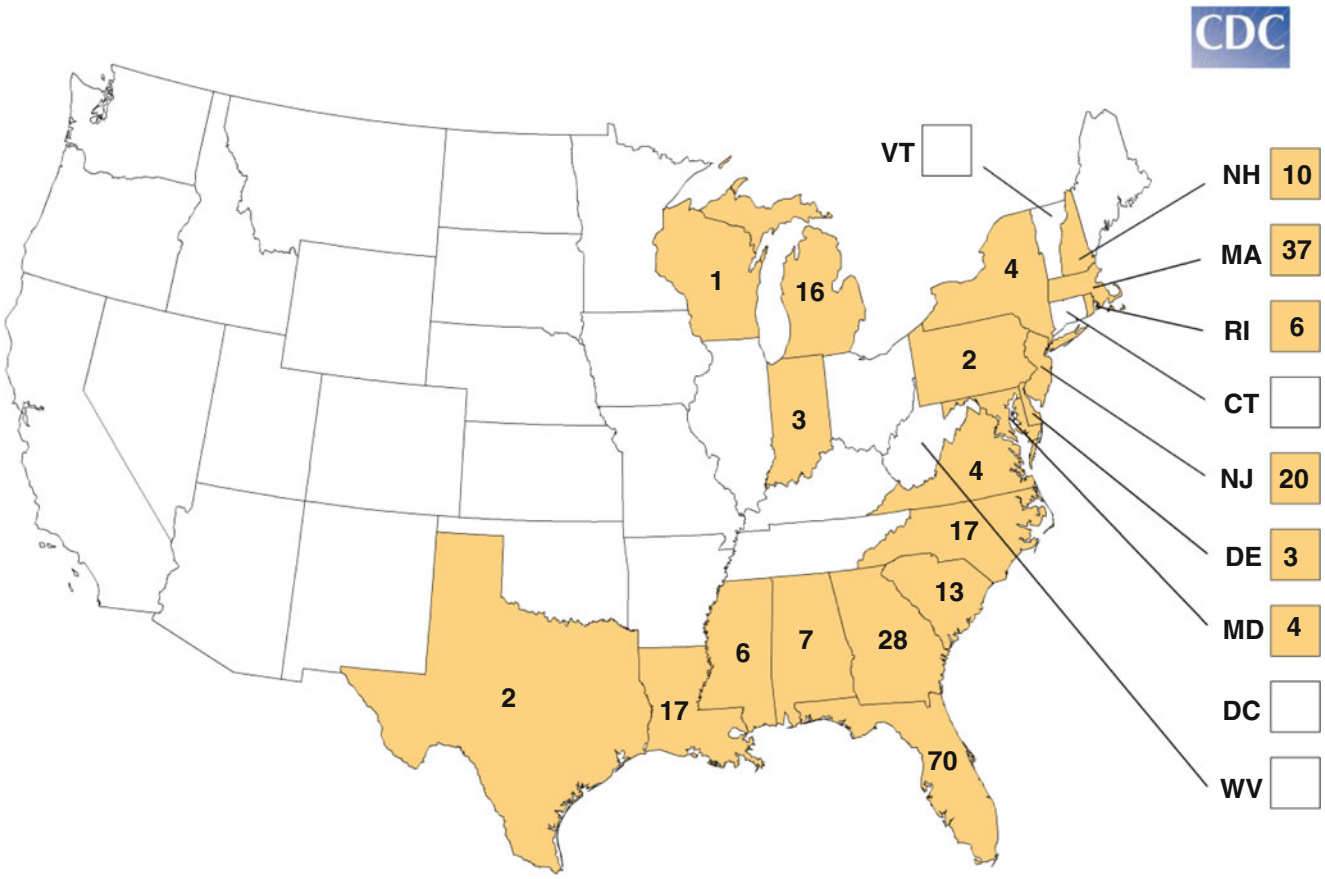

Fig. 7.2 Eastern equine encephalitis virus neuroinvasive disease cases reported by state, 1964–2010 (Adapted from http://www.cdc.gov/easternequineencephalitis/tech/epi.html)

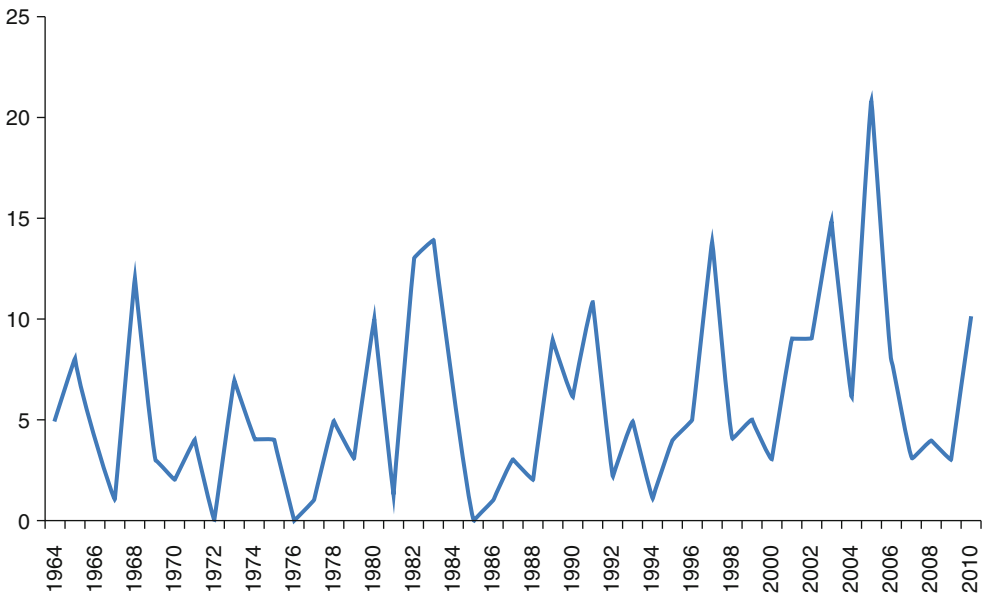

Fig. 7.3 Eastern equine encephalitis virus neuroinvasive disease cases reported as encephalitis, meningoencephalitis, or meningitis, by year, 1964–2010 (Adapted from http://www.cdc.gov/easternequineencephalitis/tech/epi.html)

15.2.2 Impact on Human and Animal Health

Clinical EEE in humans has remained sporadic with little evidence of any overall change in incidence during the past several decades. Although some epidemics from the 1930s to the 1950s involved more than 30 people [42], an average of only five to ten human cases has been documented annually in the USA since the 1970s (Fig. 7.3). Historic records suggest that the principal enzootic vector of EEE virus (EEEV),

Cs. melanura, may have become more abundant along the Atlantic seaboard following reforestation during the twentieth century, leading to increased virus circulation and periodic epidemics. Although the average number of human cases remains low, the high EEE case-fatality rate, usually exceeding 50 %, leads to fear in the affected populations and considerable expenditures for mosquito vector control to reduce the risk of infection [16]. Because EEE survivors often suffer permanent, debilitating neurologic sequelae, health care and institutionalization costs were estimated at $3 M per case in 1994 ($4.7 M in 2012 dollars) [43]. EEE occurs predominantly in eastern and Gulf Coast states and in northeastern inland states in the USA and Canada (Fig. 7.2).

15.2.3 Human Disease

The incubation period in human beings is short, usually 4–10 days [16, 44]. Gender is not a major factor in risk for disease [45], but patients <10 years of age are more likely to suffer severe sequelae than older age groups. The ratio of inapparent or mild infections to severe infections has been described as low in some studies based on very low human seroprevalence after epidemics [44]. However, following the 1959 New Jersey epidemic, 3.1–3.6 % of persons surveyed exhibited complement fixing antibodies, suggesting many inapparent infections [46], with inapparent (no recognized central nervous system disease)—apparent ratios of 8–26 % in different age groups, or 23 % overall [47]. Abrupt onset of severe fever, nausea, myalgias, and intense headache are followed by encephalitis in 1–2 days. Infants and children often present with convulsions. The encephalitis rapidly progresses to a coma, particularly in small children. Between 30 and 70 % of clinical cases are fatal. Patients with a short prodrome are more likely to develop encephalitis than those with a long prodromal illness accompanied by nonspecific signs and symptoms [48] a pattern observed in some animal models [49]. Pathological features include diffuse encephalitis with evidence of scattered neuronal destruction. Intensive supportive care is essential, since there is no specific treatment for EEE. Immunity is probably lifelong, with no reinfections being described.

15.2.4 Diagnosis

EEEV infection is typically suspected based on the proximity of the patient to known swamp habitats that support enzootic circulation in the eastern USA. Differential diagnoses include a wide variety of noninfectious (e.g., tumor, stroke, Alzheimer's disease, long-term alcohol abuse, and other dementias, although these conditions are not usually accompanied by fever) as well as infectious (herpes simplex virus, enterovirus, influenza virus, adenovirus, lymphocytic choriomeningitis virus, respiratory syncytial virus, rabies, or Epstein–Barr virus infection, bacterial meningitis, mycoplasma, *Bartonella henselae*, Rocky Mountain spotted fever, leptospirosis, Lyme disease, HIV, syphilis, tuberculosis, and other mycobacterial or fungal diseases) etiologies. After initial clinical suspicion, diagnosis is ideally established by virus isolation from the serum, cerebrospinal fluid (CSF), or brain tissue or the detection of viral RNA from these samples through reverse-transcription polymerase chain reactions [16]. However, human viremia is not typically of high titer, so serological diagnosis is more common, including demonstration of seroconversion (a fourfold or greater rise in antibody titers between acute and convalescent serum) or the finding of high-titer IgM in serum. Because the background prevalence of EEE in endemic areas is very low, a single serum demonstrating high-titer antibodies is highly suggestive of etiologic infection. Evidence of an outbreak of disease involving birds and mosquitoes, and more particularly equids or other domesticated animals in the region, should alert clinicians.

15.2.5 Prevention and Control

Because there are no licensed human vaccines or treatments, prevention of exposure to infected mosquitoes is the only way to prevent the disease. Passive surveillance relies on the finding that equine or avian disease usually precedes human infections. More active approaches include the use of chickens to detect EEEV circulation through seroconversion and monitoring mosquito populations for infection; [50] early season detection of the latter is predictive of late season epizootics and human cases [51]. When high levels of EEEV circulation are detected, the control of adult female mosquito populations is often attempted using aerial insecticide applications. Avoidance of mosquito contact relies on wearing long pants and long-sleeved shirts, applying repellents to exposed skin, and remaining indoors during the evening and nighttime when mosquito vectors are most active. An inactivated virus vaccine has been administered to laboratory personnel at risk through the US Army Special Immunizations Program [52]. Equids and other domesticated animals can be immunized with commercially available inactivated vaccines [53]. However, these vaccines are poorly immunogenic, requiring annual boosters, and can carry the risk of disease from residual live, virulent virus [54]. Several recently developed vaccine candidates have shown promise in preclinical trials [55–57], but because of the small numbers of natural human infections, they are unlikely to attract a commercial market.

15.2.6 Viral Genetics and Evolution

Genetically, aside from the recombinant portion of WEEV that was derived from an ancestral EEEV strain, EEEV is most closely related to VEE complex alphaviruses [58, 59]. Antigenically, it is also most closely related to the latter group. In North America and the Caribbean, EEEV is highly conserved genetically across time and space, with only less than 2 % nucleotide sequence divergence among all strains isolated since 1933 [60] (Fig. 7.4).

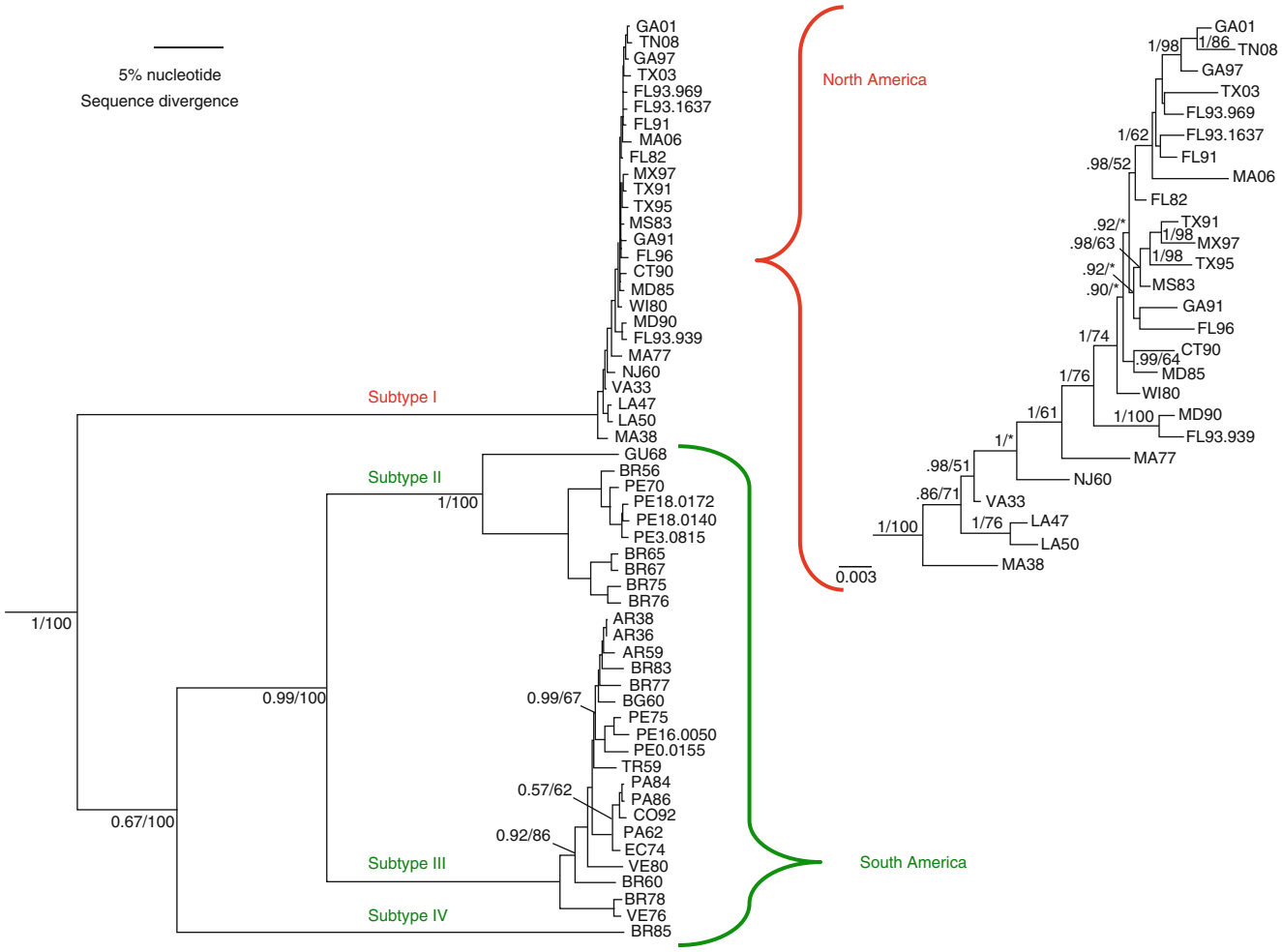

Fig. 7.4 Phylogenetic analyses of EEEV isolates using Bayesian methods, with the complete structural polyprotein open reading frames. (**a**) *Left*, tree of NA and SA EEEV. Bayesian posterior probability (PP) values and maximum parsimony (MP) bootstrap values are noted for all major nodes of lineage divergence (PP/MP values). Within each SA EEEV (MADV) lineage, values for PP/MP are shown only if either value is less than or equal to 0.90 (PP) or 90 (MP) for the adjacent node. *Scale bar* shows a genetic distance of 5 % nucleotide sequence divergence. (**b**) Magnified version of NA EEEV phylogeny. Values for PP/MP are shown only if either value is greater than or equal to 0.90 (PP) or 90 (MP) for the adjacent node. *Asterisks* indicate a polytomy in MP bootstrap analysis. *Scale bar* shows a genetic distance of 0.3 % nucleotide sequence divergence (Adapted from) [60]

This conservation and the evolution of EEEV as a single ongoing lineage in North America presumably reflect the efficient transport of strains via infected birds [61, 62], leading to periodic selective sweeps. However, phylogenetic groupings of strains isolated from temperate foci over several years also suggest that EEEV overwinters in the northeastern USA, where transmission does not occur during the winter because *Cs. melanura* survives only in the larval stage [62, 63]. In South America, EEEV strains (MADV) are much more diverse genetically and antigenically, with regionally defined viral clades that suggest the use of vertebrate amplifying hosts with limited dispersal potential [34, 60]. There is no evidence of mixing of EEEV strains between the continents. However, as with North American strains, two distinct lineages have been identified in the same location at the same time [64], suggesting that EEEV population structure is not completely spatially defined.

15.3 Venezuelan Equine Encephalitis

15.3.1 Natural History/Transmission

Venezuelan equine encephalitis (VEE) virus (VEEV) is one species in the VEE antigenic complex of alphaviruses, which comprises several species, subtypes, and varieties that can be distinguished genetically and antigenically [9, 65]. Most of these viruses, including VEEV subtypes ID and IE, circulate in enzootic transmission cycles involving rodent

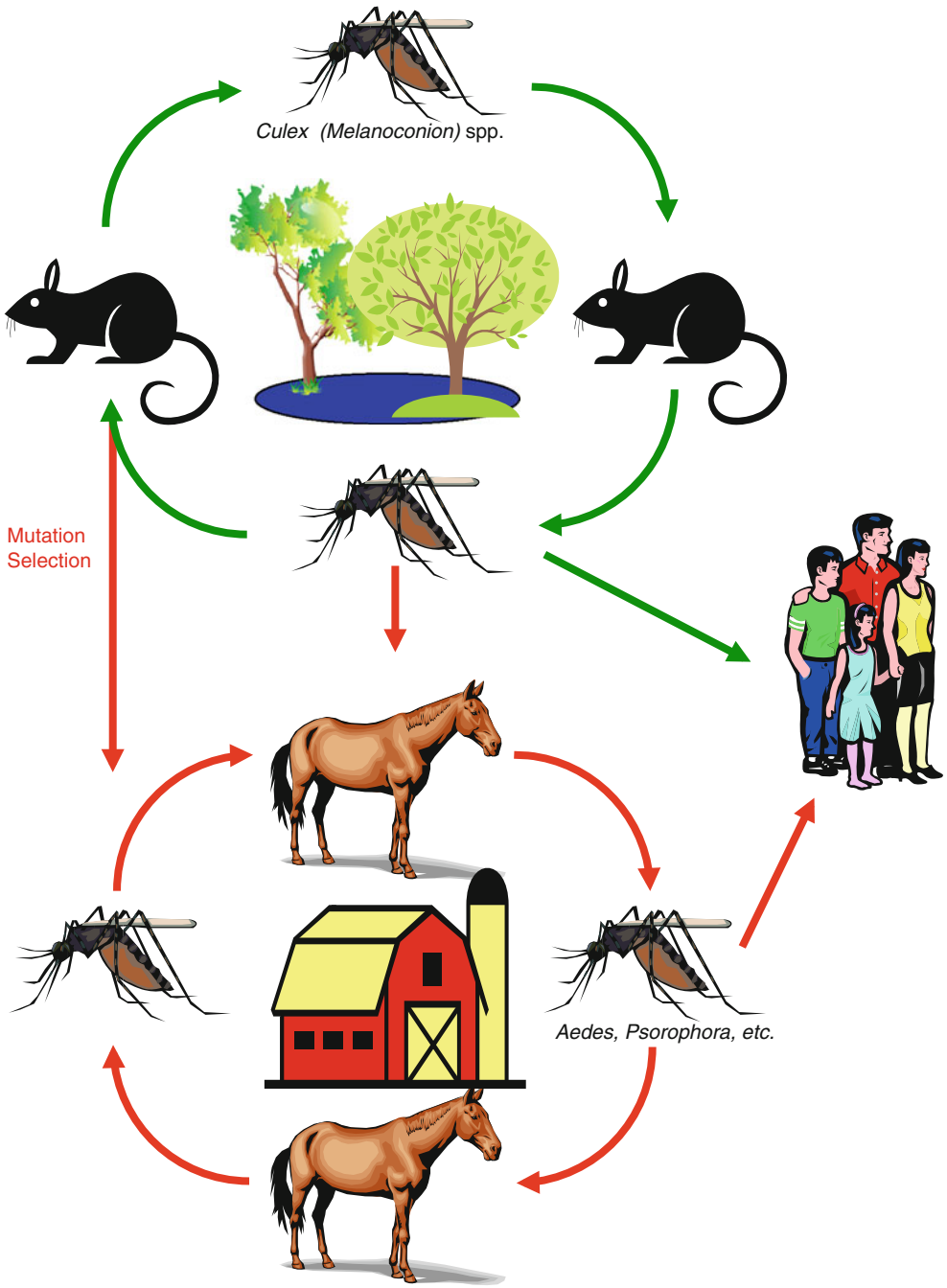

Fig. 7.5 Enzootic (*above*) and epizootic/epidemic (*below*) transmission cycles of most VEE complex alphaviruses (enzootic) and VEEV subtypes IAB and IC (epizootic/epidemic). *Arrows* show transition from the enzootic VEEV subtype ID strains to the epizootic/epidemic VEEV subtype IAB or IC strains via adaptive mutations that enhance equine viremia or from enzootic subtype IE strains in Pacific Coastal Mexico to an epizootic subtype IE phenotype that infects more efficiently *Aedes* (*Ochlerotatus*) *taeniorhynchus* mosquito vectors

reservoir/amplification hosts and mosquito vectors in the subgenus *Culex* (*Melanoconion*) (Fig. 7.5).

Exceptions include the VEE complex alphaviruses Tonate and Bijou Bridge, which appear to utilize birds as vertebrate hosts in South and North America, respectively. Although many members of the VEE complex have only been isolated in one or a few locations and at one or a few time points when surveillance activities were implemented (Fig. 7.6), they are probably widespread in South America.

Enzootic strains of VEEV, subtypes ID and IE, have been more intensively studied and are important causes of endemic disease among people who live in regions where they circulate in forested or swamp habitats [66–70]. Studies of dengue-like illness suggest that they may cause a burden of

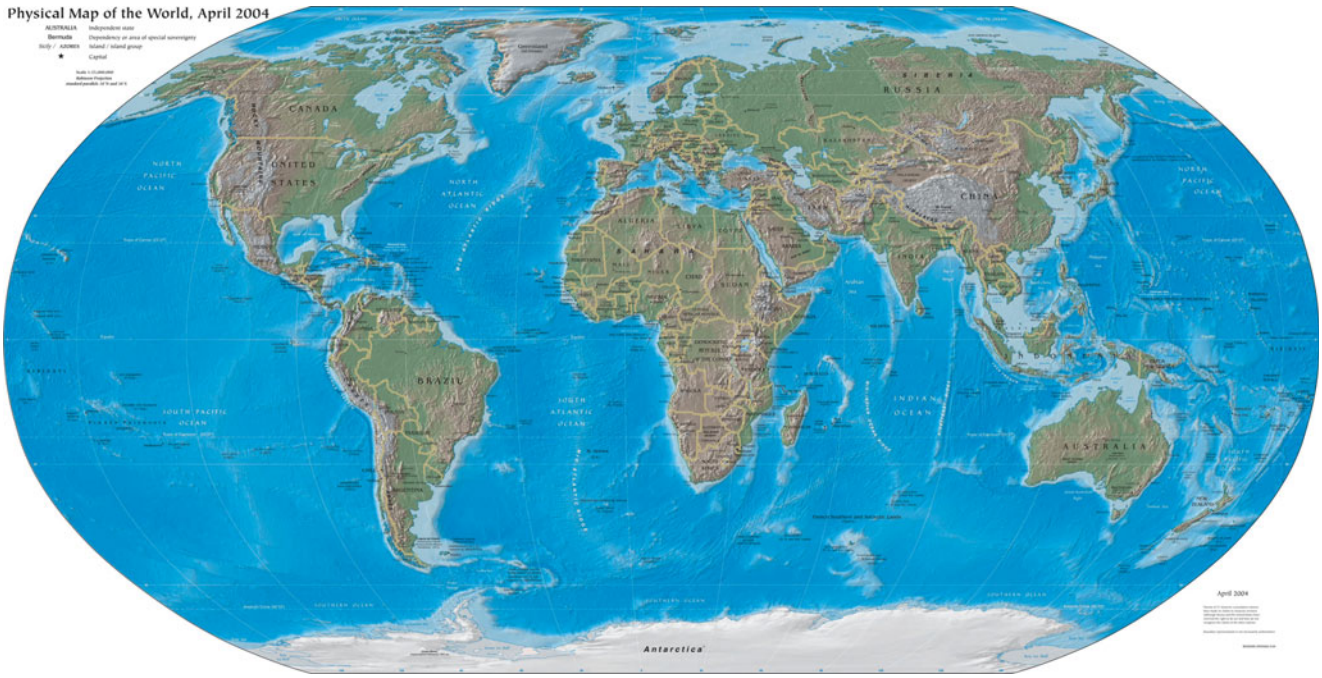

Fig. 7.6 Known distributions of alphaviruses in the VEE antigenic complex

disease that is approximately 1/10 that of dengue in Latin America and that fatal human infections occur regularly [66]. Everglades virus, which is only found in Florida, occasionally causes human disease [71] but has never been associated with equine outbreaks.

In addition to the enzootic strains that circulate continuously and in a widespread manner, VEEV variants in subtypes IAB and IC, often called "epizootic" or "epidemic" strains, arise periodically from the enzootic strains to cause major outbreaks of disease in people and equids [72, 73]. Phylogenetic and reverse genetic studies indicate that these strains arise when enzootic forms adapt via positive selection to increase their viremia and virulence in equids, leading to highly efficient amplification in rural locations [74], and/or to mosquito vectors that undergo seasonal expansions [75]. However, most of these epizootic/epidemic strains are short lived, presumably due to their reliance on equids for amplification and the limited population turnover of these relatively long-lived hosts.

15.3.2 Impact on Human and Animal Health

VEE complex viruses have a severe impact on both human and animal health. The former is affected both by spillover of enzootic transmission cycles and by equine-amplified epidemics, while equids and other domesticated animals are affected primarily during epizootics [72]. Because equids remain important for agriculture and transportation in areas of Latin America where epidemics occur, epizootics also have significant economic effects. Epidemics, which usually involve VEEV subtypes IAB and IC, typically affect hundreds to hundreds of thousands of people, with low rates of fatality but with many long-term effects on survivors due to high rates of permanent sequelae, especially among infected children. Endemic human VEE due to spillover directly from enzootic cycles is rarely diagnosed because most infections closely resemble dengue fever, which is endemic in most of Latin America [66, 67, 69]. However, if estimates that about 3 % of dengue-like illness are caused by VEEV in Iquitos, Peru [67], can be extended to other parts of Latin America, there could be tens of thousands of human cases annually, with hundreds of fatalities assuming a case-fatality rate of about 0.5 % [66].

15.3.3 Human Disease

Infection with VEEV or many VEE complex alphaviruses typically leads to an undifferentiated acute febrile illness characterized by an abrupt onset of headache and fever, often accompanied by gastrointestinal signs and symptoms [16]. Unlike those of many other arboviruses such as EEEV, most human VEEV infections are symptomatic and attack rates during outbreaks can exceed 50 % [65]. A small fraction of infections, primarily those in children, progress to central nervous system disease typically including convulsions, disorientation, and drowsiness and occasionally to coma and death. Children who survive neurologic disease often experience lifelong sequelae that affect motor function and cognition. In addition to its direct effects, VEEV is lymphotropic and causes immune suppression, which leads to secondary infections at elevated rates for up to months after infection.

There is no evidence that different strains of VEEV differ greatly in human virulence, although virulence differs markedly in equids [76, 77].

15.3.4 Diagnosis

Diagnosis of VEEV infection largely follows the procedures outlined above for EEEV [16]. However, in addition to the basic diagnosis that generally relies on virus isolation, IgM detection, or seroconversion (fourfold or greater ride in IgG measures by ELISA or other assay), it is important to determine the serotype circulating because subtypes IAB and IC can spread rapidly due to equine amplification. Virus isolation followed by complete or partial genome sequencing with a focus on the E2 envelope glycoprotein is most informative [78], but more rapid, ELISA-based methods are also available to diagnose the serotype of infection [79]. Human viremia usually lasts 3–4 days and is relatively high in titer, so virus isolation or viral RNA detection by RT-PCR is useful for definitive diagnosis. Once viral clearance from the blood has occurred, usually 5–6 days after infection, a blocking ELISA can still be used to determine the VEEV serotype of infection [80].

15.3.5 Prevention and Control

Prevention of endemic VEE caused by spillover of enzootic strains is challenging because the enzootic cycle involving rodents and forest-dwelling mosquitoes would be difficult to control due to its relatively remote location and widespread occurrence in the neotropics [66]. Prevention of exposure to enzootic vectors, which generally are active during crepuscular time periods or during the nighttime, relies on wearing appropriate long-sleeved shirts, long pants, and other garments to reduce skin exposure, applying repellents, and staying inside during the evening and night. Prevention of equine-amplified epidemics can be achieved by vaccinating equids with the live-attenuated TC-83 VEEV strain, which received its first widespread use during a 1971 Texas epidemic, where it may have limited spread of VEEV to other parts of the USA [81]. However, TC-83 has several deficiencies including reactogenicity in experimentally vaccinated laboratory workers and poor immunogenicity in many people and its reliance on only point mutations for attenuation [82]. Because it can be transmitted from vaccinated horses by mosquitoes, which occurred in 1971 [83], reversion to virulence could occur and might allow equine vaccination to initiate an epidemic. An inactivated version of TC-83, called C84 in human formulations, is administered to laboratorians and horses in the USA. Several other vaccines have been developed during the past decade, and one, live-attenuated strain V3526, was tested in Phase I clinical trials but was associated with some reactogenicity and thus has been developed as an inactivated formulation [84, 85]. Other DNA and live-attenuated vaccines appear promising in preclinical trials [86–90].

15.3.6 Viral genetics and Evolution

Among the alphaviruses, VEEV and related VEE complex viruses have received extensive study since the 1980s. Initial studies focused on determining the relationships between enzootic and epizootic/epidemic strains to determine the origins of the latter. RNA fingerprinting [91] followed by partial and complete viral genomic sequencing [78, 92, 93] first supported the hypothesis that epizootic/epidemic strains evolve periodically from enzootic precursors. Sequencing studies also suggested that several outbreaks caused by subtype IAB VEEV were initiated by incompletely inactivated equine vaccines made from early isolates [94]. Later, reverse genetic studies demonstrated that a single mutation in an enzootic subtype I strain was sufficient to transform the ID serotype and equine amplification incompetent phenotype to an IC epizootic phenotype [74] (Fig. 7.5). Another mutation found in recent Mexican subtype IE strains appears to have adapted the virus to more efficient transmission by the mosquito vector, *Ae.* (*Ochlerotatus*) *taeniorhynchus*. Phylogenetic studies have also revealed spatially partitioned genetic lineages of VEEV within serotypes, presumably reflecting the limited mobility of rodent and mosquito hosts.

15.4 Western Equine Encephalitis

15.4.1 Natural History/Transmission

Western equine encephalitis virus (WEEV) is one species in the WEE complex of alphaviruses [95]. Others include Highlands J Fort Morgan and Aura viruses in the New World and Sindbis virus in the Old World [96]. Western equine encephalitis virus was first isolated from a horse in California in 1930 [97], and the first human isolate was made nearby in 1938 [98]. Subsequent field studies determined that WEEV is mosquito-borne and that birds are the principal amplification hosts, especially house finches (*Carpodacus mexicanus*) and house sparrows (*Passer domesticus*) [95, 99, 100]. In most North American locations, *Cx. tarsalis*, which is often abundant in irrigated farmland, is the most important enzootic and epizootic vector mosquito, and human and equine infections generally occur in agroecosystems from June to August (Fig. 7.7) [99]. A secondary enzootic cycle involving leporids and other rodents and *Ae. melanimon* mosquitoes as vectors in arid habitats has also been described (Fig. 7.7). In South America, *Ae. albifasciatus* appears to be an important epizootic vector [101, 102].

Genetic studies (see below) indicate that WEEV is readily transported between North and South America but that local overwintering and regionally defined evolution occur in temperate regions of North America. However, experimental infections of birds have not generated chronic infections that could facilitate long-distance transport or

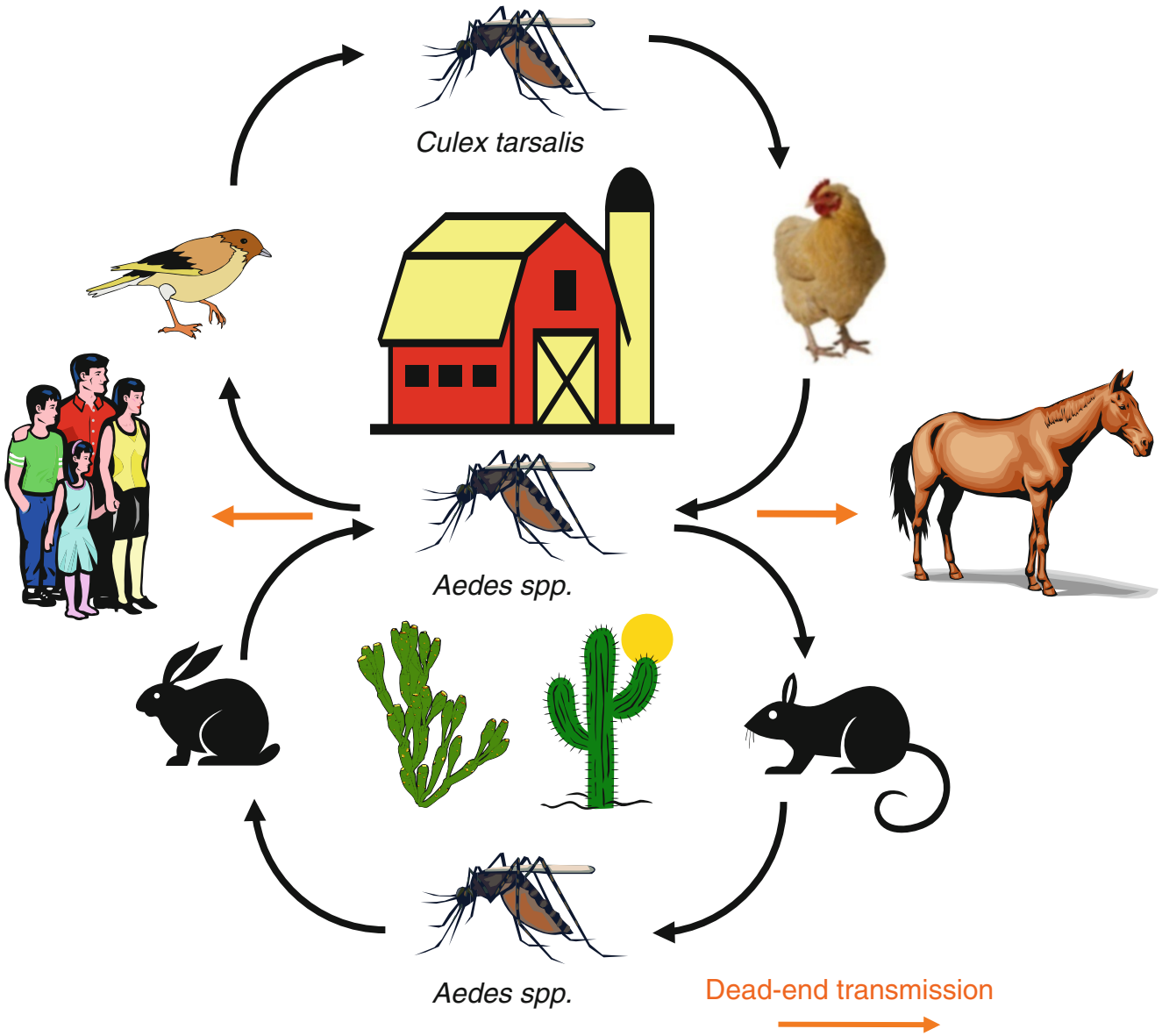

Fig. 7.7 Transmission cycles of western equine encephalitis virus in North America and epidemic or epizootic spillover to humans and domestic animals, respectively

overwintering of the virus [103, 104]. Overwintering of WEEV in infected mosquitoes undergoing reproductive diapause has been suggested by some laboratory studies but not confirmed by virus isolation from field-collected, overwintering *Cx. tarsalis* [105].

15.4.2 Impact on Human and Animal Health

During the mid-twentieth century, WEEV caused major epidemics and equine epizootics in the USA and Canada involving up to thousands of people and tens of thousands of equids during a single summer transmission season [99, 100]. Major epizootics occurred in the USA in 1930, 1937, 1938, 1941, 1944, and 1947 [95]. From 1937 to 1938, more than 300,000 equids were affected in the USA, and in 1941 alone, 2,242 human cases were documented in the Dakotas, Nebraska, and Minnesota (Fig. 7.8).

Human attack rates during this epidemic were as high as 171/100,000 persons [95]. However, the last documented human WEE case in North America occurred in 1999 in Minnesota, and only one other case has occurred since 1988 (in Colorado, 1989). Compared to the previous period from 1964 to 1988, when an average of 26 documented human cases occurred each year, this suggests a dramatic reduction in severe human disease caused by WEEV. The most recent

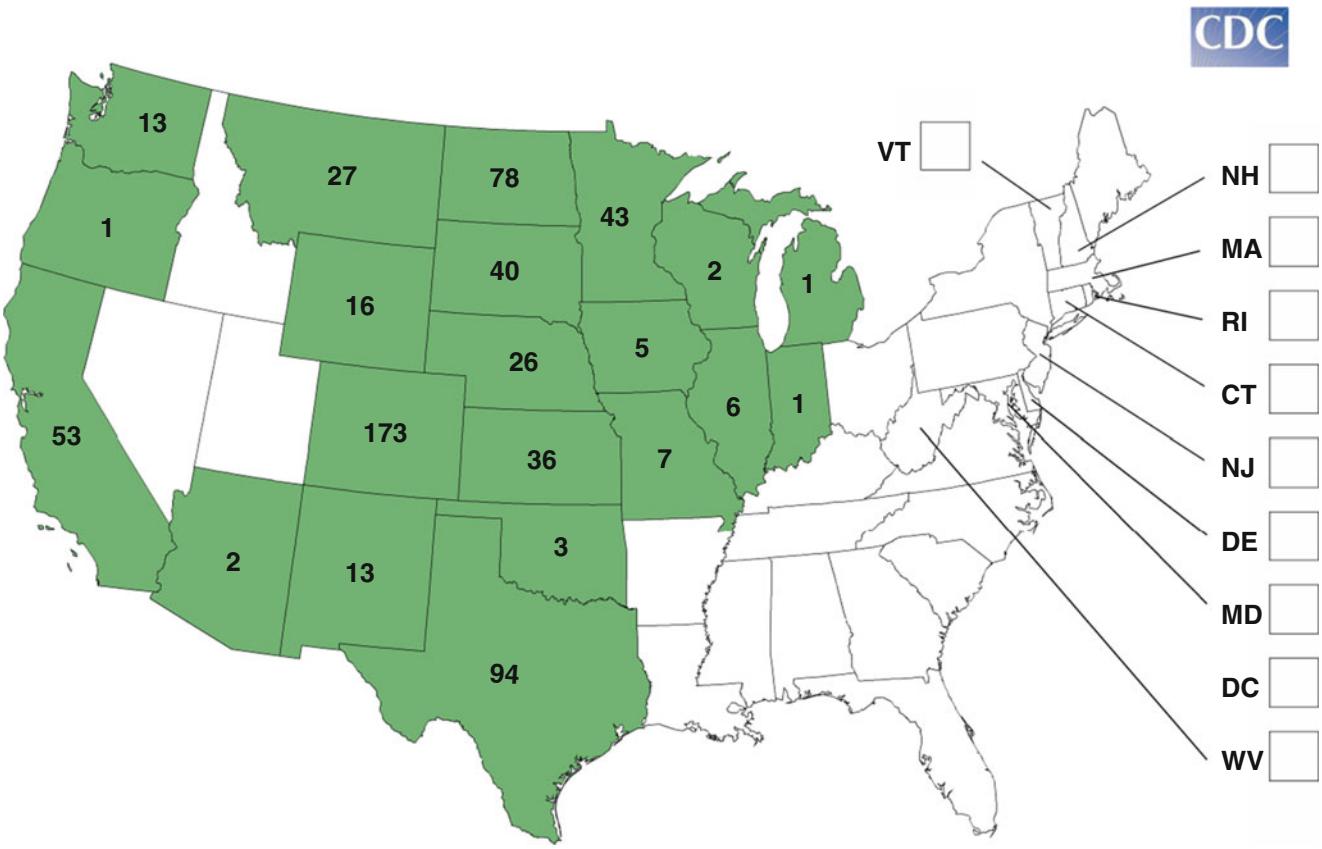

Fig. 7.8 Western equine encephalitis virus neuroinvasive disease cases reported by state, 1964–2010 (Adapted from http://www.cdc.gov/ncidod/dvbid/arbor/arbocase.htm)

estimates of human seroprevalence available from consistently enzootic regions of California were <3 %, indicating infrequent exposure [106, 107]. In Latin America, seroprevalence studies have indicated that WEEV circulation is widespread from Mexico [108] to Argentina [109], and equine epizootics have been described in Argentina since the 1930s. Human cases, which are occasionally fatal [110], have also been detected.

15.4.3 Human Disease

After an incubation period of 5–10 days, human WEE disease ranges from inapparent infection (by far the most common outcome, especially in adults where the apparent–inapparent ratio can be as low as 1:150,000) to a nonspecific flu-like illness to fatal encephalitis. The highest incidence and most severe infections generally occur in the youngest age groups, especially in infants <1 year of age. Males are more likely to be infected than females, possibly due to greater outdoor exposure in rural locations where the risk of infection is highest [95, 99, 100]. Symptomatic infections are typically accompanied by a sudden onset of fever, headache, chills, nausea, and vomiting. A minority of adult cases progress, usually one week from onset, to central nervous system disease characterized by generalized weakness and tremulousness, especially of the hands, tongue and lips. Some cases present with lethargy, drowsiness, nuchal rigidity, and photophobia, which can be followed by coma and death. Overall case-fatality rates usually range from 3 to 4 %. Children often exhibit muscular rigidity, involuntary movements, and paralysis. Survivors of encephalitis often experience lifelong neurologic sequelae, including fatigue, irritability, progressive mental and physical debilitation, headache, and tremors, which can also follow infection in utero [95, 99, 100]. Because no specific treatment is available for any alphavirus infection, patient management focuses on supportive care and treatment of acute complications, such as increased intracranial pressure and seizures, as well as management of long-term sequelae [16].

15.4.4 Diagnosis

A diagnosis of WEEV infection is usually based on the suspected exposure to rural locations where WEEV transmission occurs, often accompanied by knowledge of equine cases, which often precede those in humans. The diagnostic

approach and methods outlined above for EEEV and VEEV generally apply equally to WEE diagnosis. Like EEEV but unlike VEEV, WEEV is rarely isolated from blood or CSF at onset of symptoms because viremia titers are generally low and the incubation period is long, but can be isolated from brain samples taken at autopsy or from biopsies [95, 99, 100]. Antibodies are often detectable at first presentation due to the relatively long incubation period preceding signs and symptoms.

15.4.5 Prevention and Control

As for EEEV and VEEV described above, there are no licensed vaccines or antiviral drugs to prevent or treat WEE, so avoidance of contact with potentially infected mosquito vectors, especially in rural, agricultural settings, is critical.

15.4.6 Viral Genetics and Evolution

Initial sequencing and phylogenetic studies of WEEV revealed at least four major lineages [111]. Two of these were restricted to South America (Argentina and Brazil, respectively), while the others were widely distributed in both North and South America, suggesting that the latter are readily transported between the continents, probably via migratory birds. Enzootic WEEV strains from northern Argentina include an antigenic subtype [96] that is more attenuated in certain laboratory animal models than others [112]. More detailed studies of WEEV in California suggest that introductions from outside the state are not common, and little mixing of viral lineages occurs north versus south of the Tehachapi and San Bernardino Mountains, indicating regionally independent evolution [113]. However, within southern California, WEEV appears to disperse more freely. Finally, all WEEV strains are descendents of a recombinant alphavirus that derived its nonstructural protein and capsid protein genes from an ancestral EEEV and the envelope glycoprotein genes from an ancestral Sindbis virus strain [111, 114]. All other members of the WEE complex of alphaviruses, including Highlands J and Fort Morgan viruses, but not Aura virus, are also descendents of this ancient recombination event [111].

16 Alphaviruses of Public Health Importance Outside of the USA

16.1 Chikungunya Virus

16.1.1 Natural History/Transmission

Chikungunya virus (CHIKV) is transmitted in two distinct cycles. The enzootic maintenance cycle utilizes forest-dwelling mosquitoes such as *Ae. furcifer/taylori* and *Ae. africanus* and nonhuman primates. This cycle has only been identified in Africa [115]. In contrast, the epidemic transmission cycle occurs when humans are bitten by infected *Ae. aegypti* or *Ae. albopictus*. In this cycle, no other vertebrate reservoir is required (Fig. 7.9).

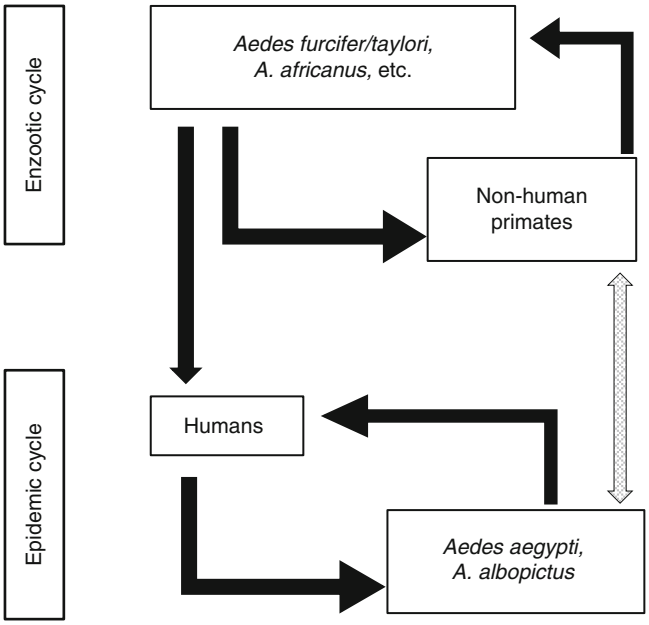

Fig. 7.9 Enzootic and epidemic transmission cycles of chikungunya virus

This is the only cycle that has been detected in Asia to date, where a limited number of field studies have failed to identify an independent enzootic cycle. It is possible that appropriate enzootic vectors are not present in Asia to establish a zoonotic cycle such as that found in Africa.

16.1.2 Impact on Human and Animal Health

The first detection of CHIK fever occurred in 1952–1953 during a small epidemic in what is now Tanzania [116]. While there were periodic human infections in population centers of East Africa, the first major urban outbreaks were recorded in Bangkok and India in the 1960s and 1970s [117–119]. During the next 30 years, there were sporadic emergences of CHIKV resulting in febrile illness, but major epidemic activity returned only after the turn of the century. At this time, reports of human cases began to increase in frequency in areas with historical activity such as Indonesia, while reports of the virus in new areas such as Central African Republic were also occurring [120, 121]. A major reemergence began in coastal Kenya in 2004 and moved to the islands of Comoros and Reunion off the coast of Tanzania in 2005/2006 [122–124]. Over 1/2 million cases were estimated in these three areas in less than 2 years. The virus then spread from Africa to India (over 1 million cases) and throughout Southeast Asia (Fig. 7.10).

A viremic traveler also transported the virus from India to Italy, where autochthonous transmission was detected for the first time in a temperate region [125]. This dramatic

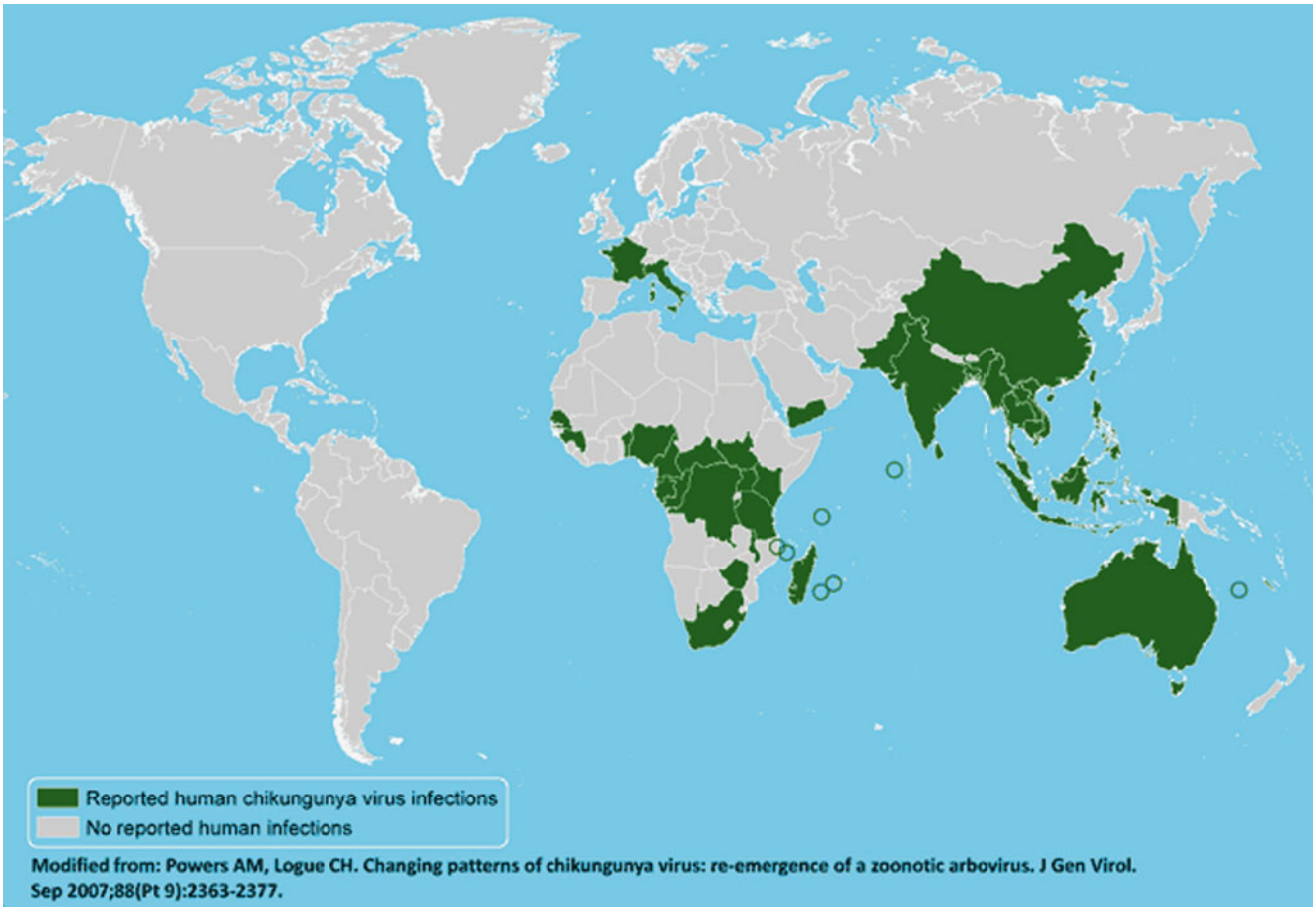

Fig. 7.10 Approximate known geographic distribution of CHIKV, 2007–2012 (Map obtained from CDC: http://www.cdc.gov/chikungunya/map/index.html)

geographic spread in less than 5 years with an estimated two million cases led to serious concern that a global epidemic could ensue. Significantly, during this massive and lengthy epidemic, the severe and chronic nature of CHIKV infection was revealed, as was the tremendous economic drain on affected countries [126].

16.1.3 Human Disease

Clinical disease begins between 3 and 7 days postinfection with the abrupt onset of a high fever (>101 °F). The fever typically lasts several days but usually subsides within 2 weeks. Shortly after fever onset, additional signs and symptoms appear which almost always include a severe bilateral arthritis of the small joints and may include a maculopapular rash. The rash, if present, lasts only a few days, while the arthralgia may be highly incapacitating and can persist for weeks or even months. Occasional reports of chronic joint pain (>1 year) have been documented, but these are atypical and may be associated with comorbidities.

Most CHIKV infections are symptomatic (75–97 %) and involve only the signs and symptoms noted above. However, recent outbreaks have revealed occasional, more serious conditions associated with infection including meningoencephalitis, hepatitis, myocarditis, and uveitis. Additionally, mortality was associated with infection for the first time in 2006, and intrapartum transmission was shown to result in neonatal complications [127].

16.1.4 Diagnosis

Because the symptoms associated with CHIKV infection are so nonspecific (fever, joint pain, rash) and similar to those caused by other sympatric pathogens (including dengue and malaria), laboratory diagnosis is critical for confirmation of infection. Fortuitously, viremia in humans is reasonably high (up to ~10^7 pfu/ml of blood) and persists for up to 1 week, allowing easy detection of the virus by either isolation or RT-PCR methods [18]. For serum samples collected more than 1 week after the onset of illness, serological assays such as ELISA or plaque-reduction neutralization tests (PRNT) can be used to detect anti-CHIKV-specific antibodies. Typically, both acute and convalescent samples must be tested, and a fourfold change in titer (for PRNT) or a change from negative to positive (in ELISA) must be detected for definitive diagnosis [128]. Commercial CHIKV diagnostic assays are now

available in limited markets, but their quality and utility are not universally accepted [129, 130]. Because CHIKV has such a broad geographic range, a complete travel history should also be considered with samples submitted for laboratory testing.

16.1.5 Prevention and Control

Like all mosquito-borne viruses, infection prevention can be accomplished using a limited number of approaches. These include mosquito control, personal protective measures, or vaccination. In general, mosquito control is extremely challenging as efforts must be broadly distributed, sustained, and coordinated with surveillance efforts. While there is historic evidence to show that CHIKV vector mosquitoes can be controlled and/or eliminated with intensive efforts, the mosquitoes rapidly return when control measures are discontinued [131]. Similarly, encouraging the use of personal protection such as repellents or long-sleeved shirts and pants presents challenges because most individuals do not maintain rigorous efforts to prevent mosquito bites. This is particularly difficult in the primarily tropical habitats where CHIKV circulates because repellents may not be widely available and the wearing of long sleeves is uncommon because of the hot and humid climate.

The ideal approach to control CHIKV would be by the use of a vaccine. However, there are no commercially available options at this time. A live-attenuated vaccine developed by the US Army was evaluated as an investigational new drug [132], and several newer vaccine candidates have been generated by genetic engineering. These include virus-like particles, chimeric viruses, genetically modified attenuated strains, DNA vaccines, and adenovirus-vectored options and others [133–136, 137]. To date, none of these have advanced into commercial development.

16.1.6 Viral Genetics and Evolution

Three distinct genotypes of CHIKV have been identified that, prior to the 2004 outbreak, were primarily aligned geographically (Asian, East/Central/South African (ECSA), and West African genotypes). Strains within each genotype are up to ~22 % genetically divergent from the others, while strains within a genotype are much more similar genetically, with a maximum of ~5 % divergence [138, 139].

The large number of complete CHIKV genome sequences publically available allowed close monitoring of the movement of the virus during the 2004–2009 outbreak. All strains from the 2004 to 2009 epidemics were the ECSA genotype, thus expanding the distribution of this genotype into Oceania, India, and Southeast Asia [139]. Studies of these novel isolates also allowed identification of mutations associated with altered vector infectivity and provided insights into the microevolution of the virus [140–143].

16.2 O'nyong-nyong Virus

16.2.1 Natural History/Transmission

O'nyong-nyong virus (ONNV) is unique among the alphaviruses in its transmission cycle; this virus is transmitted by anopheline mosquitoes (primarily *Anopheles gambiae* and *An. funestus*) during epidemics. Similarly, the few interepidemic isolates have also come from anophelines, suggesting the virus is maintained enzootically by this genus as well [144]. A single isolate of the virus was obtained from *Mansonia uniformis*, but this species was not abundant to determine its role in epidemic transmission [145]. The vertebrate reservoir host for ONNV has not been determined, but serosurveys suggest a role for domestic livestock and several species of rodents.

16.2.2 Impact on Human and Animal Health

The history of epidemic ONNV is one of the most intriguing of all alphaviruses. The virus was identified during the first recorded outbreak that lasted 3 years beginning in 1959. The magnitude of the outbreak was staggering, with an estimated two million cases spanning the duration of the epidemic across Uganda and other parts of East Africa [146]. With significant absenteeism due to the debilitating nature of infections, the outbreak had major impact on both the economies and public health resources of the numerous affected countries.

After this first epidemic subsided, there was virtually no detection of ONNV for ~40 years with the rare exception of isolates from mosquitoes or febrile humans. A subtype of ONNV, designated Igbo-Ora, was identified in West Africa, expanding its known geographic range [147]. However, no epidemic activity has been reported due to this strain. The next major epidemic of ONN fever occurred in Uganda in 1995–1996. Like the earlier outbreak, the virus moved rapidly through affected communities, impacting up to 70 % of the population [148]. No significant epidemic activity has been reported since 1996, although serological evidence of infection in vertebrates has been documented.

16.2.3 Human Disease

Like its close relative CHIKV, ONNV causes a febrile arthralgic syndrome. However, there are some distinctions from CHIKV infection such as the more prominent and frequent appearance of cervical lymphadenopathy in ONN cases. Additionally, the rash associated with ONNV infection is reported to be highly irritating but to cause little discomfort in CHIKV cases [149]. No mortality has ever been reported to be associated with ONNV infection and long-term sequelae appear to be rare.

16.2.4 Diagnosis

As noted for CHIKV, laboratory diagnosis is critical due to the lack of specific symptomology associated with ONNV infection, which precludes diagnosis based on clinical criteria. Diagnosis is primarily accomplished by serological testing, which includes testing for CHIKV infection since the two viruses have shared a close antigenic relationship and cross-reactions can be distinguished in only one direction, i.e., CHIKV immune serum inhibits the formation of ONNV plaques to the same extent as CHIKV plaques, but the inverse reaction is weaker [150]. Thus, when performing PRNT assays for detection of ONNV, additional test using CHIKV antigen must be performed. To confirm ONNV infection based upon these results, the neutralization titer for ONNV must be higher than that of CHIKV, and typically, the CHIKV titer will be lower or undetectable. Molecular techniques including RT-PCR approaches may also be used on acute samples. However, the duration and level of viremia in infected individuals is not well characterized, so the timing of the sample collection may preclude this as a feasible diagnostic option.

16.2.5 Prevention and Control

Prevention and control strategies are virtually the same for ONNV as described above for CHIKV. However, there may be fewer options for prevention of ONNV infection when compared with CHIKV. Mosquito control includes many of the same challenges but may be even more problematic for ONNV because the natural larval habitats of anopheline larvae are generally more diverse than those of CHIKV vectors. Personal protective measures include some of the same options as for CHIKV (repellent and long sleeves) but are likely to be used to a more limited degree in resource-poor settings typical of ONN epidemics. One personal protection approach that can be used to protect against ONNV infection is insecticide-treated bednets, as biting by anopheline mosquitoes primarily occurs at night. No vaccines have been developed to protect against ONNV. However, there is evidence that a recently developed CHIKV vaccine may induce cross protection against ONN disease [151].

16.2.6 Viral Genetics and Evolution

ONNV is genetically most closely related to CHIKV, with 28 % divergence at the nucleotide level [138, 139]. Antigenically, these viruses are also closely related, but a unique unidirectional relationship exists: antisera raised against CHIKV neutralize both viruses, but ONNV-antisera neutralize only the homologous virus as described above. Evolutionarily, this suggests that ONNV emerged from a CHIKV-like ancestor, followed by the evolution of unique surface epitopes.

Among the very few strains of ONNV that are available for evaluation, the degree of genetic diversity appears to be limited. Isolates obtained from the original outbreak have fewer than 100 amino acid differences from isolates obtained during the second Ugandan outbreak over 40 years later [152]. This indicates that the virus may be maintained in a zoonotic cycle involving efficient virus dispersal, which would retain a high degree of sequence similarity across a broad geographic range.

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8 Arenaviruses: Lassa Fever, Lujo Hemorrhagic Fever, Lymphocytic Choriomeningitis, and the South American Hemorrhagic Fevers

Daniel G. Bausch and James N. Mills

1 Introduction

The family *Arenaviridae* (genus *Arenavirus*) derives its name from the Latin "arena" for "sand," referring to the grainy appearance of infected host cell ribosomes seen on electron microscopy (Fig. 8.1) [1]. Arenaviruses are zoonotic, maintained in nature, with a few possible exceptions, by chronic infection in rodents of the superfamily Muroidea [2]. The viruses are grouped serologically, phylogenetically, and geographically into Old World/lymphocytic choriomeningitis (i.e., Africa, Europe, Asia, and Oceania) and New World/Tacaribe (i.e., the Americas) complexes. Over 40 arenaviruses have been identified, although less than half of these are clearly recognized as human pathogens (Table 8.1 and Figs. 8.2 and 8.3). Arenaviruses continue to be discovered, and at a quickening pace in recent decades [3–11]. Factors in disease emergence and the increased incidence of human cases often relate to anthropogenic disturbance of natural habitats, such as conversion of forest to cultivated fields, resulting in loss of biodiversity and selection for opportunistic rodent species that are frequently hosts for zoonotic pathogens [12]. Conversion to agriculture also prompts incursion of reservoir rodents from the surrounding bush seeking food in croplands, with resultant increased human exposure to rodents [13, 14]. Some reservoir species may also enter towns and homes, further increasing risk to humans.

Arenaviruses are perhaps best known as agents of viral hemorrhagic fever (HF), an acute systemic illness classically involving fever, a constellation of initially nonspecific signs and symptoms, and a propensity for bleeding and shock [15, 16]. A few arenaviruses have been associated with aseptic meningitis and other central nervous system (CNS) diseases, including congenital malformations when transmitted in utero. Many arenavirus infections are asymptomatic or result in a nonspecific febrile illness difficult to distinguish from many more common diseases.

Seven arenaviruses have been clearly established as causative agents of HF via natural infection (i.e., excluding laboratory infections), Lassa and Lujo in Africa and Junín, Machupo, Guanarito, Sabiá, and Chapare in South America (Table 8.1). Similar HF syndromes are caused by members of the virus families *Bunyaviridae*, *Filoviridae*, and *Flaviviridae* (see Chaps. 10, 15, and 17). As with other HF viruses, arenaviruses are generally named after the geographic location of the first recognized case or place of first virus isolation. For the New World arenaviruses, the

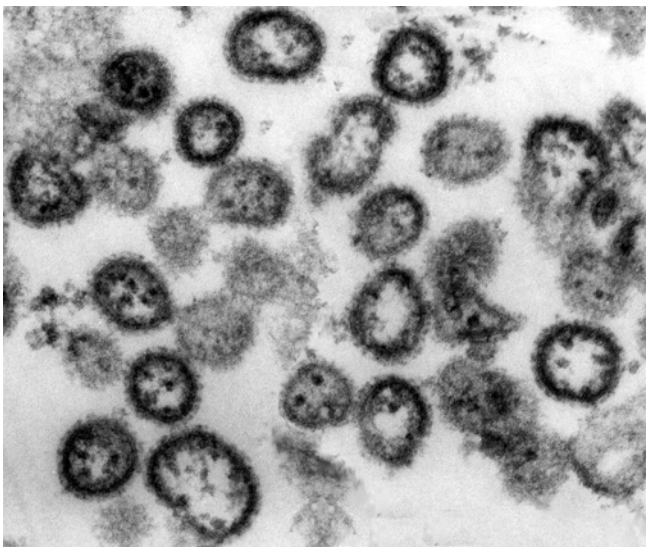

Fig. 8.1 Electron micrograph of Lassa virus. The typical sandy appearance of arenaviruses from the internal ribosomes is evident, as well as the surface glycoprotein spikes. Magnification approximately ×55,000 (Micrograph courtesy of F.A. Murphy, University of Texas Medical Branch, Galveston, Texas)

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Table 8.1 Arenaviruses known or suspected to cause human disease

Virus	Associated human disease	Annual incidence of human disease	Human disease-to-infection ratio	Human-to-human transmissibility	Case fatality
Dandenong	Fever with encephalopathy and multiorgan system failure	3 cases recognized	Unknown	All known cases infected through organ transplantation	100 % of three known cases
Lassa	Lassa fever	Poor surveillance. Estimated 30,000–50,000	1:5–10	Moderate	25 %
Lujo	Lujo fever	5 cases recognized	Unknown	Moderate to high	80 % of five known cases
Lymphocytic choriomeningitis	Nonspecific febrile illness/ aseptic meningitis	Poor surveillance. Estimated hundreds to thousands	Unknown but appears to be low	Congenital transmission as well as transmission through organ transplantation	<1 %
Chapare	Chapare HF	Unknown	Unknown	Unknown	1 fatality among a "small cluster" of cases. Few details reported
Flexal	Nonspecific febrile illness	2 laboratory infections	Unknown	Unknown	2 known infections were nonfatal
Guanarito	Venezuelan HF	<50	1:1.5	Low	30–40 %
Junín	Argentine HF	50–100	1:1.5	Low	15–30 %
Machupo	Bolivian HF	<50	1:1.5	Low	15–30 %
Pirital	Nonspecific febrile illness	1 laboratory infection	Unknown	Unknown	Only known case was nonfatal
Sabiá	Brazilian HF	1 natural and 2 laboratory infections known	1:1.5	Low?	33 % of three known cases
Tacaribe	Febrile illness with mild CNS symptoms	1 laboratory infection	Unknown	Unknown	Single nonfatal case
Whitewater Arroyo	Pathogenic potential unclear. Three putative cases with fever and some signs of hemorrhagic fever	Unknown	Unknown	Unknown	Unknown

diseases are often named after the country where they were first detected, with the virus named after the town or some other local geographic feature. For example, Junín virus was first isolated from a human in the town of Junín, in central Argentina, and subsequently designated as the causative virus of Argentine HF. Two Old World arenaviruses, lymphocytic choriomeningitis virus (LCMV) and an LCMV variant named Dandenong virus, are typically associated not with HF but rather with febrile illness and CNS disease [17]. Whitewater Arroyo virus has been detected in three sick persons in California but its role as a pathogen is controversial. Tacaribe, Pirital, and Flexal viruses have caused nonspecific febrile illnesses after laboratory accidents but no natural infections have been recorded. Numerous arenaviruses are thought to be nonpathogenic in humans, or at least pathogenicity has yet to be recognized. Pichinde and Pirital viruses are frequently used in animal models (guinea pig and hamster, respectively) of arenaviral HF.

Because of their potentially high lethality, risk of secondary spread (although this is often overestimated), and tendency to cause public panic and social disruption, some HF-causing arenaviruses are considered "Select Agents" that could possibly be used as bioweapons. Attempts to weaponize various arenaviruses were reportedly made by the Soviet Union during the Cold War era [18]. Many arenaviruses are classified as biosafety level four (BSL-4) or "maximum containment" agents [19].

2 Biological Characteristics

2.1 Physical Properties

Arenavirus virions are pleomorphic, ranging from 60 to 300 nM, and have a lipid envelope. On electron microscopy, host cell ribosomes show a grainy appearance inside the cell, with surface glycoproteins seen as club-shaped projections or spikes protruding from the envelope membrane (Fig. 8.1) [1].

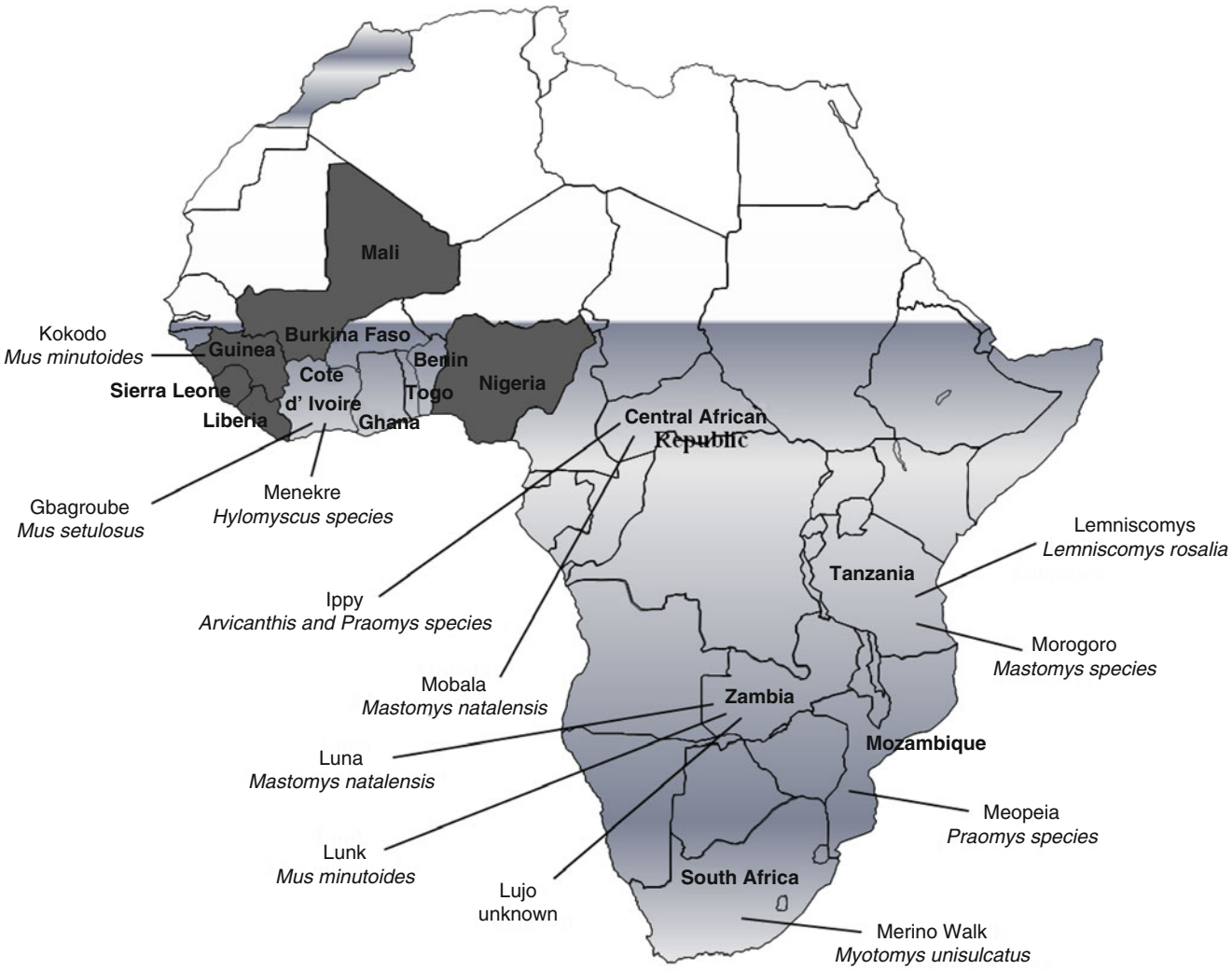

Fig. 8.2 Geographic distribution of Old World arenaviruses. The virus name and known or suspected rodent reservoir are listed. Although the virus has been isolated from the animal listed, the status of that animal as the natural reservoir is not established in all cases. Countries where Lassa fever, the major arenaviral disease in Africa, has been definitively shown are depicted in *dark gray* and the distribution of rodents of the genus *Mastomys* is shown in *light gray*. Only Lassa, Lujo, lymphocytic choriomeningitis, and Dandenong viruses have been definitively associated with human disease. The distribution of the virus and incidence of associated disease may vary significantly within each country. With the exception of Lassa and lymphocytic choriomeningitis viruses, most Old World arenaviruses have been isolated on single or very few occasions and the precise distribution of the virus beyond the place of first identification is unknown. Not shown on the map: lymphocytic choriomeningitis virus (reservoir *Mus musculus*), which has a worldwide distribution, and Dandenong virus (unknown reservoir), which is thought to be found in Eastern Europe

2.2 Gene Organization, Replication, and Transcription

Arenaviruses are bisegmented with a ~11 kb genome comprised of two single-stranded RNA segments denoted small (S) and large (L) of 3.4 and 7.2 kb, respectively [20, 21]. The S RNA encodes the viral nucleoprotein (NP) and a precursor glycoprotein (GPC) that is posttranslationally cleaved into GP1 and GP2 by the cellular subtilase SK1-1/S1P. Proteolytic processing of GPC is necessary for arenavirus infectivity. GP1 is a peripheral membrane protein while GP2 is transmembrane. Both are involved in receptor binding and cell entry. The L RNA encodes the viral polymerase (L protein) and a small zinc-binding protein (Z), which appears to play a regulatory role in virus replication, particle formation, and budding, as well as having a structural function as a matrix protein. The genes on the two RNA segments are separated by an intergenic region that folds into a stable secondary structure.

Arenavirus genes are oriented in both negative and positive senses on the two RNA segments, a coding strategy called ambisense. Through this mechanism, GPC and NP gene expression are independently regulated. Viral RNA must be transcribed before GPC can be expressed, which

Fig. 8.3 Geographic distribution of New World arenaviruses. *Left Panel*: the virus name and known or suspected rodent reservoir are listed, with viruses associated with natural infection and disease in humans in *bold*. Although the virus has been isolated from the animal listed, the status of that animal as the natural reservoir is not established in all cases. Countries with arenaviruses definitively associated with human disease are shaded *gray*. The distribution of the virus and incidence of associated disease may vary significantly within each country. Many of the New World arenaviruses have been isolated on single or very few occasions and the precise distribution of the virus beyond the place of first identification is unknown. *Right Panel*: close-up of administrative regions in which Venezuelan, Bolivian, Chapare, and Argentine hemorrhagic fevers have been recognized. The distribution of the viruses and incidence of disease may vary significantly within the region

may be fundamental to the maintenance of persistent infection in the animal reservoir. Replication and transcription of the genome occur in the cytoplasm and require the association of viral proteins with the viral RNA in the form of ribonucleoprotein (RNP) complexes. The NP and L proteins, together with virus RNA, are the minimal components of the RNP complex and are sufficient for genome replication and transcription. Purified RNPs are competent for RNA synthesis in vitro and can initiate virus replication after transfection into cells. Naked RNA is not infectious.

During genome replication, a full-length copy of the genome is synthesized yielding the corresponding antigenomic S and L RNAs. Due to the ambisense coding strategy, both genomic and antigenomic RNA serve as templates for transcription of viral mRNA. The transcripts contain a cap but are not polyadenylated. The viral RNA species that are packaged into virions are defined as the genomic RNAs; however, smaller amounts of antigenomic RNA and Z gene mRNA are also packaged. In addition to viral RNA, ribosomal RNA is present within virions. Virions mature by budding from the plasma membrane.

2.3 Phylogenetics

Both Old and New World complexes are further divided into major lineages or clades (Fig. 8.4). Genetic diversity within virus species may vary with the host and geographic region. Sequence diversity is generally higher among strains obtained from rodents than from humans; that pattern is consistent with chronic infection and frequent intraspecific transmission among rodents [22, 23].

The Old World complex is grouped into two monophyletic lineages that correlate with monophyletic genera within the rodent family Muridae, subfamily Murinae (see Sect. 3). The most attention has been paid to characterizing Lassa viruses, which show considerable sequence heterogeneity across West Africa, with four recognized lineages—3 in Nigeria and 1 in the area comprising Sierra Leone, Liberia, Guinea, and Ivory Coast [24, 25]. There is also considerable genetic heterogeneity within lineages (up to 26 % on the nucleotide level of the L and Z genes), especially in Nigeria [26]. Nigerian strains appear to be ancestral to those found further west in Africa, with limited recombination in the evolution of Lassa virus.

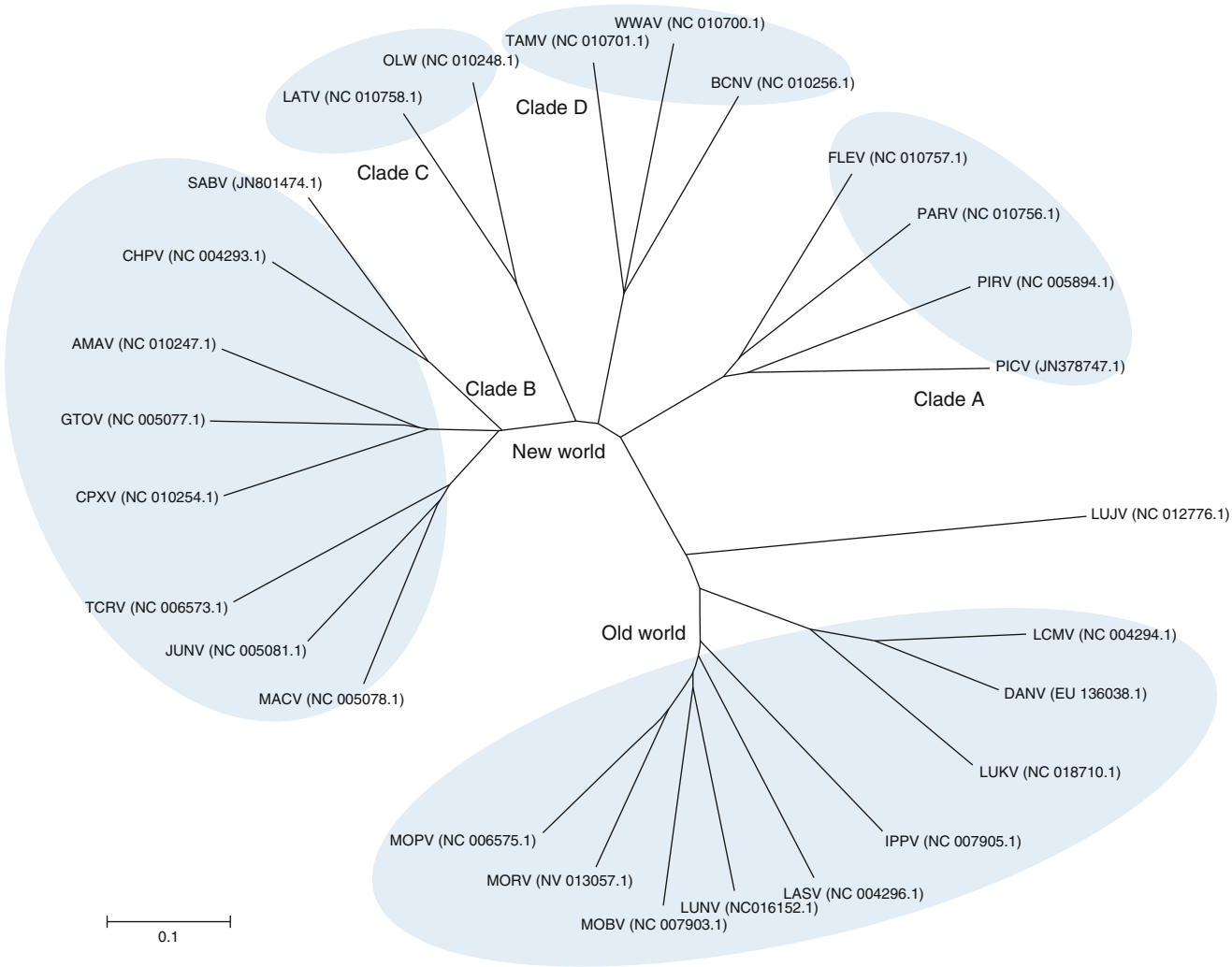

Fig. 8.4 Phylogenetic relationships of arenaviruses inferred based on full S segment nucleotide sequences. Phylogenies were reconstructed by neighbor-joining analysis applying the Jukes–Cantor model (1,000 replicates). The *A*, *B*, *C*, and *D* clades of the New World and Old World arenaviruses are delineated. Lujo virus (LUJV) falls between the two groups but appears to be closest to the Old World viruses, thus corresponding to its site of isolation in Zambia. The scale bar indicates substitutions per site. GenBank accession numbers are shown after each virus. Similar relationships are demonstrated based on L segment analysis (not shown). *Abbreviations*: *AMAV* Ampari virus, *BCNV* Bear Canyon virus, *CHPV* Chapare virus, *CPXV* Cupixi virus, *DANV* Dandenong virus, *FLEV* Flexal virus, *GTOV* Guanarito virus, *IPPV* Ippy virus, *JUNV* Junín virus, *LASV* Lassa virus, *LATV* Latino virus, *LCMV* lymphocytic choriomeningitis virus, *LUJV* Lujo virus, *LUKV* Lunk virus, *LUNV* Luna virus, *MACV* Machupo virus, *MOBV* Mobala virus, *MOPV* Mopeia virus, *MORV* Morogoro virus, *OLVV* Oliveros virus, *PARV* Paraná virus, *PICV* Pichinde virus, *PIRV* Pirital virus, *SABV* Sabiá virus, *TCRV* Tacaribe, *TAMV* Tamiami virus, *WWAV* Whitewater Arroyo virus. Viruses not shown due to incomplete sequence data: Allpahuayo, Big Brushy Tank, California Academy of Sciences, Catarina, Collierville, Gbagroube, Golden Gate, Kodoko, Lemniscomys, Menekre, Merino Walk, Ocozocoautla de Espinosa, Pinhal, Real de Catorce, Skinner Tank, and Tonto Creek

Field and laboratory data suggest variation in virulence among the four lineages and strains of Lassa virus. In laboratory experiments, the virulence of Lassa virus strains in guinea pigs roughly correlated with the severity of disease in the humans from whom the viruses were isolated [27]. Strains isolated from pregnant women and infants were benign in guinea pigs, suggesting that host factors such as immunosuppression play a role in human disease. Strains of Lassa virus from Nigeria may be more virulent than those from further west in Africa, but data to support or refute this theory are lacking. The virulence factors of the Lassa virus genome are not known, although for LCMV they are suspected to map to the L segment [28].

The New World complex is classified into 4 distinct lineages, A, B, C, and D, that generally correlate with monophyletic genera of the rodent family Cricetidae (see Sect. 3). All of the pathogenic New World viruses are in lineage B. Viruses of lineage D appear to be the product of recombination between viruses of lineages A and other arenaviruses and are thus also known as lineage A/Rec. Lineage A, B, and

C viruses are restricted to South America, while lineage D is exclusively North American.

There is also considerable genetic diversity among strains of LCMV, Guanarito, and Mopeia viruses, but no clear relationships between strain and pathogenicity in humans have been sought or recognized. Sequence diversity may also exist for other arenaviruses but the matter has not been extensively studied. Although pathogenic viruses generally cluster phylogenetically, recently identified Lujo virus illustrates the limitations in predicting clinical syndromes based on genetic sequence alone; Lujo and Lassa viruses cause almost identical HF syndromes, despite being genetically distinct (up to 38.1 % on the nucleotide level) [29]. Lujo virus is an outlier between New World and Old World arenaviruses but appears to be closest to the Old World viruses, corresponding to its site of isolation in Zambia.

2.4 Receptors

Arenaviruses enter cells by attachment of the GP1 to one or more cellular receptors [30]; α-dystroglycan, a protein found ubiquitously on primate and rodent cells, is a principal receptor for Old World viruses and pathogenic viruses of the New World clade C, while human transferrin receptor 1 protein is a receptor for the New World clade B arenaviruses [31]. Orthologs of transferrin receptor 1 protein appear to be the major receptors in the respective rodent host of each New World arenavirus and likely dictate species specificity [32].

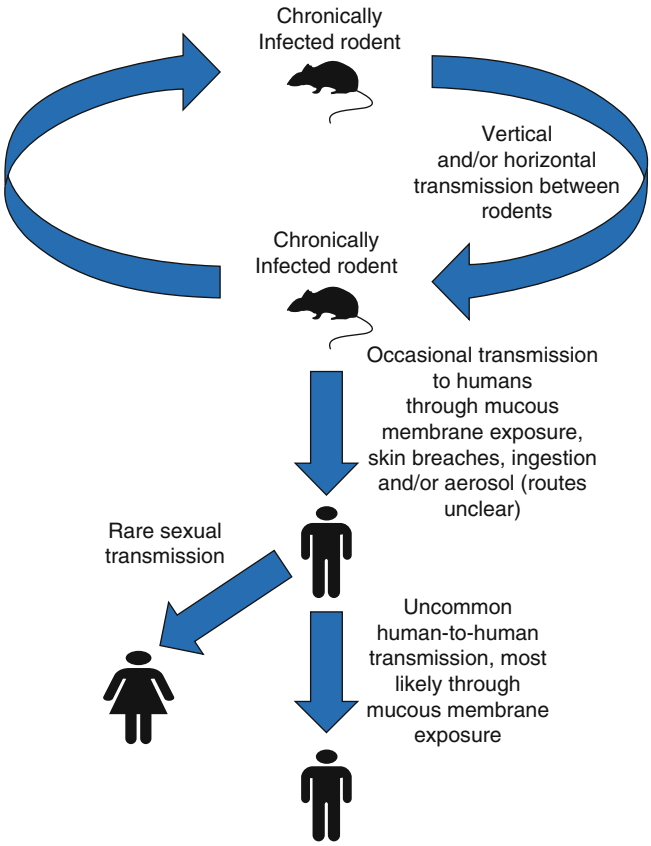

Fig. 8.5 Transmission cycle of arenaviruses illustrating chronic infection in rodents. Transmission between rodents may be vertical or horizontal depending upon the specific arenavirus. Humans are incidental hosts who play no role in virus maintenance in nature (Adapted with permission from Enria et. al. [17])

3 Reservoirs and Patterns of Host Response

Arenaviruses are generally maintained in nature by chronic infection in rodents of the superfamily Muroidea (Fig. 8.5) [2]. Transmission may be vertical (dam to progeny) or horizontal, depending on the specific arenavirus. Infected animals may chronically shed virus in urine, feces, and saliva. Old World arenaviruses are maintained in rodents of the family Muridae, subfamily Murinae, and New World arenaviruses in the family Cricetidae, subfamilies Sigmodontinae and Neotominae. There is a tight host–virus species pairing, thought to be the result of long-term rodent–virus coevolution [2]. Similar taxonomic classifications and host–virus relationships exist for the rodent-borne hantaviruses, suggesting a similar evolution. Occasional findings of a given arenavirus in species other than its recognized rodent host are usually considered to result from spillover infection (i.e., incidental transient infection of a non-reservoir host). These incidental and transient animal hosts are not thought to play a role in long-term virus maintenance.

The endemic area of each arenaviral disease is restricted to the geographic distribution of its rodent reservoir. Rodent populations are usually not uniformly infected across their entire geographic range; the distribution of the virus and disease is usually restricted to a small portion of the host range. The reasons for this are unclear but may relate to evolutionary bottlenecks in dispersal of the virus, rodent reservoir, or both. Landscape features are the most likely barriers to host migration. Humans are dead-end hosts who play no role in the natural maintenance of arenaviruses. There are generally few human cases relative to the frequency of infected rodents. The incidence of human infection may vary with changes in rodent abundance that relate to both climatic and seasonal weather changes and human-induced habitat alterations.

3.1 Old World Arenaviruses

3.1.1 Lymphocytic Choriomeningitis Virus

The common "house mouse" (*Mus musculus*) is the reservoir of LCMV, with infection maintained through vertical transmission [33]. Infection causes glomerulonephritis in mice and shortens their life-span by a few months, especially when

infected after birth. Renal deposition of virus–antibody complexes is believed to be the underlying cause of the kidney disease [34]. Although house mice and LCMV are phylogenetically of Old World origin, the rodent and virus have been disseminated worldwide as a result of rodent passage, usually on ships, over the last few centuries. Lymphocytic choriomeningitis virus may also transiently (but for months) infect laboratory mice and pet rodents, especially Syrian hamsters (*Mesocricetus auratus*) but also guinea pigs (*Cavia porcellus*), which can transmit the virus to humans [17, 35–41]. Whether Dandenong virus shares the same reservoir as LCMV is unknown.

As its name implies, the house mouse is frequently found living in association with humans within homes and other man-made structures in both rural and urban environments. The prevalence of LCMV and LCMV antibody in house mouse populations is highly variable (0–60 %) and focal [42]; in inner-city Baltimore, antibody-positive mice were clustered within residential blocks [43]; the prevalence of infected mice on four farms in California only several miles apart was 0–27 % [44].

3.1.2 Lassa Virus

Lassa virus is maintained in nature in the natal mastomys (*Mastomys natalensis*), also called the multimammate rat [45, 46]. Presumed spillover infection has been reported in other rodent species, including the reddish-white mastomys (*M. erythroleucus*), the roof rat (*Rattus rattus*), and the southern African pygmy mouse (*Mus minutoides*), although the animal species could not be definitively confirmed in all cases [47–50]. (L. Moses, manuscript in preparation). Reddish-white mastomys were reported to also be a natural host of Lassa virus but recent findings refute this. One explanation is the historical difficulty in identifying *Mastomys* to species; at least five morphometrically identical species exist in sub-Saharan Africa that, until recently, could only be distinguished through karyotyping, a technically cumbersome and perhaps error-prone technique. A reliable PCR-based assay to genotypically identify *Mastomys* to species has been recently developed and become the standard [51]. Studies using this technique have shown Lassa virus only in natal mastomys [50, 51].

Natal mastomys are almost always found in close association with humans in rural villages and surrounding cultivated fields and, less commonly, in grasslands and at the forest edge [52–54]. In highly endemic regions over 50 % of rodents caught in houses are natal mastomys, with the prevalence of Lassa virus infection ranging from 6 to 50 % [48, 50, 51, 54–56]. Because the incidence of Lassa fever is similar in men and women, and because children are frequently infected, peridomestic rodent exposure is thought to be most important [57]. Abundance of natal mastomys may be higher in houses than in surrounding agricultural fields or bush [48]. Open food storage and closed shutters during the day (a practice that favors prolonged rodent activity) may favor natal mastomys colonization of houses [48].

Despite the occurrence of *Mastomys* species throughout sub-Saharan Africa, for unknown reasons, Lassa virus and Lassa fever have been found only in West Africa (Fig. 8.2). Confusion over species identified in various regions as well as potential differences in the competency of *Mastomys* subspecies could account for this finding. Natal mastomys are not typically found in large urban centers, so the risk of rodent transmission of Lassa virus to humans in these environments is negligible.

Studies of transmission and maintenance of Lassa virus in natal mastomys are few. Consequently, many conclusions are based on extrapolations from experiments using LCMV in laboratory mice [58]. It is unknown how well this system represents Lassa virus. Available data suggest that Lassa virus is maintained in natal mastomys via vertical transmission. In addition, the age of the animal, host genotype, and route of inoculation affect the outcome of infection [59]; in the only published laboratory investigation of Lassa virus transmission in *Mastomys* species (reported to be natal mastomys), Lassa virus persistence was achieved when animals were infected as neonates, while most animals inoculated as adults cleared the virus [60]. Infected neonates did not produce Lassa virus antibody. Importantly, some laboratory strains of rodents identified as natal mastomys were later determined to be southern African mastomys (*M. coucha*), although it is unclear which species was used for the aforementioned study [61].

In wild-caught *Mastomys,* Lassa virus antibody and antigen are usually mutually exclusive [56]. Antibody-positive animals usually outnumber antigen-positive rodents, with a J-shaped curve of antibody prevalence (high at birth, decreasing in early adolescence, and then gradually rising as animals age). This pattern is consistent with transmission of maternal antibody to offspring, which is then lost as animals wean, to be regained by animals exposed to Lassa virus as they age. Although no pathology related to Lassa virus infection was observed in *Mastomys* infected in the laboratory, Lassa virus-infected wild natal mastomys were smaller in size and weight and had higher frequencies of myocardial and perivascular inflammatory lesions compared to their uninfected counterparts [62].

3.2 New World Arenaviruses

3.2.1 Junín Virus

Junín virus is maintained in the drylands vesper mouse (*Calomys musculinus*), also called the corn mouse. Occasional findings of antibody in (or even virus isolation from) species sharing the same habitat, including *Calomys laucha* and *Akodon azarae*, are likely due to virus spillover. Although drylands vesper mice are common and widely distributed in central and northwestern Argentina [63],

Junín virus is restricted to a small fraction of the host range (Fig. 8.3). The mice prefer more stable linear border habitats such as roadsides, fence lines, and railroad rights of way but may move into more mature and post-harvest crop fields in summer and early autumn to feed on abundant corn and soybeans [64, 65]. Unlike the natal mastomys, the drylands vesper mouse lacks peridomestic affinities and very rarely enters human habitations or peri-urban areas. Population densities of drylands vesper mice vary spatially and seasonally and appear to be dependent upon environmental conditions; the combination of a relatively mild winter followed by a cool, moist summer was followed by extremely high mouse abundance and a subsequent 14-year peak in cases of Argentine HF in 1990 [66]. The prevalence of Junín virus infection in reservoir populations is also highly variable temporally (0–50 % monthly prevalence over 3 years of surveillance) and spatially (0–8 % average long-term prevalence among sites within 30 km of each other) [67].

Field studies have shown that male drylands vesper mice are more frequently infected with Junín virus than females, that the prevalence of infection increases with age, and that infection is positively associated with the presence of scars [67]. These characteristics suggest that transmission between rodents is largely horizontal, between adult males, and may involve aggressive encounters and biting. In the laboratory, vertical infection resulted in highly deleterious effects on fitness [68]. Mice that were experimentally infected as adults showed a "split response"; half quickly developed antibodies and cleared the virus. The other half became chronically infected and persistently shed virus in urine and saliva [69], a characteristic that would facilitate horizontal transmission via aggressive encounters with biting, allogrooming, venereal contact, contact with infected nest materials, and perhaps inhalation of infectious aerosols.

3.2.2 Machupo Virus

The host for Machupo virus has been described as the big laucha (*Calomys callosus*), also called the large vesper mouse. Recent studies have shown, however, that *C. callosus* sensu lato (s. l.) is a clade of related but independently evolved taxa [70]. The "Beni Department clade" is one of those taxa and would be the host of Machupo virus [70, 71]. This new phylogenetic work helps to explain why Machupo virus and Bolivian HF are restricted to a small area of Beni Department, northeastern Bolivia, where *C. callosus* of the Beni Department clade are found, while *C. callosus* s. l. occurs in a much wider area of Bolivia, Brazil, Paraguay, and Argentina [72].

The big laucha is an opportunistic species that frequents disturbed habitats where forest and grasslands meet. They often seek higher ground during seasonal inundations, thus increasing contact with humans [73]. Unlike its congener *C. musculinus*, *C. callosus* will readily enter human habitations, sometimes in large numbers, and these events have been associated with devastating outbreaks of Bolivian HF [74, 75]. This same commensal characteristic contributes to relatively quick control of outbreaks by intensive trapping within and around homes in outbreak areas [73]. Both rodent abundance and prevalence of infection are highly variable over space and time, with the prevalence of Machupo virus infection in reservoir populations likely linked to population density. The prevalence of infection in rodents was 35 % in sites where human disease was present but much lower in disease-free areas [76]. No long-term studies of host or host–virus dynamics have been conducted. Therefore, the specific factors influencing population dynamics and Machupo virus transmission within host populations are unclear but likely include environmental factors such anthropogenic disturbance of natural ecosystems, which favors opportunistic species [12]; flooding, which concentrates host and human populations together on high ground [77]; and rainfall, which influences habitat quality and food availability.

In the laboratory, infection with Machupo virus in big laucha follows a similar pattern as Junín virus in drylands vesper mice; females inoculated at birth were chronically infected and sterile, indicating that vertical transmission would have a highly detrimental effect on the population. When infected as adults, again there was a split response, with half of the animals clearing the infection and half developing chronic infection and shedding virus in urine, feces, and saliva. It has been proposed that this split response is genetically controlled [78]. The mechanism of transmission among adult hosts in nature has not been studied, but presumably involves many of the same modalities described above for Junín virus and drylands vesper mice [78]. The split response caused by Machupo and Junín virus infections in their respective reservoirs has led to speculation that seasonal fluctuations in the incidence of Bolivian and Argentine HFs may be attributed to decreased populations of rodents caused by lowered fecundity in subsequent generations following chronic infection with these viruses.

3.2.3 Guanarito Virus

Guanarito virus is strictly hosted by the short-tailed zygodont mouse (*Zygodontomys brevicauda*), also referred to as the cane mouse, on the plains of northwestern Venezuela. A second *Sigmodontine* rodent species, Alston's cotton rat (*Sigmodon alstoni*), shares the same habitat with short-tailed zygodont mouse and hosts a second arenavirus (Pirital virus), yet spillover from one host to the other is rare [79, 80], illustrating the high specificity of rodent host–arenavirus relationships. It is not known whether this lack of cross-infection is due to a lack of physical interactions between the two species, despite their sharing the same habitat, or the poor ability of each virus to infect the other host. Within the endemic area for Venezuelan HF, both rodent species are commonly captured in crop fields, borders, and roadside habitats but

rarely in the peridomestic environment. Although the short-tailed zygodont mouse is a savanna species ranging from southeastern Costa Rica through most of Brazil north of the Amazon [81], the distribution of Guanarito virus (as determined by the occurrence of human cases) is restricted to a 9,000-km^2 area in Portuguesa and Barinas States in northwestern Venezuela [14]. Rodent community analysis [82] suggests that the development of intensive mechanized agriculture is associated with a relatively depauperate rodent assemblage dominated by the two arenavirus host species, the short-tailed zygodont mouse and Alston's cotton rat.

Laboratory studies [83] as well as detailed studies of tissue and fluid samples from wild short-tailed zygodont mice captured in the disease-endemic area [80] have provided a basic understanding of Guanarito virus infection dynamics in the host population. As with Junín and Machupo viruses, laboratory infection of newborn and juvenile rodent hosts resulted in chronic infection and persistent viral shedding in urine and saliva. There was no apparent detriment of infection to these animals after 3 weeks. Inoculation of adults again resulted in a split response, with some animals quickly clearing the virus and others developing a chronic infection, shedding virus in urine, saliva, and respiratory secretions. However, infection in adult females was associated with significantly reduced reproductive success [83]. In field-collected mice, infection was positively associated with host age, suggesting horizontal transmission. However, virus was restricted to lung tissue (not spleen or kidney), suggesting infection via the respiratory tract rather than wounding or venereal routes as suggested for Junín and Machupo viruses. Also unlike the Junín virus–drylands vesper mouse system, both sexes were equally infected, again arguing against infection by fighting among males [80]. Studies involving temporal patterns of host population dynamics and virus transmission and the influence of environmental factors on these processes are needed.

3.2.4 Whitewater Arroyo Virus

Numerous viruses antigenically and phylogenetically related to Whitewater Arroyo virus are widely distributed throughout the southwestern United States in association with several species of the genus *Neotoma*, including the southern plains wood rat (*N. micropus*), Mexican wood rat (*N. mexicana*), Stephen's wood rat (*N. stephensi*), and bushy-tailed wood rat (*N. cinerea*).

3.3 Other Arenaviruses

The reservoirs of Sabiá, Chapare, Lujo, and Dandenong viruses are unknown but are presumed to be muroid rodents. Most of the other arenaviruses from both the Old and New World complexes (Figs. 8.2 and 8.3) have been found in nature on relatively few occasions, often as a serendipitous result of field studies targeting other diseases. The animal from which the virus was isolated is assumed to be the natural reservoir, but systematic confirmatory field or laboratory studies have generally not been conducted. A few arenaviruses have been found only in animals other than rodents but the role of these animals as true natural hosts remains to be confirmed; Tacaribe virus has been isolated only once, from *Artibeus* species bats captured on the island of Trinidad [84]. However, a definitive role of these bats as the natural reservoir of Tacaribe virus is far from certain and placed in further doubt by failure to produce stable, nonlethal infection after laboratory inoculation of these bats with Tacaribe virus [85]. Three highly genetically divergent arenaviruses were recently discovered and putatively linked to inclusion body disease, a common infectious disease of captive snakes [86].

4 Rodent-to-Human Transmission

Transmission of arenaviruses to humans is believed to occur via exposure to rodent excreta, either from direct inoculation to the mucous membranes or broken skin or from inhalation of aerosols produced when rodents urinate [2, 45, 48, 87]. The relative frequency of these modes of transmission is unknown. Although the infectious dose for arenaviruses is thought to be low, transmission from rodents to humans appears to be inefficient, occurring infrequently even where infected rodents are common [56]. Transmission through aerosolized rodent urine or virus-contaminated dust particles is often mentioned in the scientific literature, but there are few data to support or refute its occurrence [88]. Although household clusters of arenaviral HF cases occasionally occur, single cases are much more common. This suggests that aerosol transmission to humans is not common, since it would logically often simultaneously infect multiple people in proximity to the aerosol source. Secondary aerosol generation, such as what might be produced through sweeping an area contaminated by rodent urine, is inefficient and is thus a less likely mechanism of infection. However, infectious and moderately stable aerosols of Lassa, LCMV, and Junín viruses have been artificially produced in the laboratory, so the possibility of primary aerosol transmission cannot be discarded [59, 89]. Regardless of their role in natural infection, the artificial production of infectious aerosols has obvious implications for the potential use of arenaviruses as bioweapons [19, 90].

Experimental data illustrate that arenavirus infection may also occur by the oral route, perhaps through a gastric portal [91, 92]. Lassa virus has been contracted when rodents are trapped and prepared for consumption, a common practice in some parts of West Africa, although it is rarely possible to determine whether infection resulted from exposure during

preparation or consumption [93]. Since arenaviruses are easily inactivated by heating, eating cooked rodent meat should pose no danger [2]. Various arenaviruses have been found in rodent saliva, although there are no reports of human infection from bites [60].

5 Transmission and Disease in Humans

With the exception of the LCMV reservoir, all arenavirus reservoirs occupy almost exclusively sylvatic rural habitats. Consequently, primary arenavirus infection in humans is seen almost exclusively in rural and often remote settings. Secondary human-to-human transmission may occur with some arenaviruses, most often to those caring for sick persons either at home or in healthcare centers. The combination of the remote and geographically restricted endemic areas (often in resource-poor countries), the apparent rarity of most arenaviral infections in humans, and the challenges to both clinical and laboratory diagnosis (see Sect. 8) make surveillance for arenavirus infection difficult. Consequently, reliable estimates of incidence are rare, except for Argentine HF, for which intensive surveillance with supporting laboratory diagnosis is applied in its circumscribed endemic area.

5.1 Old World Arenavirus Diseases

5.1.1 Lymphocytic Choriomeningitis and Other Central Nervous System Disease (LCMV and Dandenong Virus Infections)

Isolated during an investigation of encephalitis in St. Louis, United States, in 1933, LCMV was the first recognized arenavirus as well as the first recognized cause of aseptic meningitis in humans [94]. Ironically, despite being the prototype arenavirus, LCMV is one of the only pathogenic arenaviruses not associated with viral HF. Nevertheless, the virus has been extensively employed in mouse models to study arenavirus immunology. Given the great differences in associated clinical syndromes and pathogenesis, the applicability of these studies to other arenavirus infections is unknown [58].

Most human LCMV infections are thought to occur in homes, corresponding with the propensity of house mice to inhabit human dwellings [42]. Although cases are sporadically reported worldwide, accurate estimates of incidence are impeded by the usual presentation as a nonspecific febrile illness for which [95] diagnostic testing is not commonly requested or available. Most antibody surveys show a prevalence in humans of 5–10 % [36, 95–98]. The incidence of disease appears to have declined in recent decades, perhaps as housing construction and sanitation have improved, decreasing human exposure to house mice (although there is perhaps a bias toward data from more developed settings where cases are more likely to be noted, laboratory confirmed, and reported) [42]. Older reviews of patients with CNS disease showed LCMV infection in 8–11 % [42]. Infection with LCMV was the confirmed diagnosis in about 10 % of patients with febrile neurological syndromes admitted to a tertiary care hospital in Washington, DC, from 1941 to 1958, but the diagnosis was much less common recently [99, 100]. An investigation of cases of encephalitis in California (91 cases from 1998 to 2000) and England (2,574 cases from 1989 to 1998) revealed LCMV infection in zero and less than 1 %, respectively [101, 102]. Antibody surveys showing much lower prevalence in younger than in older patients support the notion of declining incidence, although this could also be explained by other factors that result in varied risk between age groups [97]. The incidence of LCMV infection peaks in the fall and winter in the northern temperate zones, presumably reflecting seasonal invasion of homes by house mice. The risk of infection is higher among rural dwellers and, in the United States, in low-income groups and, as expected, varies with the degree of rodent infestation in a home [36, 97, 103].

In 2003 and 2005, small clusters of LCMV infection and highly fatal (seven of eight cases died) neurological disease with organ failure occurred in solid-organ transplant recipients in the United States [104]. A third transplant-related cluster of three fatal cases occurred in Australia in 2009, leading to the discovery of the LCMV variant Dandenong virus [105]. The donor died of cerebral hemorrhage 10 days after returning to Australia from a 3-month visit to the former Yugoslavia, where he had traveled in rural areas. None of the donors in these outbreaks had a recent history of acute febrile disease and no virus could be identified in them, although the presence of IgG and IgM antibodies in the Australian donor confirmed recent infection. One donor in the United States had been exposed to a pet hamster from which LCMV was isolated and corresponded to the virus detected in the transplant recipients. With the exception of congenital transmission and through transplanted organs, there is no human-to-human transmission of LCMV.

5.1.2 Lassa Fever (Lassa Virus Infection)

Lassa fever is named after a village in northeastern Nigeria where the first case was recognized in 1969 [106]. However, hospital records and anecdotal reports describe cases consistent with Lassa fever in Nigeria as early as 1952 [107]. The disease is endemic exclusively in West Africa (Fig. 8.4). After first recognition in Nigeria, Lassa fever was noted in Sierra Leone, Liberia, and Guinea in the wake of hospital outbreaks and field studies in the early 1970s [57, 106, 108, 109]. Subsequent antibody prevalence studies and case reports indicate that Lassa virus is present in other West Africa countries [110, 111]. However, the risk of exposure to Lassa virus varies significantly across a given country and often between regions or even villages within endemic areas.

The highest incidence of disease appears to be in areas of eastern Sierra Leone, northern Liberia, southeastern Guinea, and central and southern Nigeria (Fig. 8.4) [48, 57, 112, 113]. Reasons for the extreme heterogeneity in incidence are not clear, especially considering that natal mastomys are often common in areas where little or no Lassa fever has been recognized. Varied intensity of surveillance may contribute to the apparent heterogeneous distribution, but cannot completely explain it.

Lassa fever is an extremely common infection among adults and children of both sexes in endemic areas of West Africa. An annual incidence of 300,000–500,000 Lassa virus infections with up to 5,000 deaths is often quoted in the scientific literature, but these figures are extrapolations from surveillance data collected in the 1970s and 1980s in eastern Sierra Leone where Lassa fever is clearly hyperendemic [48]. Estimating the true incidence and mortality is extremely difficult due to the nonspecific clinical presentation [57, 114], logistical impediments presented by civil unrest [115], unstable governments with underdeveloped surveillance systems [116], extensive human migration and perturbation of the physical landscape, and lack of reagents and laboratories for diagnostic confirmation in West Africa [117].

The incidence of Lassa fever is consistently highest during the dry season [57, 118]. The reasons for this are not clear but may relate to greater stability of Lassa virus at lower humidity [87] and seasonal fluctuation in host abundance and the prevalence of Lassa virus infection [54]. Natal mastomys may enter human habitations more frequently during the dry season, when food in agricultural fields is scarce, but stored rice and other staples in homes are abundant [54]. In a hospital in Guinea, the seasonal fluctuation in the incidence of Lassa fever mirrored the general pattern of other febrile diseases and hospital admissions, suggesting that nonbiological factors may be involved [57]. These may include cultural, economic, and other logistical impediments, such as poor road conditions, the need to attend to crops, and lack of funds prior to the seasonal harvest that often limit inhabitants' ability to seek medical care during the rainy season.

Mild or asymptomatic Lassa virus infection appears to be frequent. In a hyperendemic area of Sierra Leone in the 1980s, less than 20 % of persons who recently developed antibody reported recent febrile illness [48]. However, since antibody reversion (loss of antibody after infection) was also frequent, some of the seroconversions and asymptomatic infections may have represented re-exposure in persons with preexisting immunity from past Lassa virus exposure. The possibility of cross-reacting antibodies from previous infection with other nonpathogenic arenaviruses in West Africa, such as Kodoko, Gbagroube, or Menekre viruses, cannot be excluded (Fig. 8.4). Studies of the rate of asymptomatic transmission bear repeating with newer more sensitive and specific diagnostic modalities [119, 120].

Consumption of rodents and poor-quality housing, which may reflect ease of rodent access to the home, are risk factors for Lassa fever [93, 121]. In Sierra Leone, the incidence is consistently high in diamond mining areas, presumably due to increased contact with rodent excreta in the soil, although poor hygiene in mining camps and consumption of rodents may contribute. Large nosocomial outbreaks of Lassa fever have occasionally occurred in Africa following lapses in proper infection control, including reuse of non-sterilized needles and use of contaminated multiuse antibiotic vials, but secondary transmission associated with imported cases from returning travelers is extremely rare [122].

5.1.3 Lujo Fever (Lujo Virus Infection)

The name "Lujo" reflects the two geographic areas involved in the only outbreak recognized to date—*Lu*saka, Zambia, and *Jo*hannesburg, South Africa. The first recognized case was infected near Lusaka and subsequently medically evacuated to Johannesburg, initiating a chain of four nosocomial infections (a medical evacuation attendant, two nurses, and a cleaner) in South Africa in 2008 (Fig. 8.4). Four of the five (80 %) cases were fatal. The virus and disease have not been seen since and no antibody or ecologic surveys have been conducted to yield a greater understanding of the epidemiology or epizoology of Lujo virus.

5.2 New World Arenavirus Disease

5.2.1 Argentine Hemorrhagic Fever (Junín Virus Infection)

In 1959, the first of the HF-causing arenaviruses, Junín (named after a village about 240 km west of Buenos Aires), was described after a new disease, subsequently named Argentine HF, emerged in the central Argentine pampas, an area of intensive agriculture. Annual outbreaks of up to 1,500 cases continued in subsequent years. The endemic zone initially appeared confined to an area of approximately 16,000 km^2 but has steadily expanded to about 150,000 km^2 in parts of northern Buenos Aires, southern Santa Fe and Entre Rios, and southeastern Córdoba Provinces as agriculture expands (Fig. 8.5) [123]. The present endemic area is home to an estimated three million people. Transmission is typically high in the first few years of expansion into a new area and then declines.

The endemic area is largely a mosaic of crop fields and some cattle ranches crisscrossed by linear islands of relatively stable border habitats that are the preferred habitat of drylands vesper mice. As expected, the annual incidence is positively correlated with local population densities of this rodent. Young adult males are generally at highest risk due to their traditional involvement and exposure in agricultural activities [66]. Although cases occur throughout the year, the

incidence is usually highest from March to June, corresponding to periods of peak agricultural activity. In the early years the men affected worked in close contact with the land and harvested crops by hand, activities that presumably carried a high risk of exposure to rodents and their excreta. Later, mechanized agricultural practices became common, but cases continued to occur in field-workers, especially tractor drivers, probably through inhalation of infectious aerosols generated during the mechanized harvesting process.

An effective vaccine (see Sect. 10.6) was introduced in 1991, decreasing the incidence of Argentine HF from 837 cases that year to an average of 122 cases yearly from 1992 to 2009 [124]. The incidence has been steadily declining, with less than 100 cases in most years since 2000, despite increasing intensity of surveillance and quality of laboratory diagnosis. As young male agricultural workers in the high-risk provinces are increasingly protected by vaccination, the proportion of cases fitting this demographic is decreasing.

5.2.2 Bolivian Hemorrhagic Fever (Machupo Virus Infection)

An outbreak of what was subsequently named Bolivian HF occurred in Bolivia's El Beni Department in 1959. The etiologic agent, Machupo virus, named after a river in the region of the outbreak, was described in 1965. Community outbreaks of Bolivian HF continued during the 1960s but were eventually brought under control, in part by rodent trapping. There were then no reported cases for decades, followed by sporadic cases and small outbreaks reported again in the 1990s, which continue through the time of this writing in 2012.

Although Bolivian HF may be seen throughout the year, like Argentine HF, the incidence is usually highest during the dry season (June–August) at the peak of agricultural activity. Young men engaged in agricultural activities in rural areas are at highest risk. However, family and community clusters of Bolivian HF cases affecting both sexes and all age groups have also occurred in towns and hospitals, either as a result of epizootic conditions fostering high rodent population densities with invasion of towns and human dwellings [13] or through person-to-person transmission [125].

5.2.3 Venezuelan Hemorrhagic Fever (Guanarito Virus Infection)

In 1989, an outbreak of viral HF occurred in Guanarito District in the southern tip of Portuguesa State, Venezuela. Although originally thought to be dengue HF, in 1991 the disease was attributed to a new arenavirus named Guanarito, the causative agent of Venezuelan HF [126]. Outbreaks of Venezuelan HF have been reported every 4–5 years since its discovery, suggesting some cyclic climatic or social influence. Similar to Argentine and Bolivian HFs, epidemics appear to have a seasonal peak in November to January, coinciding with maximum agricultural activity, with highest incidence in male agricultural workers [14]. Recent surveys have demonstrated the presence of Guanarito virus genotypes (some closely matching genotypes from case patients in the disease-endemic area) in host populations over a wide area of the Venezuelan plains (western llanos), including areas in five states up to several hundred kilometers from the recognized disease-endemic area [22].

5.2.4 Brazilian Hemorrhagic Fever (Sabiá Virus Infection)

In 1990, a fatal case of HF was reported from outside of Sao Paulo, Brazil [127]. The victim was an agricultural engineer, although she worked primarily in the office. Although originally thought to be a case of yellow fever, a new arenavirus was subsequently isolated and named Sabiá after the community where the engineer was staying when she fell ill, although the name Brazilian HF has not been formally assigned. Only two cases of Sabiá infection, both nonfatal and due to laboratory accidents, have occurred since: one in Brazil and one in the United States. The case in the United States resulted from a centrifuge accident, illustrating the potential for aerosol transmission of arenaviruses in the laboratory. Despite the infected individual not reporting the incident for 12 days after the accident, no secondary transmission occurred.

5.2.5 Chapare Hemorrhagic Fever (Chapare Virus Infection)

The latest HF-causing New World arenavirus to be recognized is Chapare virus, discovered in 2003 after a small outbreak in Chapare Province in Cochabamba Department, Bolivia. Few details have been reported and no cases have been reported since then, although surveillance is limited.

5.2.6 Whitewater Arroyo Virus Infection

In 2000, in California, Whitewater Arroyo virus was detected via RT-PCR in three fatal cases with symptoms consistent with viral HF, with infectious virus isolated from one patient. However, not all laboratory results could be confirmed, leaving doubt about the pathogenicity of this virus. No further cases have been recognized. Nevertheless, findings from recent antibody surveys, including persons with significant increases in antibody titer associated with febrile illness, suggest a pathogenic role for Whitewater Arroyo or a similar arenavirus in the southwestern United States [128].

5.3 Human-to-Human Transmission

Most arenaviruses can be transmitted between humans through direct contact with infected blood or bodily fluids although, contrary to popular concept, secondary attack rates are generally low, probably on the order of 5 % or less as long as strict barrier nursing practices are observed. Tertiary

transmission is unusual. Large outbreaks are almost always fueled by nosocomial transmission, usually in resource-poor regions where barrier nursing practices may not be maintained [109, 118, 122, 129].

Viremia and infectivity of persons infected with arenaviruses generally parallels the clinical state, with highest infectivity late in the course of severe disease, especially when bleeding is present. Data on the precise modes of human-to-human transmission are lacking, but infection presumably results from oral or mucous membrane exposure, most often in the context of providing care to a sick family member (community) or patient (nosocomial transmission). Although aerosol spread of Lassa virus was speculated in the first recognized outbreaks in Nigeria, extensive field experience since then has not suggested aerosol transmission of arenaviruses between humans in natural settings [118]. African funeral rituals that entail the touching of the corpse prior to burial may also result in transmission of HF viruses, although this has not been specifically recognized with arenaviruses [130]. Arenavirus clearance from immunologically protected sites such as the kidney and gonads may be delayed for weeks to months, potentially resulting in transmission during convalescence [131–134]. Excretion may be intermittent [135]. Lassa virus has been isolated from human urine up to 67 days after onset of illness. Nevertheless, only a few incidences of transmission during convalescence have been reported, all consistent with sexual transmission of Lassa, Machupo, and Junín viruses months after recovery from acute disease [48, 136]. The risk of transmission during the incubation period or from asymptomatic persons is negligible.

New World arenaviruses appear to be less transmissible between humans than their Old World counterparts, although human-to-human transmission of Machupo virus has been reported in both community and nosocomial settings [137, 138]. Only one family cluster of Argentine HF is described, with the index case presenting with atypical skin lesions that may have facilitated transmission. Person-to-person transmission or nosocomial infection has not been observed with Guanarito virus despite the fact that patients with Venezuelan HF are usually admitted to open wards with minimal isolation precautions. It is unknown whether the perceived differences in transmissibility between the arenaviruses reflect true biological properties or varied cultural and infection control practices in the endemic areas of each virus.

Contrary to popular concept, the risk of imported arenavirus infections initiating outbreaks in industrialized countries, where barrier nursing techniques are usually maintained, is generally low. Because of the considerable travel between the United Kingdom and their former West African colonies, which also often happen to be the hyperendemic areas, Lassa fever is easily the most exported arenavirus infection. Foreign military personnel, peacekeepers, aid workers, and tourist in rural settings are occasionally infected, sometimes importing Lassa virus back to their countries of origin, most frequently the United Kingdom and Europe, but occasionally as far away as Japan [139]. Despite Lassa virus being one of the most communicable arenaviruses, in over 25 imported cases of Lassa fever reported since 1969 with at least 1,500 cumulative identified contacts, only a single putative and asymptomatic instance of secondary transmission has been noted [140]. Nevertheless, the risk is highlighted by the three secondary cases and one tertiary case following an index case of Lujo fever imported to South Africa; this transmission occurred in settings in which proper infection control practices may not have been maintained.

6 Clinical Presentation

6.1 Central Nervous System Disease

Despite the name, a minority of patients infected with LCMV manifest meningitis or CNS disease. Rather, most LCMV infections are asymptomatic or result in a nonspecific febrile illness [141].

The incubation period is about 1–2 weeks (typically 8–13 days). After 7–14 days of nonspecific illness (fever, headache, malaise), and often with a brief period of effervescence, CNS manifestations may ensue in a minority of patients, running a spectrum from aseptic meningitis (the most common) with headache, stiff neck, and photophobia to fulminant encephalitis with cranial nerve palsies, abnormal reflexes, focal seizures, polyneuritis, flaccid paralysis, and papilledema. CNS symptoms can also occur without recognized febrile illness. Rarer manifestations and complications of LCMV infection include hydrocephalus, transverse myelitis, Guillain–Barré syndrome, hearing loss, arthritis, parotitis, orchitis, myocarditis, mucosal bleeding, and pneumonia. Congenital infection has been associated with spontaneous abortion in early pregnancy and, when occurring later in pregnancy, a variety of neurological deficits, including psychomotor retardation, microcephaly and macrocephaly, hydrocephalus, chorioretinitis with visual loss, and seizures [17]. LCMV and Dandenong virus infection in immunosuppressed organ transplant recipients has resulted in a highly fatal syndrome with graft dysfunction, CNS symptoms, and multiorgan system involvement, in some cases resembling viral HF, within 3 weeks after transplantation, with case fatality approaching 90 % [104].

6.2 Arenaviral Hemorrhagic Fever

Although there are differences in the pathogenesis and clinical manifestations produced by the various arenaviruses, they are usually too subtle to allow for distinction on clinical grounds, at least in the early stages of disease. Nevertheless,

some notable distinctions can be made between the syndromes caused by Old World and New World viruses. The disease caused by the various New World arenaviruses is usually referred to simply as "South American HF." Asymptomatic transmission and milder nonspecific febrile illness appear to be relatively frequent in Old World arenavirus infections, at least for LCMV and Lassa virus, while infection with the pathogenic New World arenaviruses much more often results in disease that can ultimately be recognized as HF.

Arenaviral HF may be seen in both sexes and all age groups, with a spectrum ranging from mild disease to shock, multiorgan system failure, and death. Most patients present with nonspecific signs and symptoms difficult to distinguish from a host of other febrile illnesses common in the tropics. The incubation period is usually about 1 week (range 3–21 days). Illness typically begins with the gradual onset of fever and constitutional symptoms, including general malaise, anorexia, headache, chest or retrosternal pain, sore throat, myalgia, arthralgia, lumbosacral pain, and dizziness [57, 114]. The pharynx may be erythemic or even exudative in Lassa fever, a finding which has at times led to misdiagnosis of streptococcal pharyngitis [114]. Gastrointestinal signs and symptoms occur early in the course of disease and may include nausea, vomiting, epigastric and abdominal pain and tenderness, and diarrhea. Lassa fever has sometimes been mistaken for acute appendicitis or other abdominal emergencies. A morbilliform, maculopapular, or petechial skin rash is very frequent in South American HFs. Interestingly, although rash almost always occurs in fair-skinned persons with Lassa and Lujo fever, it rarely occurs in blacks. The reasons for this observation are unknown, but prior infection with partial immunity and genetic differences have been postulated. Conjunctival injection or hemorrhage is frequent but is not accompanied by itching, discharge, or rhinitis. A dry cough, sometimes accompanied by a few scattered rales on auscultation may be noted, but prominent pulmonary symptoms are uncommon early in the course of the disease. Jaundice is not typical and should suggest another diagnosis.

In severe cases, patients progress to vascular instability, which may be manifested by subconjunctival hemorrhage, facial flushing, edema, bleeding, hypotension, shock, and proteinuria. Swelling in the face and neck and bleeding are particularly specific but not sensitive signs in Lassa and Lujo fevers [114]. Despite the term "viral HF," clinically discernible hemorrhage is not always seen, being less frequent in disease produced by Old World than New World arenaviruses, and never in the first few days of illness [57, 114]. Hematemesis, melena, hematochezia, metrorrhagia, petechiae, epistaxis, and bleeding from the gums and venupuncture sites may develop, but hemoptysis and hematuria are infrequent. Significant internal bleeding from the gastrointestinal tract may occur even in the absence of external hemorrhage. Neurological complications are more common in the South American HFs and include disorientation, tremor, ataxia, seizures, and coma, particularly in the late stages, and usually portend a fatal outcome [142]. The cellular and chemistry profile in CSF is often normal. Pregnant women with arenaviral HF often present with spontaneous abortion and vaginal bleeding, with maternal and fetal mortality rates approaching 100 % in the third trimester [143]. Anasarca has been described in a single report of children with Lassa fever (termed the "swollen baby syndrome") but may have been related to aggressive rehydration [144]. Various clinical manifestations of arenaviral HF are illustrated in Fig. 8.6.

Typical clinical laboratory findings in arenaviral HF include early leukopenia and lymphocytopenia, sometimes with atypical lymphocytes, followed later by leukocytosis with a left shift; mild-to-moderate thrombocytopenia, hemoconcentration; elevated aspartate aminotransferase (AST), alanine aminotransferase (ALT), and amylase; electrolyte perturbations; and proteinuria [134, 145]. Unlike classic viral hepatitides, in arenaviral HF the AST is typically much higher than the ALT, suggesting that its source is not exclusively the liver. Since a broad range of tissues can release AST, it should be considered a marker of systemic organ damage [134]. Radiographic and electrocardiographic findings are generally nonspecific and correlate with the physical examination [146, 147].

7 Clinical Management

Although data from clinical trials are sparse, most arenaviruses show in vitro and in vivo sensitivity to the antiviral drug ribavirin, which should be considered in all severe arenavirus infections. Convalescent immune plasma has demonstrated efficacy in Argentine HF and may be indicated, when available, in other New World arenavirus infections, although it is associated with a late neurological syndrome in 10 % of cases. Detailed reviews of the clinical management of viral HFs are available elsewhere [17, 145].

8 Diagnosis

8.1 Clinical Diagnosis

Difficulties in diagnosing arenavirus infection, both clinically and in the laboratory, pose a major impediment to surveillance and control. Even if more clearly recognized HF or neurological syndromes eventually develop, most patients present with a nonspecific febrile syndrome difficult to distinguish from a host of other diseases that are usually more common in the area. The differential diagnosis of arenaviral HF includes a broad array of febrile illnesses, including malaria, typhoid fever, leptospirosis, bacterial septicemia, rickettsial infections, dengue fever, and other viral HF

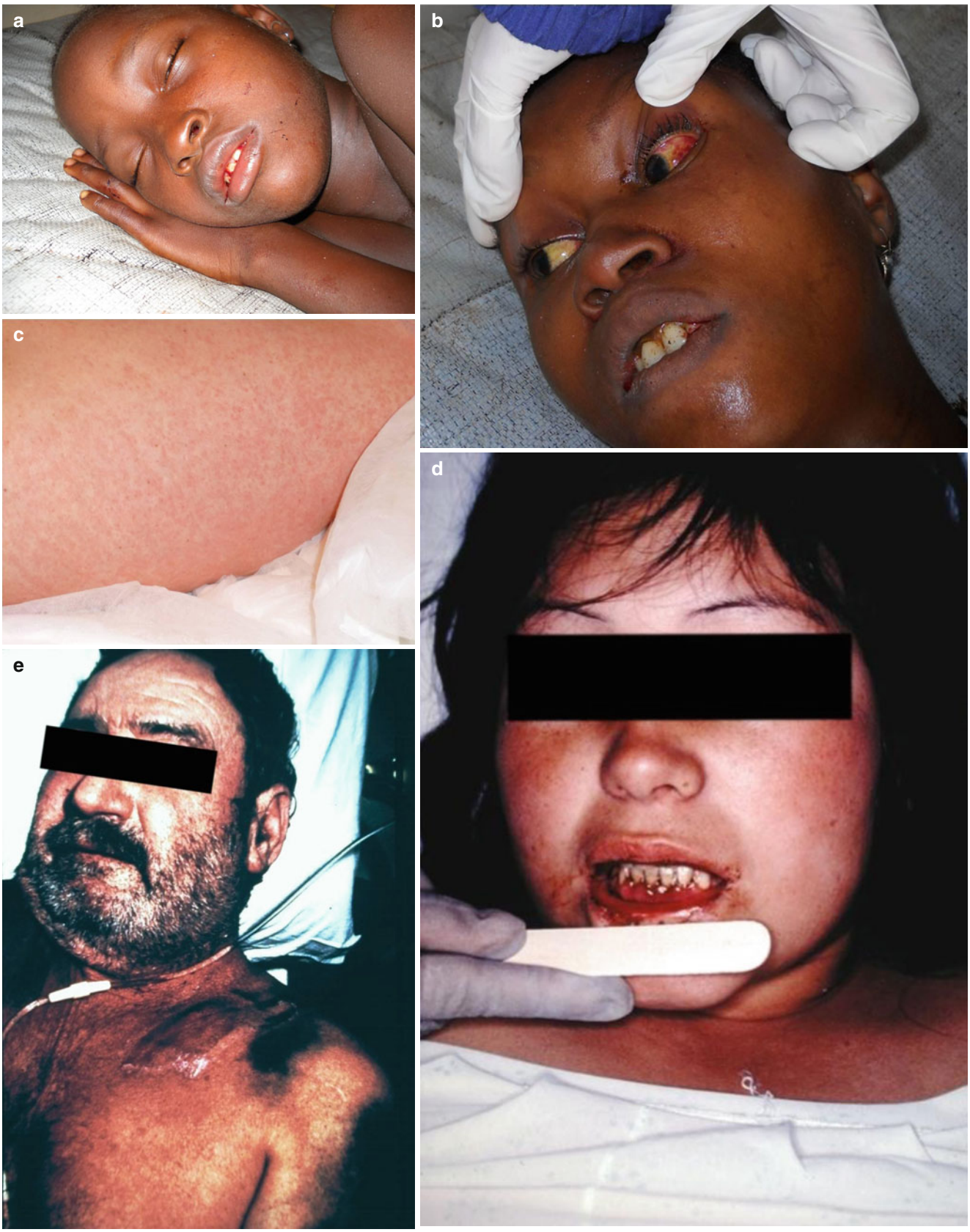

Fig. 8.6 Clinical manifestations of arenaviral hemorrhagic fever. (**a**) Facial swelling and mild gum bleeding in Lassa fever (photo by Donald Grant), (**b**) conjunctival injection in Lassa fever (photo by Donald Grant), (**c**) maculopapular rash in Lujo hemorrhagic fever (photo by TH Dinh), (**d**) gum bleeding in Argentine hemorrhagic fever, (**e**) petechial rash in Argentine hemorrhagic fever (Photos used with permission from Blumberg et al. [210])

syndromes, depending upon the specific geographic region and patient history of exposures [145]. The classic presentation of LCMV infection is aseptic meningitis occurring in the fall, particularly if an initial prodromal period of fever, perhaps with a remission, occurs before the CNS phase. The differential diagnosis includes the arboviral meningitides and herpes encephalitis and, for congenital manifestations, the classic TORCH organisms (*to*xoplasma, *r*ubella, *c*ytomegalovirus, and *h*erpes virus). The presence of thrombocytopenia and leukopenia should enhance suspicion of arenavirus infection.

A diagnosis of arenavirus infection should be considered in patients with a clinically compatible syndrome who, within 3 weeks prior to disease onset, (1) live in or traveled to an endemic area (Figs. 8.2 and 8.3), (2) had potential direct contact with blood or bodily fluids of a person with arenaviral HF during their acute illness (this group most often is comprised of healthcare workers or persons caring for family members at home), (3) worked in a laboratory or animal facility where arenaviruses are handled, or (4) had sex with someone recovering from arenaviral HF in the last 3 months. Recognized direct contact with rodents in endemic areas, including laboratory mice and pet hamsters and guinea pigs if LCMV infection is suspected, should heighten suspicion but is rarely noted even among confirmed cases. Acts of bioterrorism must be considered if arenaviral HF is strongly suspected in a patient without any of the aforementioned risk factors, especially if clusters of cases are seen [19]. Even persons who meet the above criteria most commonly have a disease other than arenaviral HF, so alternative diagnoses should always be aggressively sought.

8.2 Laboratory Diagnosis

The difficulty in clinical diagnosis makes prompt laboratory testing imperative. Unfortunately, no commercial assays are available, a situation further complicated by the BSL-4 designation and Select Agent status of many arenaviruses that limit access and research potential even to many legitimate scientists. Various recombinant-protein- and virus-like-particle-based assays are being developed which may eventually relieve the diagnostic bottleneck, as well as improve sensitivity and specificity, although interpretation and validation of these assays are proving challenging [148, 149]. Meanwhile, various "in-house" assays have been developed and are performed in a few specialized laboratories. Common diagnostic modalities include cell culture (restricted to BSL-4 laboratories for most pathogenic arenaviruses), serologic testing by enzyme-linked immunosorbent assay or immunofluorescent antibody assay, and the reverse transcriptase polymerase chain reaction. Serum is the usual substance tested, but these assays may also be applied to cerebrospinal fluid in LCMV infection [38, 95, 150, 151]. Postmortem diagnosis of some arenavirus infections may be established by pathology examination with immunohistochemical staining of formalin-fixed tissue [152]. Although extensive standardization and validation of these assays has not been conducted, they generally appear to have high sensitivities and specificities when conducted by experienced technicians [119, 153]. Cross-reactions between antigenically similar arenaviruses may pose a problem with the serologic assays, which sometimes can be resolved using neutralization tests [59]. The diagnostic methodologies and their distinct advantages and disadvantages are reviewed elsewhere [154].

9 Pathogenesis and Immune Response in Humans

9.1 Central Nervous System Disease

Overt cell damage by arenaviruses is minimal or modest, but they alter cell function and induce mediators of shock, directly or by immunopathologic mechanisms. Immune cell activation is also the fundamental process in the pathogenesis of LCMV, although without the same effects on endothelial cell function and hemostasis as in HF (see Sect. 9.2) [17]. CNS involvement typically occurs a week or two after disease onset, after virus has cleared from the blood, and is thus thought to be immune mediated, although virus can still be recovered from the CSF at this time.

9.2 Arenaviral Hemorrhagic Fever

As with all viral HFs, microvascular instability and impaired hemostasis are the hallmarks of arenaviral HF. Unchecked viremia appears to be central to the pathogenesis of arenaviral HF [134]. Viremia is usually present at patient presentation (presumably starting with disease onset, although patients are rarely available for testing at this time), peaking between days 4–9 and clearing within 2–3 weeks in survivors. Cell-mediated immunity is thought to be the primary arm of recovery in Old World arenavirus infection, while the humoral arm is important in disease caused by New World viruses [155–157]. Antibody titers are significantly lower in fatal cases relative to survivors [119, 134, 158] Neutralizing antibodies may be produced in Old World arenavirus infection, but usually months after recovery and often at a low titer. The continued increase in antibody titer to Lassa virus months after infection suggests a sustained B cell response that might be attributable to low-level virus persistence; in vaccine experiments in monkeys, replication-competent virus was cleared within 14 days after Lassa virus challenge,

but detection of RNA up to 112 days suggested low-level viral persistence or the presence of defective interfering particles [159]. The delayed clearance of Lassa virus from immunologically protected sites could explain the finding.

In survivors, IgM antibodies begin to appear after about a week and progressively increase as virus clears, lasting at least some months [119]. There are conflicting reports regarding the timing of IgM antibody appearance; this discrepancy may reflect differences in the production of reagents and their target epitopes, variations in assay sensitivity and specificity, and experience of the persons conducting the assay [119, 134, 160]. IgG antibody begins to appear 2–3 weeks after onset and recovery and lasts for years, including being found in persons over 40 years after Lassa virus infection who left the endemic area for Lassa fever and had no opportunity for re-exposure [161].

A long-standing mystery of arenaviral HF, especially Lassa fever, is the extreme range of clinical severity. The reasons for this variation are unknown but may relate to heterogeneity in the virulence of infecting Lassa virus strains, route and dose of inoculation, genetic predisposition, underlying coinfections or premorbid conditions (e.g., malaria, malnutrition, or diabetes), or misclassification of reinfection as new infection due to waning of antibody. Most studies in nonhuman primates use challenge doses designed to produce uniformly fatal disease and thus are not particularly illustrative regarding the pathogenesis of the full spectrum of arenaviral HF. In a monkey model of Lassa fever using the WE strain of LCMV, intravenous inoculation resulted in fatal viral HF while monkeys inoculated via the gastric route mostly had an attenuated infection with no disease [92, 155]. Human genes encoding "like glycosyltransferase" (*LARGE*), dystrophin (*DMD*), and IL-21 have apparently undergone positive selection in populations in endemic areas for Lassa fever in Nigeria, suggesting a protective effect [162, 163]. Readers are referred to recent reviews for more details on arenavirus pathogenesis [21].

10 Prevention and Control

10.1 Rodent Control

In the absence of effective vaccines for most arenavirus infections, effective and sustainable control relies on avoidance of known reservoir host habitats or, when this is not feasible, implementation of measures to prevent direct contact with rodents and their excreta. Most successful control programs entail principles of integrated pest management, integrating biological and chemical control measures with education or regulation to change human behaviors that put them at risk [164, 165]. Structural improvements to homes may be important for arenaviruses whose rodent hosts invade houses [166].

For LCMV, Lassa, and Machupo viruses, whose hosts often colonize human dwellings, measures to improve "village hygiene" are advocated to discourage rodent invasion, including assuring proper waste disposal, eliminating unprotected storage of garbage and foodstuffs, reducing clutter and vegetation around houses that give rodents shelter, and sealing holes that allow entry into homes [55]. These measures may not always be possible with the rudimentary construction of houses in some regions, especially with regard to Lassa fever in areas of West Africa that have been ravaged by war or civil unrest.

Although complete elimination of rodents from the environment through trapping or poisoning is not feasible or desirable, considering their importance to overall healthy ecosystems, short-term intensive rodent elimination in defined areas may occasionally be useful to control outbreaks; an outbreak of Bolivian HF in 1963–1964 in the village of San Joaquin ended abruptly after 2 weeks of continuous trapping in homes during which 3,000 big laucha were captured [73, 167]. However, a single trapping session in houses in Sierra Leone did not diminish the incidence of human Lassa virus infection [55]. A sustained elimination program would be necessary for effective long-term control because rodents from surrounding fields and forests may quickly recolonize villages and homes. Rodent elimination programs are unlikely to control transmission of Junín and Guanarito viruses to humans because their reservoir hosts are widespread in agricultural fields, although targeting the linear habitats preferred by drylands vesper mice might be plausible [2]. Crop replacement or periodic burning of tall grassy areas that are in close proximity to agricultural fields and human habitation has been recommended to control these viruses, although the measures are untested [42].

10.2 Environmental Shedding and Disinfection

Little published literature exists on transmission dynamics or viability of arenaviruses after shedding into the environment, especially data applicable to settings other than research laboratories. However, the lipid envelope of all HF viruses, including arenaviruses, is easily disrupted, limiting their viability outside a living host. Little concern is required regarding HF viruses seeping into groundwater or posing any long-term risk through casual exposures in the general environment, where harsh thermal and pH conditions would likely readily inactivate them. The survival of HF viruses in animal excreta is likely affected by factors such as the animal's diet and urinary pH and the presence of protein. When shed naturally in animal excreta or human body fluids, which would then usually dry, infectivity appears to last from a few hours to days, varying with the specific virus and environmental conditions

such as temperature and light [168–170]. However, other non-arenavirus HF viruses have been isolated from samples kept for weeks at ambient temperatures if stored hydrated in a biological buffer, such as blood or serum. This would likely hold true for arenaviruses as well. The viruses are also stable when frozen or freeze-dried [171].

Routine environmental surveillance (i.e., in soil, water, or air) for the arenaviruses is generally not feasible or routinely conducted. However, concerns about bioterrorism have spurred interest in this area in recent years, especially around specific events attracting large crowds, such as sporting events, that might be potential bioterrorist targets. Detection of an arenavirus outside its usual endemic area should certainly raise suspicion about bioterrorism. The specificity of any test employed would have to be high to avoid the considerable alarm and expenditure of resources that a false-positive result would likely initiate.

When contamination is suspected, such as in homes or hospitals treating persons with arenaviral HF, disinfection is warranted and effective. Arenaviruses can be inactivated by exposure to temperatures above 60 °C for 1 h, acidic or basic pH, gamma irradiation, ultraviolet light (surface disinfection only), surfactant nanoemulsions, and a host of chemicals and detergents, including chlorine compounds, alcohols, phenols, quaternary ammonium compounds, formaldehyde, cupric and ferric ions, zinc finger-reactive compounds, photoinducible alkylating agents, and various proprietary detergent-containing lysis buffers [172–184]. Toxicity and corrosion may be concerns with some of these compounds depending upon the frequency of use and concentrations.

Sodium hypochlorite (i.e., household bleach) is the most readily available effective inactivation method [185]. Bleach solutions should be prepared daily; starting with the usual 5 % chlorine concentration, a 1:100 (1 %) solution should be used for reusable items, such as medical equipment, patient bedding, and reusable protective clothing before laundering. A 1:10 (10 %) bleach solution should be used to disinfect excreta, corpses, and items to be discarded. Workers cleaning areas potentially contaminated by the excreta of small mammals should let the area aerate before entering, then spray the area with the 10 % bleach solution and let it sit on the surface for at least 15 min before mopping or wet sweeping [186]. A site with appropriate security should be dedicated for waste disposal if routine autoclaving is not available.

Specific guidelines exist regarding handling and burial of corpses of victims of viral HF [185]. The question of the safety of exhuming remains of persons who died of arenaviral HF overseas for transport back home occasionally arises. No data regarding the viability of arenaviruses under these circumstances are available, but it is unlikely that the viruses would survive long under the harsh pH conditions of a decomposing corpse unless the ambient temperature is below freezing, which is unlikely in the endemic areas of most arenaviral diseases. If corpses are to be exhumed, the same protective measures used for burial of victims of viral HF, including placing the cadaver in a sealed body bag, should be used.

10.3 Occupational Exposure and Personal Protective Equipment

10.3.1 Healthcare Workers

Since transmission of arenaviruses between humans requires direct contact with blood and body fluids, infection can be prevented by the isolation of the patient and use of routine barrier nursing. However, due to the high fatality rates associated some arenaviral HFs, so-called viral HF precautions (surgical mask, double gloves, gown, protective apron, face shield, and shoe covers) are advised for added security [185]. Powered air-purifying respirators and other small-particle aerosol precautions should be used when performing procedures that may generate aerosols, such as endotracheal intubation. Items that were in direct contact with the patient should be decontaminated (see Sect. 10.2).

10.3.2 Laboratory Workers

Fortunately, since most arenavirus infections are rare in humans and routine laboratory safety procedures are generally adequate to prevent transmission, infection in hospital laboratories from clinical samples is extremely rare. Of more concern is the risk to laboratorians specifically working with cultured arenaviruses, especially the highly pathogenic agents of HF. However, these agents are largely restricted to work in BSL-4 laboratories. No arenavirus infections have been reported in laboratorians working in BSL-4 laboratories, although numerous accidents, sometimes fatal, have occurred in lower-level containment laboratories. Recognition of LCMV transmission associated with laboratory mice has led to guidelines and regulations in these settings that have markedly decreased risk [187].

10.3.3 Field-Workers

Field-workers such as agricultural workers, field biologists, and pest control workers are potentially exposed to rodents and their excreta in an occupational setting. Inadvertent exposure to contaminated rodent excreta by agricultural workers is difficult to prevent. Routine use of masks is probably not feasible or cost-effective, but should be considered when the risk of aerosols is high. Common dust or surgeon's masks will not protect against small-particle aerosols; specifically designed N-100 or P-100 face masks must be used for this purpose [188].

Field biologists and pest control workers who trap rodents merit special consideration; although the risk would seem to

be high, the limited evidence available suggests that arenavirus infection in this group is rare, even in the absence of the use of extensive personal protective equipment. Field biologists who handled rodents in North America showed a prevalence of exposure to HF viruses of less than 1 %, and not all infection could be clearly attributed to occupational exposure [189]. The risk may vary depending upon the prevalence of the pathogen in the animals and the specific procedure conducted. For example, simple elimination of pests via snap-trapping and disposal of intact dead animals would be less risky than live-trapping with subsequent euthanasia and necropsy to procure samples for scientific research.

In the absence of a clear consensus on the risk of infection, a wide variety of biosafety practices are employed by field-workers, ranging from no personal protective equipment to use of gloves, gowns, safety glasses, and HEPA filter respirators or even positive-pressure respirators [189]. While the inclination of those responsible for workers' safety is to err on the side of caution, and thus require the full complement of protective personal equipment, significant cost and practical implications must be considered. More research is needed to assess the true risk to field-workers and thus the cost–benefit balance of the various protective measures if a widely agreed-upon standard procedure is to be set. Because risk depends upon geographic location, pathogens expected to be encountered, species handled, specific tasks, and worker training, any such standards will have to address multiple situations. In the meantime, it seems prudent to follow established safely guidelines when handling known viral HF reservoir host species [190]. Full precautions, including positive-pressure respirators, are warranted when sample centrifugation or other potentially aerosol-generating procedures are performed.

10.3.4 Pet Store Workers and Pet Owners

Recognition of LCMV transmission associated with pet animals has led to guidelines and regulations in these settings [187]. Pregnant women and immunosuppressed persons in particular must be protected from infection because of the potentially severe consequences in these groups [36]. Serologic testing of individual pet rodents is generally unreliable and not recommended. Educational materials on safe handling of pet rodents should be made available at pet stores. Pet stores with potentially infected rodents in stock should contact public health authorities for additional information and guidance. Human infection with arenaviruses other than LCMV from pet contact has not been reported. However, although not an arenavirus, monkeypox virus, traced to the importation of infected Gambian rats from Ghana, has infected humans in the United States and highlights the risk to those importing and keeping exotic pets [191].

10.4 Case Identification and Patient Isolation

Control of most arenaviral HF relies on classic public health principles of identification and isolation of cases and monitoring of their contacts. However, the early nonspecific presentation of arenaviral HF poses a serious challenge to case identification. Fortunately, the low secondary attack rate affords a measure of reassurance even when cases go unrecognized as long as proper barrier nursing is maintained. Furthermore, since mild cases are not very infectious, missed or delayed diagnosis of these patients is unlikely to pose a problem from an infection control standpoint. All patients with a clinically compatible syndrome should be presumed infectious and kept under "viral HF isolation precautions" (see below) until a specific diagnosis is made [185]. If available, placement in a negative airflow room is prudent, but hermetically sealed isolation chambers are not required and may have severe adverse psychological effects on both patient and staff. Access to the patient should be limited to a small number of designated staff and family members with specific instructions and training on the implementation of viral HF isolation precautions.

Since the clinical status of patients with viral HF generally correlates with the level of viremia and infectivity, patients who have recovered from their acute illness can safely be assumed to have cleared viremia and can be discharged without concern of subsequent transmission. However, because of potential delayed virus clearance in urine and semen, abstinence or condom use is recommended for 3 months after acute illness. Although transmission through toilet facilities has not been noted, simple precautions to avoid contact with excretions in this setting are prudent, including separate toilet facilities and regular hand washing. Breastfeeding should be avoided during convalescence unless there is no other way to support the baby.

10.5 Contact Tracing

Persons with unprotected direct contact with a patient during the symptomatic phase of arenaviral HF should be monitored daily for evidence of disease for three weeks (the longest known incubation period) after their last contact. Contacts should check their temperature daily and record the results in a log. Despite the lack of evidence for transmission during the incubation period, exposed persons should avoid close contact or activities with household members that might result in exposure to bodily fluids, such as sex, kissing, and sharing of utensils. Hospitalization or other confinement of asymptomatic persons is not warranted, but persons who develop fever or other signs and symptoms consistent with arenaviral HF should immediately be isolated until the diagnosis can be excluded.

10.6 Vaccines

In Argentina, the use of a live attenuated vaccine for Argentine HF called Candid #1 began in 1991 and has resulted in a steady decrease in the incidence of cases, as described above. The vaccine may also protect against Bolivian HF, although it does not appear to cross-protect against other arenaviruses [192]. However, Candid #1 is generally not available or approved outside of Argentina, and even within Argentina supplies are insufficient to cover the population at risk. Furthermore, despite the efficacy and generally excellent safety profile of Candid #1 for Argentine HF, fear of reversion to virulence with live-virus vaccines is a major disincentive to exploring its application to other arenavirus infections.

Other arenavirus vaccines are in the experimental stages. A number of vaccine platforms have been explored in animal models of arenaviral HF, primarily aimed at preventing Lassa fever. Approaches include inactivated or attenuated viruses [193, 194], recombinant vaccinia viruses [159], RNA replicon vectors derived from an attenuated strain of Venezuelan equine encephalitis virus [195], recombinant *Salmonella* Typhimurium [196], arenavirus protein subunits [197], naked DNA [198], chimeric viruses using the yellow fever 17D vaccine strain [199, 200], reassortant Lassa/Mopeia viruses [201–203], viruslike particles [204], and recombinant vesicular stomatitis virus [205, 206]. The arenavirus GP1 appears to be the most important immunogenic protein.

The recombinant vesicular stomatitis virus platform is perhaps the most promising candidate, providing 100 % protection after a single dose in a monkey model of Lassa fever. Furthermore, this vaccine may be effective when given by the nasal or oral route, potentially facilitating its use in epidemics or as postexposure prophylaxis [207]. Although there are concerns over the safety of an unproven live-vector vaccine in populations in Africa with a high frequency of immunosuppressed persons due to HIV/AIDS or malnutrition, preliminary studies of the recombinant vesicular stomatitis virus vaccine platform in immunocompromised monkeys have not indicated problems, perhaps because the vector virus lacks the neurovirulence genes and properties of the wild-type virus [208]. The social, economic, and political barriers to development of arenavirus vaccines are at least as formidable as the scientific ones, considering that most arenavirus diseases exist in geographically restricted areas with resource-poor populations.

10.7 Postexposure Prophylaxis

Ribavirin has been used as postexposure prophylaxis for Lassa fever, although there are no data on efficacy, dose, or duration of administration of the drug for this purpose. Nevertheless, postexposure prophylaxis with oral ribavirin should be considered for persons with direct unprotected contact with blood or bodily fluids from a person with confirmed or highly suspected arenaviral HF. Persons who develop manifestations of arenaviral HF should be immediately converted to the intravenous form. Prophylaxis should not be given if the only exposure was during the incubation period. A review and specific guidelines have been published [209].

11 Unresolved Problems and Future Challenges

The progress in our understanding of the arenaviruses in recent decades has primarily been driven by concern over the use of these agents as bioweapons [19, 117]. The focus has been largely in the realm of the laboratory and basic sciences, with promising developments in treatments and vaccines. However, much remains to be elucidated regarding arenavirus epizoology and epidemiology if improved prevention and control are to be achieved. The ultimate goal is to understand the complex epizoologic, epidemiologic, and ecologic interactions of arenaviruses and predict and prevent their emergence and increased incidence of disease. Significant barriers exist, including inadequate surveillance and lack of commercially available diagnostic reagents. Population-based, longitudinal studies in both humans and rodents are necessary to understand transmission dynamics, including reasons for patchy endemicity and seasonality, and the genetic and evolutionary relationships between viruses and reservoirs. However, the often remote and sometimes politically unstable endemic areas and the difficulty in securing long-term funding are significant challenges to long-term studies. Laboratory studies on precise modes of arenavirus transmission between rodents and humans are needed, especially the role of aerosol transmission. While logical in terms of biosafety and biosecurity, the BSL-4 and Select Agent status of many arenaviruses poses a significant impediment to research. Little attention has been paid to community control, including the efficacy of measures such as regular rodent trapping or the use of rodenticides, perhaps timed to coincide with the breeding season, rodent-proofing of homes, and the introduction of predators, such as cats. Interdisciplinary programs bridging human, animal, and ecosystem health and conservation offer further opportunities to understand ecosystem regulation of arenavirus transmission and how human alterations of the environment and biodiversity loss affect the risk of infection. Lastly, major challenges include global economic distress that threatens to blunt research funding, the lack of economic incentive to invest in research on agents that are primarily endemic in resource-poor regions of the world, and the relatively weak research and training infrastructure in developing countries that continue to experience loss of their scientists to more lucrative jobs elsewhere.

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Bunyaviruses: Hantavirus and Others

9

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1 Introduction

The *Bunyaviridae* family is the largest family of RNA viruses with more than 350 named isolates [70]. Viruses in the family are divided into five genera (*Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Hantavirus*, and *Tospovirus*) on the basis of serological, molecular, antigenic, and structural characteristics. The *Bunyaviridae* are a unique group of viruses whose members are able to infect invertebrates, vertebrates, and plants, and they can be found worldwide. Multiple members are significant pathogens with the ability to cause severe disease in humans, such as encephalitis, hepatitis, or hemorrhagic fever. Most bunyaviruses are spread through sylvatic transmission cycles between susceptible vertebrate hosts and hematophagous arthropods, including ticks, mosquitoes, and phlebotomine flies. Unique are the members of the *Hantavirus* genus, in that they are not infecting insect vectors but are maintained in nature through persistent infection of rodents. Human and animal pathogenic bunyaviruses can be found in four of the five genera, with tospoviruses being plant pathogens. The large family of bunyaviruses is a pool not only for many emerging and reemerging viruses, such as Rift Valley fever virus, Crimean-Congo hemorrhagic fever virus, and the hantaviruses, but also for recently emerged pathogens, such as the newly identified severe fever with thrombocytopenia syndrome and Heartland, Shuni, and Schmallenberg viruses. This chapter will discuss the most important human pathogens of the *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, and *Hantavirus* genera.

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2 Overview of the *Bunyaviridae* Family

2.1 Morphology and Morphogenesis

The morphological properties among the viruses in each of the five bunyavirus genera vary; however, in general, virions appear to have a spherical to pleomorphic morphology, with diameters ranging from 80 to 120 nm. Embedded in the lipid bilayered envelope are the two surface glycoproteins, Gn and Gc, which can be up to 10 nm in length and form protrusions or spikes. Recent structural studies have revealed that the two surface glycoproteins of phleboviruses form the so-called capsomeres (built by Gn/Gc heterodimers) and are arranged on an icosahedral lattice with a triangulation number of 12 [86, 112, 176, 204, 223]. For hantaviruses each glycoprotein spike complex is arranged in a square-shaped assembly with fourfold symmetry [19, 111]. In contrast to most other negative-strand RNA viruses, bunyaviruses do not contain a matrix protein, which facilitates the interaction between the surface glycoproteins and the ribonucleoprotein (RNP) core. It has been demonstrated that the cytoplasmic tails of at least one of the glycoproteins mimic the function of a matrix protein [177, 194, 224]. The virion envelope is usually derived from cellular Golgi membranes but can occasionally also be derived from the cell surface membrane and contains 20–30 % lipids. The membrane encloses the viral tripartite single-stranded negative-sense RNA genome. The viral genomic RNA segments are only found encapsidated by the nucleoprotein (N) in the form of RNP complexes and

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not as naked RNA molecules, indicating that encapsidation and RNA synthesis occur concomitantly. The RNA-dependent RNA polymerase (L) is associated with the RNP complexes and they display a helical symmetry. At least one of each of the three segments must be contained in a virion for infectivity. Virions contain 2–7 % carbohydrate by weight, and asparagine-linked sugars on the glycoproteins Gn and Gc are largely of the high mannose type when viruses are grown in vertebrate cells.

2.2 Biochemical and Physical Properties

Bunyavirus buoyant densities in sucrose and CsCl gradients are 1.16–1.18 g/cm^3 and 1.20–1.21 g/cm^3, respectively. The molecular mass of the virions ranges from 300 to 400×10^6 and have a sedimentation coefficient of 350–500 S. Treatment with lipid solvents or nonionic detergents results in loss of infectivity due to the removal of the envelope [173]. Bunyaviruses are also sensitive to heat and formaldehyde.

2.3 Genome Organization and Viral Proteins

All members of the *Bunyaviridae* share a common tripartite single-stranded negative-sense RNA genome. The three RNA molecules are designated as S (small), M (medium), and L (large) segments (Table 9.1). Each genome segment can be divided into three regions, the 3′ noncoding region (3′ NCR), coding region, and 5′ NCR. The terminal nucleotides at the 3′ and 5′ NCRs of each viral RNA segment are highly conserved and invertedly complementary to allow base pairing, resulting in stable panhandle structures and non-covalently closed circular RNAs [105]. These terminal sequences are conserved among viruses within each genus but are different to members in the other genera. The 5′ NCRs of the genomic viral RNA segments are not modified. Viral mRNAs are not polyadenylated and are truncated relative to the 3′ NCR of their genomic RNA counterpart. The 5′ ends of viral mRNAs have methylated caps and 10–18 non-templated nucleotides which are derived from host cell mRNAs. The NCRs do contain signals not only for the N protein encapsidation but also for the viral promoters, mRNA transcription termination, and probably the RNP-packaging signals [65, 81, 175, 258].

The four bunyavirus structural proteins (Table 9.2) are encoded in the complementary sense RNA of the three viral segments. All bunyavirus L segments use conventional negative-sense coding strategies and encode for the RNA-dependent RNA polymerase. The two envelope glycoproteins, Gn and Gc, are encoded in a single, continuous open reading frame (ORF) in the mRNA, and the primary gene products are then co-translationally cleaved to give mature Gn and Gc proteins (referring to the amino-terminal or carboxy-terminal coding of the protein). Orthobunya- and phleboviruses encode an additional nonstructural protein (NSm) in the virion-complementary-sense RNA. Nairoviruses encode two proteins of unknown function, a mucin-rich protein and glycoprotein GP38, which are the products of posttranslational cleavage of glycoprotein precursor preGn. The N protein is encoded on the S segment. Orthobunyaviruses and some hantaviruses encode a nonstructural protein (NSs) in an overlapping ORF to that encoding the N protein in the 3′ half of the virion-sense S RNA. Phleboviruses encode a NSs protein in an ambisense ORF in the 5′ half of the virion-sense S RNA. The NSs proteins of orthobunya-, phlebo-, and hantaviruses have been shown to act as interferon antagonists (reviewed in [131]). The NSm protein of phleboviruses can act as an apoptosis antagonist [196, 240, 262].

Table 9.1 Sizes of viral RNA segments in the bunyavirus genera (sizes are given in kb)

	RNA segment		
Genus and virus	S	M	L
Phlebovirus	1.7	3.5	6.4
Nairovirus	1.7	4.9	12.2
Orthobunyavirus	1.0	4.5	6.9
Hantavirus	1.7	3.6	6.5
Tospovirus	2.9	4.8	8.9

2.4 Antigenic and Genetic Properties

One or both of the envelope glycoproteins display hemagglutinating and neutralizing antigenic determinants. Complement-fixing antigenic determinants are principally associated with the N protein.

Table 9.2 Sizes of structural proteins in the bunyavirus genera (sizes are given in kDa)

RNA segment: viral protein	Genus				
	Phlebovirus	*Nairovirus*	*Orthobunyavirus*	*Hantavirus*	*Tospovirus*
S: N	24–30	48–54	19–25	50–54	52
M: Gn	50–70	30–45	29–41	68–76	52–58
M: Gc	55–75	72–84	108–120	52–58	72–78
L: L	238–241	459	259–263	246–247	330–332

Viruses with segmented genomes have the ability to expand their genetic diversity through (a) slow genetic drift (i.e., accumulation of individual point mutations) or (b) sudden genetic shift (i.e., new arrangement of whole genome segments in progeny viruses produced during a coinfection event). Genetic reassortment occurs in nature and in the laboratory by exchange of genomic segments and genetic relationships within the same serogroup and can affect pathogenicity of RNA viruses. Genetic reassortment between closely related bunyaviruses has been demonstrated in experimentally coinfected tissue culture cells and arthropod hosts but also by analysis of naturally occurring virus isolates [20, 26, 28, 43, 104, 140, 198, 201, 208, 212]. High-frequency reassortment can occur when arthropod vectors are infected simultaneously with two viruses. This can either happen in the same blood meal or through interrupted feeding, when blood meals are taken within a relatively close time window of each other [21].

2.5 Biological Properties

Members of the *Orthobunyavirus*, *Nairovirus*, and *Phlebovirus* genera have the capability of replicating alternatively in vertebrate and arthropod hosts. While they are not causing any cytopathic effects in their invertebrate hosts, cytolytic effects are generally observed in their vertebrate hosts. Arthropod vectors can be mosquitoes, ticks, sandflies, and phlebotomine flies, and transovarial and venereal transmission has been demonstrated. Hantaviruses are the exception, since they are the only rodent-borne bunyaviruses and are transmitted via the aerosol route of aerosolized rodent excreta. In contrast to other bunyaviruses, hantaviruses routinely establish a persistent, non-cytolytic infection in susceptible mammalian host cells, primarily in endothelial cells. This observation is consistent with their nonpathogenic persistence in their natural rodent host.

3 The Viruses

3.1 *Orthobunyavirus* Genus

The *Orthobunyavirus* genus is the largest of the five genera within the *Bunyaviridae* family and contains more than 170 named viruses, including medically important viruses, such as Oropouche, La Crosse, and Jamestown Canyon viruses. Viruses within this genus are grouped into 18 serogroups (Anopheles A, Anopheles B, Bakau, Bunyamwera, Bwamba, Group C, Capim, California, Gamboa, Guama, Koongol, Minatitlan, Nyando, Olifanstlei, Patois, Simbu, Tete, and Turlock). This classification is based on serological studies, using neutralization, hemagglutination inhibition, and complement fixation assays, and each serogroup contains viruses that are antigenically distinct but related.

At least 30 members of the *Orthobunyavirus* genus have been associated with human disease, causing a range of symptoms such as encephalitis, hemorrhagic fever, or febrile illness. In livestock, orthobunyavirus infections are mainly associated with abortions or teratogenic effects.

The majority of the orthobunyaviruses are arthropod-borne viruses and have amplification cycles in a variety of vertebrate hosts. Infection of the different species can result in fundamentally different outcomes for the host cells. Mosquitoes (and derived cell lines) are persistently infected and shed virus.

3.1.1 Oropouche Fever

Oropouche virus (OROV) is a member of the Simbu serogroup and the causative agent of Oropouche (ORO) fever, an urban febrile arboviral disease widespread in South America [193]. OROV was first isolated in 1955 in Trinidad and Tobago from the blood of a febrile forest worker from a pool of *Coquillettidia venezuelensis* mosquitoes and named after the Oropouche River [8, 134]. Recurrent epidemics have involved tens of thousands of patients and are economically significant, owing to loss of working hours. Over the past 50 years, ORO fever has emerged as a major public health problem in tropical areas of Central and South America [11]. OROV is transmitted by culicoid midges and humans develop a high viremia for an orthobunyavirus infection, such that uninfected midges can acquire the virus after biting [101].

Descriptive Epidemiology

Members of the Simbu serogroup are distributed globally and transmitted by biting midges (*Culicoides*). Since the original isolation of OROV from a febrile patient in Trinidad and Tobago in 1955, the medical importance of the virus, particularly in the Amazon basin regions of northern Brazil and Peru, has become increasingly evident [193]. OROV has been shown to be associated with more than 30 epidemics reported in Brazil, Peru, Panama, and Trinidad and Tobago during 1960 and 2009, with more than 500,000 persons estimated to be infected [193, 202]. Most of these outbreaks occurred in relatively urban areas, leading to the suggestion that OROV is maintained in two distinct cycles, an epidemic urban and a silent sylvatic cycle [61, 200]. High antibody prevalence in populations in endemic areas suggests that human infection can be common in such areas [12, 202, 257]. The principal urban vector in Brazil appears to be the tiny biting midge, *Culicoides paraensis* [190, 200]. Isolates have also been made from mosquitoes such as *Culex pipiens quinquefasciatus*, but its vector competence is poor [61, 107]. The favorite larval breeding habitats of *C. paraensis* midges are rotting banana tree stumps and cacao husks [192]. These decomposing plant materials remain moist even during dry

periods and serve as an excellent growth medium for the microorganisms on which the *C. paraensis* larvae feed. Adult midges feed predominantly in the early evening hours and are quite anthropophilic. It is assumed that humans entering the jungle become infected with OROV and develop viremia sufficiently high to transmit the virus to uninfected midges and appear capable of serving as the vertebrate amplifying host during the urban cycle [192]. When they return to their village or town, temporary urban transmission can occur, resulting in an epidemic. Serological data also suggest that forest animals (primates or sloths or even birds) may be potential vertebrate hosts in the sylvatic cycle. Factors such as deforestation, urbanization, and agricultural development are probably contributing to the emergence of ORO fever as a human pathogen in South America. Phylogenetic studies indicate that distinct OROV genotypes are present in different geographic areas: Genotype I viruses include the prototype strain from Trinidad and Tobago and Brazilian strains from the states of Acre, Amazonas, Maranhão, Tocantins, and Pará; Genotype II contains the Peruvian strains isolated between 1992 and 1998 and strains from the Brazilian states of Amapá, Pará, and Rondônia; Genotype III includes virus strains isolated in Panama and the Brazilian states of Acre, Minas Gerais, and Rondônia; and Genotype IV is formed by the Brazilian strains isolated in Amazonas state [11, 171, 207, 250, 251].

Clinical Features

Several thousand cases of acute febrile illness can occur during OROV epidemics observed throughout the Amazon basin regions of Brazil and Peru. Humans acquire the infection by bite from infected midges. In addition to large outbreaks, OROV can also cause sporadic infections in humans [243]. The incubation period is approximately 4–8 days, followed by an abrupt onset of fever, with arthralgia, generalized myalgia, anorexia, nausea, vomiting, weakness, dizziness, severe headache, chills, photophobia, and prostration. Occasionally, ORO fever patients also exhibit rash, meningitis, or meningismus [161, 191]. Most of the symptoms resolve within 3–5 days, although a period of asthenia, myalgia, and dizziness may persist for up to 9 months. Viremia is detectable in the majority of patients 2–3 days post onset of illness. No fatalities have been reported with the disease, and lifelong immunity follows recovery. Approximately half of the patients can exhibit recurrence of some disease symptoms 1–10 days after they become afebrile [252]. A recent study identified the presence of OROV in the cerebrospinal fluid of three samples from 110 meningoencephalitis patients, demonstrating the involvement of the central nervous system in OROV infections (Bastos et al. [17]). Two of the three patients presented with other diseases affecting the immune system and central nervous system (HIV and neurocysticercosis). The findings suggest that OROV should be investigated in cases of meningoencephalitis of unknown etiology in affected areas and that previous blood-brain barrier damage might facilitate the entry of OROV into the central nervous system. OROV is also suspected to be infectious by aerosol, based on reported laboratory infections [190].

Diagnosis

The diagnosis of OROV is performed by virus isolation from the blood of infected patients and by serological assays. The serological response is strongest in the acute phase against the N protein, and recombinant expressed N protein has been used to develop an enzyme immunoassay for the diagnosis of ORO fever [206]. Further, RT-PCR-based assays are available for the detection of OROV [158, 259].

Treatment and Prevention

There is no specific treatment for ORO fever and the disease is normally self-limiting. However, treatment of symptoms with anti-inflammatory drugs might be recommended. This consists of drinking plenty of fluids to prevent dehydration, as well as prescribing pain analgesics. A recent study indicated that ribavirin does not have antiviral activity on OROV [144]. With the ORO fever disease in Brazil and Peru, avoiding the buildup of rotting organic debris such as banana tree stalks or cacao husks in agricultural areas may help curtail population levels of *C. paraensis* and reduce the risk of seasonal epidemics [192]. Avoiding exposure (treated netting, DEET repellents) to these midges during their early evening feeding hours is also recommended.

3.1.2 California Serogroup

The California serogroup has great relevance from a human infection perspective, as they have been associated with influenza-like illness in central Europe and are an important cause of encephalitis in the United States. Other members of the genus are of veterinary importance. The California serogroup includes 14 viruses and subtypes. All members of the group are transmitted by mosquitoes. Although serological studies and analysis of genomic sequences have demonstrated their relationship, each virus is only prevalent in a specific geographic area delimited by the presence of its mosquito vector and mammalian host. Hammon and Reeves isolated California encephalitis virus from *Aedes melanimon* mosquitoes in 1943 in Kern County, California [100, 199]; serological studies subsequently showed high incidence of seropositivity. Nevertheless, California encephalitis virus has not been recently implicated with any human disease in California [199]. California serogroup viruses sequentially replicate in striated muscle, cause viremia, and invade and replicate in the central nervous system causing encephalitis in their mammalian hosts. Each of these viruses has a limited geographic range, and members have been isolated from several continents including North and South America, Europe, and Africa.

La Crosse Virus

In 1960, La Crosse virus (LACV) was isolated from a fatal case of a 4-year-old girl who suffered encephalitis in La Crosse, Wisconsin [245]. LACV was shown to be closely related but distinct from California encephalitis virus, and it was quickly recognized as an important virus causing human infections over a wide area of the Midwestern United States. Testing mosquitoes and more widespread serological diagnosis of individuals with encephalitis identified a number of other arboviruses that were related to California encephalitis virus and LACV, and together these were designated as the California serogroup.

Descriptive Epidemiology

Approximately 80–100 cases of LACV encephalitis occur every year; however, the disease is gravely underreported and underdiagnosed. The disease is primarily reported in the upper Midwestern states (Minnesota, Wisconsin, Iowa, Illinois, Indiana, and Ohio) from late spring through early fall; however, infrequently cases are also reported in winter in the Gulf states. Recently, more cases have been reported from mid-Atlantic and southeastern states (West Virginia, Virginia, Kentucky, North Carolina, and Tennessee) [99]. LACV is transmitted to humans through the bite of an infected mosquito. LACV is maintained in nature by infections of two alternate hosts: mosquitoes and small mammals, particularly chipmunks and squirrels. The main vector is a woodland mosquito, *Aedes triseriatus* (the eastern tree hole mosquito), which preferably breeds in tree holes and in discarded tires and feeds during the day [133]. In the United States, *Aedes triseriatus* is present in areas east of the Mississippi River, where numbers peak from June through September [57]. *Aedes albopictus*, a mosquito introduced into the United States from Asia, has been shown to be a competent vector, at least in experimental studies. LACV can be transmitted transovarially to the next generation or venereally from infected males to uninfected females [22]. In endemic areas, a high proportion of chipmunks and squirrels have been found to be seropositive. Studies have shown that these two species generate high enough viremia levels to subsequently infect mosquitoes after feeding on the experimentally infected animals [267]. Incidence of California serogroup virus neuroinvasive disease has ranged from fewer than 40 to over 160 cases per year since 1964. The highest numbers of cases reported are from Ohio, West Virginia, Wisconsin, and North Carolina, with an average annual incidence above 1.0 per 100,000 population in some endemic counties ([98]; www.cdc.gov/lac/tech/epi.html, accessed Sept 2012).

Clinical Features

LACV causes acute encephalitis preceded by a nonspecific febrile illness [47]. Based on experimental studies it is estimated that the incubation period ranges between 6 and 15 days. After mosquito bite, multiplication probably occurs in vascular endothelial and reticuloendothelial cells; dissemination occurs in the blood and lymph [203]. The symptoms of the central nervous system disease include stiff neck, lethargy, nausea, headache, and vomiting in milder cases and seizures, coma, paralysis, and permanent brain damage in severe cases. Severe disease occurs most commonly in children under the age of 16. Death from LACV encephalitis occurs in less than 1 % of clinical cases. A small proportion of patients may develop persistent paresis or learning disabilities and other cognitive deficits [203]. The cerebrospinal fluid demonstrates elevated protein in 20 % of the cases. Unlike most viral encephalitis, many of the cells found in the cerebrospinal fluid are polymorphonuclear neutrophils [97].

Diagnosis

Diagnosis is based on clinical features and patient exposure history. Laboratory testing is available in many state or local public health laboratories and in some referral hospitals and is accomplished by testing serum or cerebrospinal fluid to detect virus-specific IgM antibodies. Virus may also be detected by nucleic acid amplification and in fatal cases by histopathology and virus-specific immunohistochemistry. Virus isolation may also be useful.

Treatment and Prevention

There are no vaccines against LACV infection or specific antiviral treatment. Treatment is supportive. Prevention is based on avoidance of vector mosquitoes through use of insect repellants, wearing protective clothing, keeping screens in good repair, and effective vector control.

3.1.3 Ngari Virus

An orthobunyavirus, originally designated Garissa virus, was isolated from human hemorrhagic fever cases during a large disease outbreak in 1997 and 1998 in East Africa (Kenya and Somalia) [28]. The disease was characterized by acute onset of fever and headache, followed by hemorrhage (with gastrointestinal and/or mucosal bleeding). During the investigations, a previously unidentified orthobunyavirus was isolated from two hemorrhagic fever cases. The genetic analysis of fragments of the virus S, M, and L genome RNA segments revealed that the virus was subsequently recognized as being the same as the previously identified Ngari virus and characterized as a naturally occurring reassortant between Bunyamwera virus (S and L segment donor) and Batai virus (M segment donor) [30, 93]. Interestingly, these two viruses do not cause a hemorrhagic febrile illness. During the outbreak in 1997–1998, an estimated 89,000 human infections occurred with over 250 deaths. Of 231 febrile patients for which clinical records existed, half met the case definition of hemorrhagic fever [28]. Initially, it was thought that the

hemorrhagic fever outbreak was associated with Rift Valley fever virus (RVFV) infections [263], a human pathogenic phlebovirus endemic in Africa. Of the hemorrhagic fever cases, 23 % had evidence of acute RVFV infection, whereas 27 % had evidence of acute Ngari infection.

3.2 *Phlebovirus* Genus

The genus *Phlebovirus* comprises over 70 antigenically distinct serotypes, which are divided into two groups, the *Phlebotomus* fever viruses (sandfly group, transmitted by sandflies) and the Uukuniemi group (transmitted by ticks). Eight of these serotypes contain viruses known to cause disease in humans (Alenquer, Candiru, Charges, Naples, Punta Toro, Rift Valley fever, Sicilian, and Toscana viruses). Recently, two new human pathogenic tick-borne phleboviruses have been identified, severe fever with thrombocytopenia syndrome virus and Heartland virus [152, 266].

3.2.1 Rift Valley Fever Virus

Rift Valley fever virus (RVFV) is a member of the genus *Phlebovirus*, endemic in sub-Saharan Africa and introduced to Egypt and the Arabian Peninsula periodically. It is the causative agent of the mosquito-borne disease Rift Valley fever (RVF) in humans and ruminant animals. Infections with RVFV generally result in self-limiting febrile disease but can also manifest in more severe illness, such as hemorrhagic fever, encephalitis, or retinitis. Currently, there is no commercially available vaccine for human use.

Descriptive Epidemiology

The geographic distribution of RVFV covers much of Africa, ranging from Egypt to South Africa and from Senegal to Madagascar. The first confirmed outbreak of RVFV outside Africa was reported in 2000 in Saudi Arabia and Yemen [3, 255]. RVFV has the potential to infect a remarkable number of different vectors, including mosquitoes, ticks, and flies. However, mosquitoes, especially floodwater *Aedes* mosquitoes, are thought to be the principal vector and play an important role in RVFV epizootics, which frequently occur at times of unusual high precipitation [141]. In general, the vectors can be classified into two groups: (1) reservoir vectors (*Aedes* species (spp.)) and (2) amplifying vectors (*Culex* spp.) [82, 83, 91]. RVFV persists in the environment through vertical transmission in mosquitoes and horizontal transmission by mosquitoes among animals [174]. Dambos are thought to play a central role because they flood during heavy rainfall. During interepidemic periods RVFV was isolated from unfed mosquitoes, demonstrating that the virus can be maintained between epidemics through transovarial transmission in mosquitoes [142]. Heavy rainfall and flooding increases the hatching of eggs and explosive population growth in floodwater *Aedes* mosquitoes [234], which can initiate an epizootic leading to viremic livestock that then act as amplifying hosts and a source of the virus for feeding mosquitoes [234]. Once livestock is infected, the classical hallmark of RVF epizootics is the large number of abortions observed among pregnant ruminants. Usually 1–2 weeks after these abortions, initial human infections can be detected, especially in groups that are in close contact with livestock, such as farmers, veterinarians, and abattoir workers. RVFV infection can be acquired either through the bite from an infected mosquito or more importantly through direct contact with infected livestock. People involved in the birth or abortions of livestock, butchering process of animals, or abattoir workers are at high risk of infection during epizootics [2, 39]. Aerosol exposure has been demonstrated to be another potential route of infection, especially in a laboratory setting [14, 31, 84, 109, 159, 160, 229].

Clinical Features

RVFV is one of the most severe human pathogen among the phleboviruses and infections can result in a wide range of clinical features, varying from an asymptomatic (30–60 %), to a weeklong undifferentiated febrile illness, to the severe development of hemorrhagic fever, encephalitis, retinitis, and potentially death (the overall case fatality rate is estimated to be between 0.5 and 2 %). The most frequent course of the disease is a self-limiting febrile illness; less than 2 % of human infections result in severe disease (hemorrhagic fever, encephalitis, or retinitis). During the 1977–1978 outbreak in Egypt, approximately 200,000 human cases with 600 deaths were estimated to have occurred [153], and an estimated 27,500 RVFV infections occurred during the 1997–1998 outbreak in the Garissa district of Kenya [263].

Infected patients can develop a mild form of RVF, which is characterized by a feverish syndrome with a sudden onset of flu-like fever, muscle pain, joint pain, and headache. Additional symptoms can include neck stiffness, photophobia, loss of appetite, and vomiting. These symptoms normally last from 4 to 7 days. However, in a small percentage of patients, the development of one or more of the three distinct syndromes can occur: ocular disease (0.5–2 %), meningoencephalitis (less than 1 %), or hemorrhagic fever (less than 1 %). Ophthalmologic complications remain very important sequelae of human RVF disease and can result in long-lasting visual disturbance in affected patients [129]. It has also been described that in its most severe form, RVF can lead to acute renal dysfunction [69].

A majority of RVF patients suffer from a self-limiting and mild to often subclinical febrile illness, with an incubation time of typically 4–6 days [55, 80, 119, 147, 162, 232]. Symptoms typically include severe chills, weakness, malaise, throbbing headache, and dizziness [80, 120]. Further, the development of a sensation of fullness over the liver region

has been described [80]. Following these symptoms, patients present with fever, decreased blood pressure, proximal large-joint arthralgia (particularly shoulders, elbows, and knees), and anorexia followed by nausea and vomiting. A lowering of the body temperature can be observed between the 3rd and 4th day after onset of symptoms, which then returns back to a normal level. However, some patients can either maintain their fever as long as 10 days, or within 1–3 days after the recovery, a recurrence of high fever can be observed [80, 162]. This biphasic febrile phase is normally combined with a severe headache. During the febrile period, viremia can be detected, as well as neutralizing antibodies, which appear around day 4 after onset of symptoms [80, 205]. Patients can also develop a massive coronary thrombosis after the febrile phase [162, 219].

Patients who develop one of the severe forms of RVF (jaundice, hemorrhagic fever, or neurological disease) are at increased risk of fatality [130, 147]. Hemorrhagic manifestations are typically characterized by a sudden onset of illness, including fever, throbbing headache, lethargy, malaise, vomiting, nausea, vague mid-epigastric discomfort, and body pain. Further, these patients may present with a low blood pressure, hematemesis, diarrhea, jaundice, macular rash over the entire trunk, subconjunctival hemorrhage, and bleeding from the gums and/or gastrointestinal mucosal membrane [120, 237, 264]. Elevated aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and alanine aminotransferase (ALT) and reduction of platelet count and hemoglobin level are typical [5, 237]. Death can occur within 3–17 days after onset of these symptoms, and fatal RVF cases show diffuse hepatic and gastrointestinal necrosis [1, 237].

RVF patients developing encephalitis may present with a sudden onset of fever, rigor, retro-orbital and throbbing headache, mild arthralgia of the knees and elbows, and bilateral retinal hemorrhage [6, 120, 145]. After onset of the illness, patients eventually experience neck rigidity, confusion, hypersalivation, fatigue, malaise, stupor, and coma, and temporal or permanent vision loss has been described as well [6, 145, 249]. Indicative of a possible development of meningitis or meningoencephalitis was the increased number of white blood cells (mainly lymphocytes) in the cerebrospinal fluid (CSF). Patients with a developing RVF-induced encephalitis can also present with a decreased level of consciousness [6]. Histopathological lesions in the brains of these patients were characterized by focal necrosis associated with infiltration of lymphocytes and macrophages and perivascular cuffing [249].

As described previously, in some cases, RVF patients can develop a maculopathy or retinopathy (0.5–2 %), which can affect either one or both eyes and can occur either very early or months after infection [59, 210, 228]. Patients reported to experience a blurry vision and noticed floating black spots within their visual fields or even the loss of central vision. While in some cases a partial improvement in vision over time is established [210, 218, 226, 227], in many patients, no complete recovery of vision occurred and chorioretinal scarring remained in macular and paramacular areas [4, 10, 59, 85, 210, 226, 227].

While there is much documentation of the symptoms of severe RVF illness, much more work is still needed to better describe specific manifestations of RVF in humans. This will be necessary to allow clinicians to recognize the disease in its early stages and to be able to limit and ultimately prevent its spread.

Diagnosis

RVFV belongs to a large group of RNA viruses endemic in Africa, which have the potential to cause viral hemorrhagic fever (VHF), including Lassa, Ebola, Marburg, Crimean-Congo hemorrhagic fever, and yellow fever viruses. Infections by RVFV and other VHF viruses are clinically difficult to recognize, if no hemorrhagic or specific organ manifestations are present. Diagnosis of RVFV infection is often performed by combining the analysis of clinical signs of disease and available diagnostic testing. RVF may be suspected when there is an occurrence of abortions in domestic ruminants and death of young animals, associated with an outbreak of febrile illness in human with headache and myalgia. In the laboratory, the diagnosis of RVF is achieved by a variety of techniques, such as virus isolation from whole blood, serum, or tissue samples [7]; viral antigen detection [154, 168]; detection of specific antibodies [138]; and amplification of viral nucleic acids [64, 89, 115], which are preferable due to the rapid and reliable analysis of samples for RVFV. Multiplexed PCR, reverse transcription (RT)-PCR enzyme hybridization assays, quantitative (q)RT-PCR, and real-time detection PCR are very useful techniques, since detection can be performed simultaneously for many hemorrhagic fever viruses [25, 64, 89, 102, 115, 211]. Further, the real-time RT-loop-mediated isothermal amplification (RT-LAMP) assay demonstrated its practicability in a field laboratory, since it does not rely on thermocycler equipment and only requires a one-step, single-tube reaction [132, 188]. To prevent potentially false-negative results due to the rather short viremic phase during which RVFV genomes can be detected, diagnosis should be combined with the detection of RVFV-specific antibodies. Viral antigen (such as the RVFV nucleoprotein) can rapidly be detected in blood and tissue samples by the use of immunohistochemistry staining of tissue samples or enzyme-linked immunosorbent assays (ELISA) [117, 154, 168, 268]. Shortly after exposure to RVFV, serological tests, such as detection of type-specific IgG and IgM antibodies, can also be performed [182, 183, 185, 186]. Additional serodiagnostic tests for the detection of RVFV are hemagglutination inhibition, indirect immunofluorescence, and virus neutralization test (VNT)

[184, 185, 239]. However, VNT as a diagnostic tool has to be handled with care, because RVFV induces a long-lasting neutralizing immunity and previously infected individuals will also give a positive result.

Treatment and Prevention

Currently, no licensed vaccines and specific treatments are available for RVF in humans. Studies in monkeys and other animal models for RVF infection have demonstrated promise for ribavirin as an antiviral drug for future use in humans [187]. However, ribavirin might have unexpected side effects. Further, studies have suggested that interferon, immune modulators, and convalescent-phase plasma may also help in the treatment of patients with RVF [187].

A formalin-inactivated vaccine (TSI-GSD-200) is the only RVFV vaccine presently available for use in humans [195] but, currently, only for military personnel, veterinarians working in endemic areas, high-containment laboratory workers, and others at high risk for contracting RVFV. Another strategy of controlling RVF outbreaks is vaccination of livestock to prevent epizootics. The currently used live Smithburn vaccine is only partially attenuated and leads to high abortion rates or teratology (10–25 %) of pregnant animals [50, 121, 235] and exhibits pathogenicity in European cattle [27]. In addition, the risk of reversion to full virulence precludes its use in countries where RVFV is not known to be endemic [235]. A live attenuated vaccine (RVFV MP-12) is efficacious in livestock. However, RVFV MP-12 may have similar safety concerns associated with the Smithburn strain, since RVFV MP-12 can be teratogenic in sheep [114]. RVFV MP-12 was recently tested in human clinical trials, to determine adverse effects in humans using a single injection dose, with promising results [24]. A 95 % seroconversion rate was reported with a high titer in a plaque reduction neutralization test. In addition, a genetic analysis of RVFV MP-12 isolated from vaccinated individuals showed no reversions of vaccine virus in attenuated regions compared to wild-type RVFV. Overall, many promising RVFV vaccines (including subunit and DNA vaccines, as well as viruslike particles) and therapeutic concepts are currently employed to generate an urgently needed safe and efficacious countermeasure against RVFV.

Infection with RVFV can be prevented by decreasing the chance of contact with mosquitoes and other bloodsucking insects through the use of mosquito repellents and bed nets. An important protective measure for persons working with animals in RVF-endemic areas is avoiding exposure to blood or tissues of animals that may potentially be infected.

3.2.2 Sandfly Fever Viruses

Sandfly fever viruses are transmitted in the Mediterranean area by the sandflies *Phlebotomus papatasi, P. perniciosus* and *P. perfiliewi*. Human infections with Naples and Sicilian sandfly fever viruses cause self-limiting disease of unknown epidemiology. However, infection with Toscana virus is the third most frequent cause of aseptic meningitis between May and October in central Italy, where it was originally isolated from sandflies in 1971. Toscana virus has since been detected in many countries close to the Mediterranean area, such as France, Spain, Slovenia, Greece, Cyprus, and Turkey. In all Mediterranean countries Toscana virus should be included in the differential diagnosis of viral meningitis during the warm summer season.

Descriptive Epidemiology and Clinical Features

Toscana Virus

Toscana virus (TOSV) is an arthropod-borne bunyavirus, which is transmitted to humans by *Phlebotomus*, *Sergentomyia*, and *Lutzomyia* genera, in particular by *Phlebotomus perniciosus* and *P. perfiliewi*. The virus was isolated in 1971 from *P. perniciosus* in Monte Argentario (Tuscany) [235–255], but it was first recognized as a causative agent of neurological disease in humans only in 1983, when it was isolated for the first time from a young woman with lymphocytic meningitis [167]. Transmission of the virus occurs transovarially in the insect vectors, and it has been suggested that the reservoir of TOSV is most likely the vector itself. However, a progressive decline of vector infected rates over many generations suggests that TOSV cannot be maintained exclusively by vertical transmission [48, 49, 242], but its animal reservoir has not been identified yet. TOSV isolation from the brain of an insectivorous bat (*Pipistrellus kuhlii*) has so far been the only evidence of the possible involvement of this species in the ecology of the virus [49, 254, 255].

TOSV is the major cause of aseptic meningitis (95 %) and meningoencephalitis (4.5 %) and influenza-like illness during the summer season, with a peak of incidence in August [103, 214]. Most cases of the TOSV infections have been reported in either residents or travelers in central Italy or Spain. However, over the last years, an increased number of cases have also been recorded in other countries in the Mediterranean area, such as Portugal, France, Cyprus, Turkey, and Greece [67, 179, 189, 213, 215]. It has also been described that TOSV can result in asymptomatic infection and infection without involvement of the central nervous system, such as febrile erythema or influenza-like illness [103, 197]. Recent reports of TOSV infections included unusual clinical manifestations or severe sequelae, such as hydrocephalus, epididymo-orchitis, and ischemic complications [13, 103, 150, 197, 213].

Serological analysis conducted in Italy (particularly in Tuscany) revealed a TOSV seroprevalence of 77.2 % in forestry workers, compared to 22.7 % of seropositivity in the urban population [247]. Moreover, this serological analysis underlined the presence of asymptomatic infections of TOSV in exposed people. Preexisting immunity also seems to play

an important role in limiting TOSV-caused illness, and cases with reinfection have not been detected so far [52, 53].

A recent retrospective study on the antibody prevalence rates of TOSV among adults and children was performed in a population (n=2,737) living in Tuscany during 1999 to 2006 [241]. This study revealed that the seroprevalence rate was 19.8 % in adults and 5.8 % in children, showing an age-dependent increase in TOSV-specific immunity. Furthermore, the study indicated that asymptomatic TOSV infection is more frequent in young people (91 %) than in adults (31.4 %). A correlation of the seroprevalence to the clinical profile showed that a higher incidence of severe signs of neurological disorders was found in adults, but it is still unclear why this occurs.

Interestingly, a seroprevalence study conducted in volunteer blood donors in France showed that 12 % of sera from healthy donors and 18.9 % of sera from patients hospitalized for CNS infection were IgG positive for TOSV. These findings confirmed that TOSV circulates in southeastern France and that a significant proportion of healthy blood donors have a history of TOSV infection [56]. This indicates that a potential risk of transmitting the virus to naïve subjects is possible and can raise concerns about potential implications for blood donors.

Sandfly Fever Sicilian and Naples Viruses

The sandfly fever Sicilian virus (SFSV) and sandfly fever Naples virus (SFNV) serotypes have the widest geographic distribution which is related to the distribution of the vector (*P. papatasi*), and these viruses have been isolated from sandflies in Africa, Central Asia, and Europe [18, 51, 54, 60, 79, 116, 128, 179, 209, 244]. Serological investigations performed on the indigenous populations and tourists visiting temperate regions demonstrated that sandfly activity normally peaks during the summer months when the human population in these areas is exposed to sandfly-transmitted diseases [32, 40, 62, 66–68, 71, 72, 113, 231]. SFSV and SFNV were first described in Italy in 1943–1944 during outbreaks of influenza-like illness in US soldiers [244].

SFSV and SFNV cause the so-called 3-day fever or pappataci fever. Infected patients present with influenza-like symptoms, including fever, myalgia, malaise, and retro-orbital pain [29, 256]. Usually, these patients recover fully within 7 days. Even though infections with these viruses are mild and self-limiting, patients are highly incapacitating during the course of the illness.

Newly identified SFVs include the sandfly fever Turkey virus [33, 127], which is an SFSV variant; Punique virus, a related virus to SFNV [273]; and Massilia virus, which is a new member of the SFNV complex [41].

Diagnosis

The direct diagnosis of SFVs can be performed by virus isolation or RT-PCR from the blood or CSF but is only possible during early stages of infection (i.e., within the first 2 days after onset of symptoms and before the IgM seroconversion occurs). In most cases, the diagnosis is based on serological investigation of acute and early convalescent sera. ELISA for the detection of SFV antigen can be performed in reference laboratories. Within the SFSV and SFNV antigenic complexes, serological cross-reaction exists. VNT using early convalescent sera still remains the reference method to specifically identify the viruses or to assess the antibody response specificity.

Treatment and Prevention

There is no specific therapeutic available for infections with SFV and treatment is symptomatic. Individual protective measures such as insect repellents and insecticide-impregnated mosquito bed nets are recommended.

3.2.3 Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV)

Recently, a new member of the *Phlebovirus* genus has been identified in China (Hubei and Henan provinces), after a heightened surveillance of acute febrile and life-threatening illness in China. The novel virus was isolated from patients who presented with fever, thrombocytopenia, leukocytopenia, and multiorgan dysfunction and termed severe fever with thrombocytopenia syndrome virus (SFTSV) [266]. The clinical symptoms were initially considered to resemble those of human anaplasmosis [270]; however, neither bacterial DNA nor antibodies could be detected in blood samples.

Descriptive Epidemiology

The majority of affected patients were farmers living in wooded and hilly areas, and they were working in the fields before onset of clinical signs of disease. SFTSV RNA was detected in ticks of the Ixodidae family (species *Haemaphysalis longicornis*) that were collected from domestic animals where the patients lived, and these ticks may be a candidate vector for SFTSV. High seroprevalence of SFTSV was detected among goats (up to 83 %) and cattle (up to 32 %) but also in pigs (up to 5 %), dogs (up to 6 %), and chicken (1 %) in SFTSV-endemic regions [272]. SFTSV caused cytopathic effect in DH82 cells after inoculation with white blood cells isolated from a 42-year-old patient were subsequently isolated. In cell culture, SFTSV can infect a variety of cells, such as Vero E6, Vero, and L929 cells; however, cytopathic effect could only be detected in DH82 cells. Phylogenetic analysis of six isolated SFTSV strains indicated that all isolates cluster together and that they were nearly equidistant from the sandfly fever group and the Uukuniemi group, suggesting that SFTSV is the prototype of a third group within the genus *Phlebovirus*.

Clinical Features

The major clinical symptoms include fever (temperatures of >38 °C), fatigue, conjunctival congestion, thrombocytopenia (platelet count of <100,000 per cubic millimeter),

gastrointestinal symptoms, abdominal pain, diarrhea, proteinuria, hematuria, and leukopenia. Frequently, regional lymphadenopathy was observed. In most patients, multiorgan failure develops rapidly, indicated by elevated levels of AST, ALT, CK, LDH, and CRP [58, 88]. The appearance of CNS symptoms (including apathy, lethargy, convulsions, muscular tremor, and coma) as well as hemorrhagic fever (bleeding from the mouth mucosa, lungs, and gastrointestinal lumen) has been described [87, 88, 266, 269]. Dynamic profiling of laboratory findings in SFTS patients showed that clinical progression of the disease occurs in three stages [88]. The first stage or fever stage (days 1–7 after onset of illness) is characterized by marked thrombocytopenia and leukopenia and low platelet counts and peripheral white blood cell counts. Lymphocyte levels substantially decrease during the first 9 days, suggesting that these cells are mainly involved in leukopenia. Coagulation tests indicated that prolonged aPTT occurred on days 5–9. Cardiac and liver enzymes, such as ALT, AST, LDH, CK, and CK-MB, were elevated, indicative for heart and liver impairment. The second stage (or multiorgan dysfunction stage) of the disease is defined to occur between days 7 and 13 after disease onset and characterized by development of multiorgan dysfunction. In survivors viral load decreases, while it remains elevated (up to 10^8 viral RNA copies/mL) in patients with fatal outcome. Further, in fatal cases, platelet counts continued to decline, and serum tissue enzymes further increased, reaching maximum levels before death. A recent study by Deng and colleagues analyzed the cytokine and chemokine profile in 57 patients and found that levels of TNF-α, IP-10, and IFN-γ were elevated in patients with severe disease [58]. These findings suggest that a cytokine-mediated inflammatory response, mediated through an imbalance in cytokine and chemokine production, might result in a severe disease progression. The third stage or convalescent stage describes the recovery phase for surviving patients after day 13 and is characterized by the return of clinical parameters to normal physical levels. In summary, the key risk factors contributing to a patient's death have been identified as increased viremia; elevated AST, ALT, LDH, CK, and CK-MB levels past the fever stage; and the appearance of CNS symptoms and manifestation of hemorrhagic tendency, DIC, and multiorgan failure. The average case fatality rate is 12 % but can be as high as 30 % [266].

Initially, no epidemiologic evidence of human-to-human transmission of SFTSV was detected [266]. However, recent publications indicated that the virus can be transmitted from person-to-person through personal contact with the index patient, while no exposure to suspected animals or vectors was reported [16, 87, 143].

Diagnosis

Direct diagnosis of SFTSV RNA in patient serum can be performed by real-time PCR from the blood or by quantitative real-time RT-PCR assay [233]. Patient sera can also be tested for the presence of antibodies to SFTSV by indirect fluorescence assay (IFA), and sera reactive at a dilution of ≥1:64 were considered to be positive [143]. Virus neutralization assays (modified microneutralization assay) can be performed to detect IgG-specific antibodies to SFTSV [16, 266]. High levels of neutralizing antibodies are generated during the convalescent phase of the illness.

Treatment and Prevention

There is no specific treatment available for infections with SFTSV. Clinical management is directed toward a symptomatic treatment. Individual protective measures such as insect repellents and avoidance of areas where ticks may be common are recommended.

3.2.4 Heartland Virus

Recently, a new pathogenic phlebovirus has been identified in northwestern Missouri (USA), named the Heartland virus. Two patients were independently hospitalized in 2009 with fever, diarrhea, headache, anorexia, nausea, elevated hepatic aminotransferase levels, thrombocytopenia, moderate neutropenia, and leukopenia [152]. Both patients were bitten by ticks approximately 5–7 days prior to onset of signs of illness. After discharge from the hospital, both patients reported fatigue and difficulties with short-term memory. Blood was negative for *Ehrlichia* and *Rickettsia* and electron microscopy identified viruses isolated from patient leukocytes to be consistent with members of the bunyavirus family. Next-generation sequencing then revealed that the viruses were new members of the genus *Phlebovirus*. Isolates from both patients were highly identical, indicating that both patients were infected independently with the same virus strain. Further, phylogenetic analysis identified that the Heartland virus is closely related to the recently identified tick-borne phlebovirus SFTSV in China [266]. Interestingly, serum samples were strongly positive for IgG antibodies 2 years after the onset of illness. Future studies will need to be performed to identify the vector and potential hosts of Heartland virus, as well as epidemiologic and ecological studies.

3.3 *Nairovirus* Genus

The *Nairovirus* genus was named after Nairobi sheep disease, an acute hemorrhagic gastroenteritis in sheep, which was first recognized in the early twentieth century in Nairobi, Kenya. The genus consists of at least 34 strains/serotypes that have been divided into seven species/serogroups. All viruses in the *Nairovirus* genus are exclusively transmitted by ticks, although a few isolations have been made from *Culicoides* flies and mosquitoes.

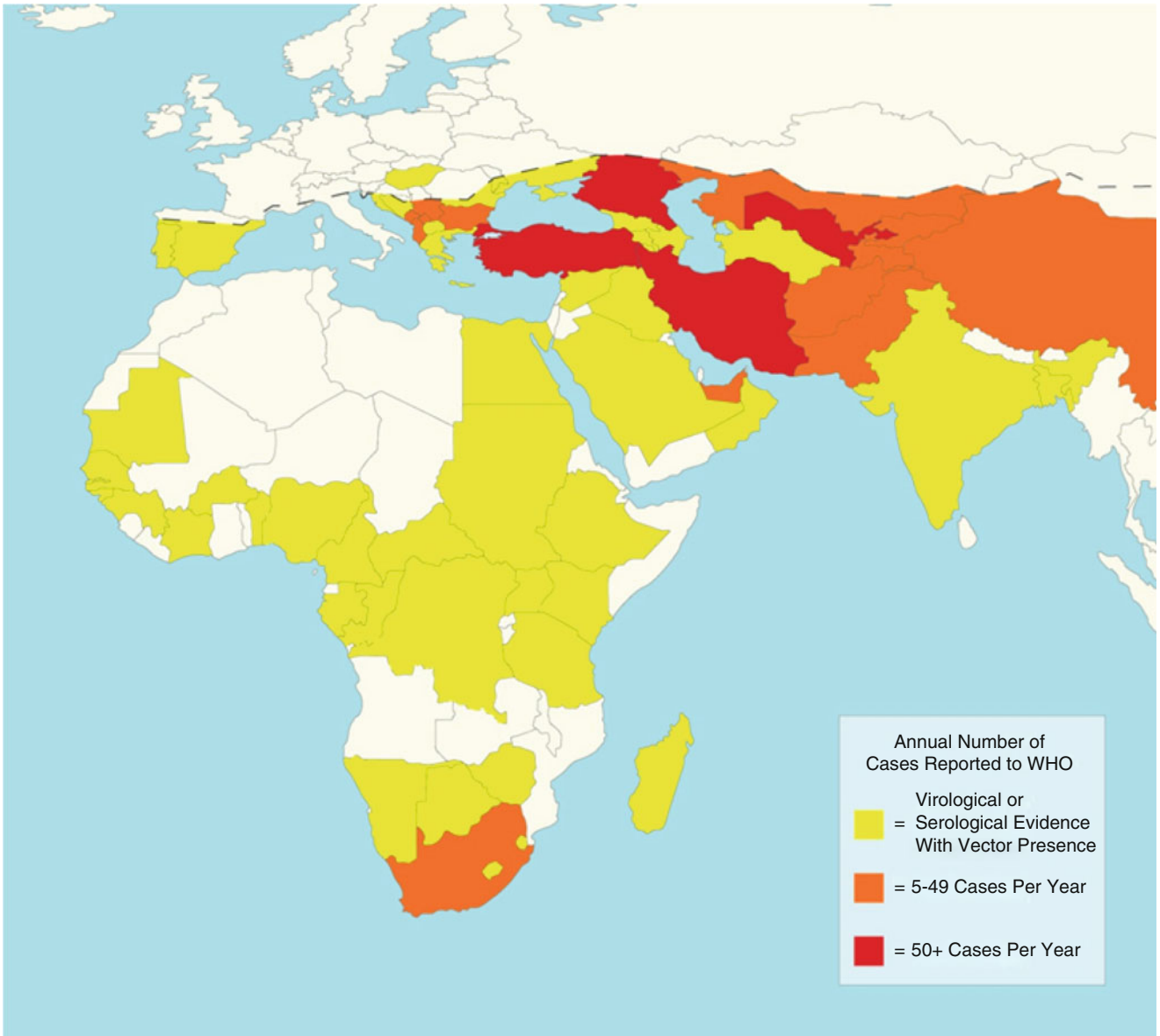

Fig. 9.1 Geographic distribution of CCHF. Countries in red report more than 50 human cases annually to the WHO, and those in orange report fewer than 50 cases. Countries in *yellow* have not reported human cases, but CCHFV has been isolated, or its presence has been inferred from serological studies, and a transmission-competent tick vector is also present. The northernmost limit of *Hyalomma margin-atum* and *Hyalomma asiaticum* is demonstrated by a *gray*, *dashed line*. The figure is based upon that created by the WHO (http://www.who.int/csr/disease/crimean_congoHF/en/) and on tick distribution maps at www.kolonin.org and http://www.efsa.europa.eu/en/efsajournal/pub/1723.htm

3.3.1 Crimean-Congo Hemorrhagic Fever Virus

The most important human pathogen in this genus is Crimean-Congo hemorrhagic fever, a zoonosis, which does not cause disease in its animal host, but results in a severe hemorrhagic syndrome in humans.

Descriptive Epidemiology

Crimean-Congo hemorrhagic fever (CCHF) is an acute, often fatal, tick-borne zoonosis and is among the most important emerging viral hemorrhagic disease of man. Crimean-Congo hemorrhagic fever virus (CCHFV) is extensively distributed in wild and domestic mammals, birds, and ticks throughout at least 30 countries in Western Asia, Southeast Europe, Middle East, and Africa regions [108, 23] (Fig. 9.1).

The distribution pattern approximates that of the predominant tick vector of the genus *Hyalomma* and is the largest among the tick-borne viruses, second of all medically important arboviruses only to that of the dengue viruses [90, 261]. The incidence of CCHF cases has increased over the past decade, including reports of cases for the first time in some countries such as

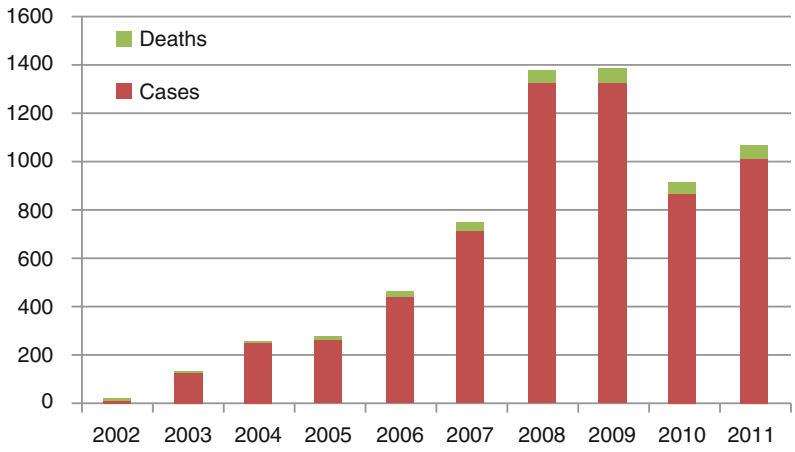

Fig. 9.2 Confirmed CCHFV cases in Turkey since 2002 (Source: Turkish Ministry of Health)

Turkey or India [123, 181]. This has been attributed to anthropogenic factors such as habitat fragmentation as well as possible climate change. In affected countries, typically a few dozen confirmed cases are reported, but annual spikes three to four times higher occur sporadically with no apparent pattern or synchrony between countries. Presumably, a combination of biological factors, both intrinsic and extrinsic, may trigger these spikes [75]. Interestingly, the emerging epidemiologic characteristics of CCHF in Turkey, where the disease was first diagnosed in 2002, appear to differ from those reported previously. Since 2002, the case numbers have increased exponentially and still continue to remain high with a total of over 6,330 cases in the last 10 years (Fig. 9.2). It is suspected that difference in ecology of the disease might have contributed to this emergent trend [76].

Clinical Features

CCHF viral apparent infection has been well documented with an onset of a mild febrile illness that may progress to severe and often fatal hemorrhagic disease [148]. CCHF has a distinct course of infection in humans with four different phases: incubation, prehemorrhagic, hemorrhagic, and convalescence period [73]. The length of the incubation period for the illness varies and precise data is difficult to obtain. The incubation period appears to depend on the mode of acquisition and the dose of the virus. Following infection via tick bite, the incubation period is usually short, ranging on average between 3 and 7 days. The incubation period following contact with infected blood or tissues is usually 5–6 days, with a documented maximum of 13 days [73]. Clinical manifestations in the prehemorrhagic period include fever, malaise, myalgia, dizziness, and, in some patients, diarrhea, nausea, and vomiting lasting for an average of about 3 days. The hemorrhagic phase usually begins on days 3–5 after the onset of illness and is usually short, lasting on average 2–3 days. The first evidence of disease is usually a flushing of the face and the pharynx and a skin rash that progresses to petechiae and ecchymoses, hemorrhage of the mucus membranes and conjunctiva, hematemesis, melena, epistaxis, hematuria, and hemoptysis [73]. Cerebral hemorrhage, gingival bleeding, and bleeding from the nose, vagina, uterus, or urinary tract may occur, as well as internal bleeding in abdominal muscles. Cerebral hemorrhage and massive liver necrosis indicate a poor prognosis. Hepatomegaly and splenomegaly may occur in up to 40 % of the patients. Deaths generally occur on days 5–14 of illness and are attributed to hemorrhages, hemorrhagic pneumonia, or cardiovascular disturbances. Lethal cases typically do not develop an antibody response. The mortality rate has been reported to average around 30 %, but may range from about 5 % to more than 80 % [261]. The variation in rates is believed to reflect the difference in diagnostic capabilities or treatment of patients between outbreak areas as well as the genetic variation of CCHFV strains resulting in different levels of virulence. The convalescence period usually begins 10–20 days after the onset of illness and recovery can take up to a year. Patients may experience generalized weakness, tachycardia, temporarily loss of all their hair, poor appetite, difficulty breathing, polyneuritis, loss of hearing and memory, and impaired vision [236]. In contrast, convalescing patients during recent outbreaks in Turkey and Iran did not present with any of the latter symptoms [148]. The mortality rate is among the highest of all the viral hemorrhagic diseases, ranging from about 5 % to more than 80 %.

Diagnosis

Preliminary diagnosis is based on clinical features and relevant travel or exposure history. Laboratory diagnosis can be made serologically by the detection of IgM-specific antibodies and detection of viral nucleic acids or through virus isolation; however, appropriate biocontainment precautions should be taken. Virus-specific immunohistochemical testing of autopsy tissues may aid in confirming the diagnosis [23].

Treatment and Prevention

At present, there is no internationally licensed vaccine, and treatment is only supportive [74]. Ribavirin has been used for treatment; however, efficacy has not been clearly established [230]. Research has been hampered due to the need of

a maximum biocontainment laboratory (BSL-4) to study the virus and lack of an animal model.

3.3.2 Other Nairovirus Infections

Dugbe Virus

Dugbe virus (DUBV) was first isolated in 1964 in Nigeria from *Amblyomma variegatum* ticks [35]. Serologically, DUBV is related to Ganjam virus, first isolated in 1954 in India from *Haemaphysalis intermedia* ticks and later also from man. DUBV has since been recovered on many occasions in Western Africa from ticks, domestic cattle, mosquitoes, and *Culicoides*. Despite the large number of isolations, serosurveys did not reveal widespread human infections, and isolations from blood of humans were only made occasionally, mainly children, with benign febrile illness including a laboratory infection. One patient had transient meningitis and virus was isolated from the cerebrospinal fluid [92, 157].

Nairobi Sheep Disease

Nairobi sheep disease (NSD) is a noncontagious, tick-borne infection of sheep and goats characterized by hemorrhagic gastroenteritis, abortion, and high mortality caused by NSD virus (NSDV). Antibodies against NSDV have been detected in human serum, but it is not known if these antibodies are the result of an NSDV infection or have been caused by a yet unidentified agent. An apparently naturally acquired clinical case was reported from Uganda in which a young man from which the virus was isolated experienced transient clinical signs [125]. However, no serological conversion was demonstrated.

Erve Virus

Erve virus (ERVEV) was isolated in 1982 from the tissues of a white-toothed shrew (*Crocidura russula*) collected in the Erve river valley in northern France and is only distantly related to the other nairoviruses [42]. The virus has been described in the Czech Republic, Germany, France, and the Netherlands. ERVEV has been suspected to cause severe headache and intracerebral hemorrhage in humans [42]. Due to the lack of commercial diagnostic test, only very little is known about the involvement in human disease.

3.4 *Hantavirus* Genus

Many distinct hantaviruses have been associated with human illness since the isolation of prototype Hantaan virus in 1978 and recognition of the new genus *Hantavirus* within the family Bunyaviridae [138, 217]. Hantaan virus is the causative agent of Korean hemorrhagic fever (KHF) in Korea and epidemic hemorrhagic fever (EHF) in China. Hantaviruses likely occur around the world; however, to date they are best known in Asia, Europe, and the Americas. Human disease caused by hantaviruses in Asia and Europe is characterized by acute fever, a tubular renal lesion, and in some instances a life-threatening capillary leak syndrome, often accompanied by hemorrhagic manifestations. Hantaviruses found in the Americas cause hantavirus pulmonary syndrome, also known as hantavirus cardiopulmonary syndrome, a severe acute disease associated with the onset of febrile illness that rapidly progresses to life-threatening respiratory failure and shock.

3.4.1 Descriptive Epidemiology

Hantaviruses are maintained in nature through chronic infection of small mammals, primarily rodents, although a number of novel hantaviruses have recently been found associated with shrews and moles [122]. Transmission to humans is by aerosol following exposure to infectious rodent excreta and occasionally by bite [94, 246]. Human infection is often seasonal, occurring in temperate zones during the fall when rodents may invade households, during warmer months when summer cabins may be first occupied, and through rural occupational exposure as seen among farmers, hunters, and field-deployed military. Hantaviruses are natural parasites of small mammals, and this genus is apparently unique in the *Bunyaviridae*, which otherwise are uniformly biologically transmitted by arthropods. Routes of transmission among small mammals are not known, although laboratory experiments have clearly documented the susceptibility of laboratory rats to aerosol exposure [172], and a strong correlation exists among free-living urban rats between increased evidence of wounding and rising antibody prevalence rates, suggesting that transmission by bite may be important [94]. Depending on the virus and host, transmission may occur in rural, urban, or occupational (such as from laboratory rats) settings. Person-to-person transmission is not known to exist among Old World hantaviruses; however, the New World cause of HPS, Andes virus, has been associated with person-to-person transmission [260]. Other New World hantaviruses do not appear to be transmitted in this manner. At least 20 genetically and antigenically distinct hantaviruses are known to cause human disease (Table 9.3), and many other hantaviruses with unknown pathogenic potential for humans have been found associated with various species of small mammals including shrews and moles.

Systematic population-based surveys to establish antibody prevalence rates for hantavirus infection have not been widely attempted; however, sufficient examination of selected "at-risk" populations has been conducted to offer a good indication of the global distribution of the hantaviruses. Methods employed include immunofluorescent antibody (IFA) assays, most often using prototype Hantaan virus-infected cell cultures as antigen; enzyme immunoassays (EIA); and radioimmunoassays (RIA). Populations surveyed are generally rural, frequently with high potential for occupational exposure to rodents (farmers, woodcutters,

Table 9.3 Old and New World hantaviruses causing hemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS) and their known distribution and hosts

Distribution and subfamily of rodent host	Virus	Distribution	Primary host species	Human disease
Old World	Hantaan virus	China, Korea, Russia	*Apodemus agrarius*	HFRS
Murinae	Dobrava (Belgrade) virus	Balkans	*Apodemus flavicollis*	HFRS
	Seoul virus	Global	*Rattus sp.*	HFRS
	Saaremaa virus	Europe	*Apodemus agrarius*	HFRS
	Amur virus	Far East Russia	*Apodemus peninsulae*	HFRS
Arvicolinae	Puumala virus	Europe, Russia	*Myodes (Clethrionomys) glareolus*	HFRS (nephropathia epidemica)
New World	Sin Nombre virus	North America	*Peromyscus maniculatus*	HPS
Sigmodontinae	Monongahela virus	North America	*Peromyscus leucopus*	HPS
	New York virus	North America	*Peromyscus leucopus*	HPS
	Black Creek Canal virus	North America	*Sigmodon hispidus*	HPS
	Bayou virus	North America	*Oryzomys palustris*	HPS
	Choclo virus	Panama	*Oligoryzomys fulvescens*	HPS
	Andes virus	Argentina, Chile	*Oligoryzomys longicaudatus*	HPS
	Bermejo virus	Argentina	*Oligoryzomys chocoensis*	HPS
	Lechiguanas virus	Argentina	*Oligoryzomys flavescens*	HPS
	Maciel virus	Argentina	*Bolomys obscures*	HPS
	Oran virus	Argentina	*Oligoryzomys longicaudatus*	HPS
	Laguna Negra virus	Argentina, Bolivia, Paraguay	*Calomys laucha*	HPS
	Araraquara virus	Brazil	*Bolomys lasiurus*	HPS
	Juquitiba virus	Brazil	*Oligoryzomys nigripes*	HPS

Condensed from Jonsson et al. [118]

foresters, and others); patients with clinically compatible or interchangeable disease (e.g., leptospirosis); or unselected blood donors. About 12,000 sera from the European parts of the Russian Federation were examined by RIA, with antibodies found in a geographically focal pattern, generally increasing in prevalence with age, and most common in oil production and forestry workers or tractor and truck drivers [163]. Men were more likely to have antibodies than women (1.3:1 to 2:1), and this ratio increased in the clinically ill to 3:1. In a subsequent study in Bashkortostan, antibody prevalence among over 9,000 persons reached over 16 % in some adult age groups. Seropositive children have been infrequently identified in virtually all serological surveys.

The distribution pattern that has emerged from various surveys suggests that Hantaan virus is most abundant in Asia, including China, where the endemic areas are thought to be expanding, and the Korean Peninsula. Puumala virus, cause of nephropathia epidemica, is most abundant across a broad band from Norway, northern Sweden, Finland, and through the Russian Federation to the Ural mountains. Antibody prevalence rates in these endemic areas approach or exceed 10 % [169, 170, 221]. Elsewhere in Western Europe, antibody prevalence rates are lower, but clinical cases and positive serological results have been found in most European countries; and in areas such as France, Belgium, and Germany, where active research programs exist and clinicians recognize the disease, NE is increasingly diagnosed as an important cause of acute disease [248]. Incidence of human infection with Puumala virus increases following mast years, and recent studies in Germany demonstrated multiple local outbreaks of distinct virus clades associated with simultaneous increases in densities and infection rates of voles in different regions [77]. Seoul virus, cause of a less severe form of HFRS, is thought to be nearly global in its distribution based on serological surveys of peridomestic rodents [135, 136], but extensive tests for this specific virus in humans have not been widely attempted outside of China. In the United States, however, surveys were conducted among residents of Baltimore, Maryland, where inner-city rats are known to be heavily infected with a Seoul-like virus [44, 45, 46]. An antibody prevalence rate of 0.25 % was found among 6,060 persons with no known risk factors for hantavirus infection except residence in Baltimore [96]. This rate was significantly different from the rate found among patients with proteinuria (1.46 %; OR, 3.23; $p<0.05$) and the rate among dialysis patients with end-stage renal disease (2.76 %; OR, 5.03; $p<0.05$). Overall, 6.5 % of patients with end-stage renal disease due to hypertension were seropositive for a hantavirus, suggesting that hantavirus infection may be associated with hypertensive renal disease. Canadian blood donors had 1.4 % prevalence of antibodies to hantaviruses (3.5 % in the Maritime Provinces), but the exact strain of infecting virus was not determined [139].

3.4.2 Clinical Features

Hemorrhagic Fever with Renal Syndrome (HFRS)

Acute hemorrhagic and nephropathic clinical syndromes were described and variously named decades before their causative agents were identified. As cited by Casals et al. [34], records of severe, often fatal, hemorrhagic fever have been discovered in Vladivostok, Eastern Siberia, as early as 1912, and it seems likely that similar syndromes, albeit often confused with other causes of fever and hemorrhage, were known in Asia and Europe several centuries prior to this time. Modern scientific descriptions are credited to Soviet and Japanese scientists working in Siberia and Manchuria in the 1930s [163]. English-speaking physicians first encountered this disease during the Korean conflict in the early 1950s and named it Korean hemorrhagic fever (KHF). Modern Chinese and Japanese authors prefer the term "epidemic hemorrhagic fever" (EHF). A milder form of illness, with minor hemorrhage and lower mortality, was recognized in Scandinavia, also in the early 1930s, and termed "nephropathia epidemica" (NE) [164]. Today, hemorrhagic fever with renal syndrome (HFRS) is generally used to refer to the diseases caused by Old World hantaviruses.

Mortality in HFRS generally ranges from <5 to 15 % and is reduced by recourse to dialysis and proper management of patients who experience renal shutdown [34]. Between 1950 and 2007, over 1.5 million cases of HFRS and over 46,000 deaths (3 %) were reported in China with the highest case fatality rates above 14 % in 1969; however, for the last decade mortality rates for HFRS in China have maintained at approximately 1 % [271]. Case fatality rates of 10–15 % were reported from the Russian Federation for the periods 1978–1992, with peak annual incidence rates of over 11,000 cases (8.0 per 100,000 population) (World Health Organization 1993) and annual incidence rates of 50 cases per 100,000 in Bashkortostan [170]. Mortality in the former Yugoslavia during an outbreak in 1989 was 6.6 % (15 deaths of 226 cases) [95]. Dobrava virus is found in Greece, Bulgaria, Albania, the former Yugoslavia, and some foci elsewhere in Europe where it occurs relatively infrequently but produces an especially severe form of HFRS with mortality rates as high as 30 % in some areas [9, 95, 135, 136]. Incidence rates have dropped in recent years for prototype Hantaan virus in Korea to reports of a few hundred cases annually. Less severe NE-like disease occurs in several hundred patients each year in Scandinavia, and increasing numbers of cases are now recognized in Western Europe. The highest incidence rates in Sweden exceeded 20 cases/100,000 population in endemic areas [169, 221], while the prevalence of serum IgG antibodies ranged from about 2 % in non-endemic areas to around 8 % in endemic regions [168] [169]. The incidence of clinical cases and antibody prevalence in an endemic area of Sweden were compared, and the antibody prevalence rate in the oldest age groups was found to be 14–20 times higher than the accumulated life risk of being hospitalized with NE, suggesting that mild or asymptomatic infections are not uncommon [124]. The case fatality rate for NE due to Puumala virus in Sweden was estimated at about 0.2 % [220]. In the Balkan region of Europe, the viruses that cause both severe HFRS and relatively mild NE coexist, although the total disease burden generally does not exceed several hundred cases annually. More than 100 clinical and subclinical infections associated with occupational exposure to laboratory rats infected with Seoul virus have been recognized in Japan, and additional cases have occurred in Korea, the former Soviet Union, Belgium, and the United Kingdom. HFRS caused by Hantaan, Dobrava, or Amur virus is more severe, while Puumala and Seoul virus infections are generally milder.

Clinical HFRS in its classic severe form is highly distinct. By far the best description of the disease in English is that written by a medical commission during the Korean conflict [222]. Distinct phases of evolution were described, and although not all patients exhibited each of them, the pattern accurately reflected significant physiological changes that characterized Hantaan virus infection (Table 9.4). The incubation period of HFRS has been variously estimated at 10–35 days. The longer intervals, in particular, are of significance both in terms of pathogenesis and for their implications with respect to occurrence of unusual illness among travelers who may be infected on one continent and sick on another.

Hantavirus Pulmonary Syndrome (HPS)

In May 1993, an outbreak of an apparently new disease characterized by unexplained adult respiratory distress syndrome occurred among residents of rural southwestern United States. This disease became known as hantavirus pulmonary syndrome (HPS) or sometimes hantavirus cardiopulmonary syndrome (HCPS). The original outbreak was centered in the Four Corners region, where the states of New Mexico, Arizona, Utah, and Colorado join, and primarily affected young, previously healthy adults. By October 1993, 42 cases had been confirmed, including one retrospectively diagnosed from July 1991. Median age of patients was 32 years (range 12–69 years) and 52 % were males. American Indians accounted for 55 % of the cases; of the remainder, 36 % were non-Hispanic whites, 7 % were Hispanic, and 2 % were black. The mortality rate was 62 %, and cases were identified from 12 states [36, 37]. Epidemiologic investigations quickly determined that small rodents in the outbreak area were especially abundant in the spring and early summer of 1993, and virological examinations of captured rodents found *Peromyscus maniculatus* to be the species most frequently infected. Route of transmission from rodents to humans was assumed to be by the aerosol route, similar to other hantaviruses. The examination of PCR-amplified products from human cases and from *P. maniculatus* captured in and around patient's homes found identical hantaviral sequences at a given site but surprisingly high levels of

Table 9.4 Characteristic course of clinical disease for hemorrhagic fever with renal syndrome

Phase	Duration	Predominant signs and symptoms	Laboratory findings
Febrile	3–7 days	Fever, malaise, headache, myalgia, back pain, abdominal pain, nausea, vomiting, facial flush, petechiae (face, neck, trunk), conjunctival hemorrhage	WBC = normal or ↑ Platelets = ↓ Hematocrit = ↑ Urine = proteinuria 1 + → 3+
Hypotensive	2 h–3 days	Nausea, vomiting, tachycardia, hypotension, shock, visual blurring, hemorrhagic signs, ± oliguria (late)	WBC = ↑ with left shift Platelets = ↓↓ Bleeding time = ↑, PT may be ↑ Hematocrit = ↑↑ Urine = proteinuria 4 + hematuria 1 + Hyposthenuria BUN and creatinine = increasing
Oliguric	3–7 days	Oliguria ± anuria BP may ↑; nausea and vomiting may persist; 1/3 with severe hemorrhage (epistaxis, cutaneous, GI, GU, CNS)	WBC = normalizes Platelets = normalize Hematocrit = normalizes, then ↓ Urine = proteinuria 4 + Hematuria 1 + → 4 + BUN and creatinine = ↑↑ Na^{+}↓, K^{+}↑, Ca^{2+}↓
Diuretic	Days to weeks	Polyuria = 4 + (3–6 l daily)	BUN and creatinine = Normalize electrolytes Possibly abnormal (diuresis)
			Urine = normalizes
Convalescent	Weeks to months	Strength and function regained slowly	Anemia and hyposthenuria = may persist for months

From McKee et al. [151] Reproduced with permission
Phases as seen in KHF. All phases may not be present in a given patient

genetic diversity among samples taken from different sites [166]. Investigators using the same PCR techniques on preserved tissues of *P. maniculatus* captured in 1983 in California likewise found infected rodents, indicating that this virus is not "new" or recently mutated [222]. *Peromyscus maniculatus* is one of the most common and widely distributed rodents in North America. Subsequently, many other hantaviruses have been identified from small mammals throughout the Americas, from Canada to Argentina and Chile, many of which cause HPS-like clinical disease, often resulting with fatalities. Each recognized hantavirus appears to be predominantly associated with a particular species of small mammalian host and occupies a distinct ecological habitat.

Hantavirus pulmonary syndrome is found in the New World and characterized by rapid onset of pulmonary edema, soon followed by respiratory failure and cardiogenic shock. Many New World hantaviruses are capable of causing HPS, with the most severe disease typically associated with Sin Nombre virus, isolated during the original North American outbreak in 1993, and Andes virus, recognized predominantly in Chile and Argentina; however, many other New World hantaviruses found elsewhere in the Americas are known to cause serious human infection similarly associated with severe morbidity and high mortality rates. Case fatality rates for HPS are usually about 40 %. New World hantaviruses infect lung microvascular endothelium causing microvascular leakage that is characteristic of HPS [146]. Incubation period is 9–33 days, with most onsets occurring between 14 and 17 days post exposure to small mammal reservoir hosts [265]. A detailed examination of HPS in Brazil was characteristic of HPS as seen elsewhere in the Americas and reported a typical clinical course associated with a febrile prodrome with fever, myalgia, and worsening thrombocytopenia, headache, backache, and other symptoms, followed by dyspnea, cough, and tachycardia along with low blood pressure, leading to shock in about a third of patients. More than half the patients developed renal failure and about half had mild hemorrhagic disturbances. Reduced platelets, metabolic acidosis, hemoconcentration, and leukocytosis were documented in more than half the patients seen. Diffuse bilateral rales were seen in the majority of cases. Patients were typically admitted 3–6 days post onset of symptoms, and fatalities occurred within a day or two after admission. Survivors typically remained hospitalized for more than a week [118].

3.4.3 Diagnosis

The serological diagnosis of hantaviral infection in humans is typically done by detection of IgM- and IgG-specific antibodies, often using recombinant N protein as antigen, which is the most abundant viral protein and induces a strong humoral response. Both traditional indirect IgG and IgM enzyme-linked immunosorbent assays (ELISAs) and IgM capture ELISAs are used routinely. Virtually all patients are

IgM positive by the onset of acute illness [137]. Rapid strip immunoblot assays have also been developed [106]. Immunoblot and neutralization tests have been used for serological diagnosis, and focus reduction neutralization assays and plaque reduction neutralization assays have been used for virus-specific serological diagnosis and classification of hantaviral isolates. Molecular diagnostics such as reverse transcription PCR can rapidly detect hantaviral genomic sequences in clinical specimens early in infection and even prior to onset of acute illness [78, 178]. Direct isolation of hantaviruses from acute human specimens or from tissues of suspect small mammalian hosts is difficult and time-consuming. Hantaviruses are slow to adapt to growth in cell culture, and indeed only a few cell lines are known to support hantaviral growth. Most infected cell cultures do not exhibit cytopathic effect, the exception being HEK293 cells [149]; consequently, the presence of infectious virus must be established by demonstration of specific viral antigen or nucleic acids, typically by direct IFA assay or polymerase chain reaction (PCR). Primary isolation has been most successful when a combination of techniques was employed [126]. A note of caution: Although asymptomatically infected, laboratory animals may shed infectious virus in their excreta and saliva. Consequently, isolation attempts and experimentation that utilizes laboratory animals should only be carried out under appropriate biocontainment conditions that minimize the risk of aerosol transmission between animals and humans. BSL-2 practices, containment equipment, and facilities are recommended for laboratory handling of sera from persons potentially infected with hantaviruses, and potentially infected serum or tissue samples should be handled in BSL-2 facilities following BSL-3 practices and procedures. Cell culture-virus propagation and purification should be carried out in a BSL-3 facility using BSL-3 practices, containment equipment, and procedures. Experimentally infected rodent species known not to excrete the virus can be housed in ABSL-2 facilities using ABSL-2 practices and procedures. Primary physical containment devices including BSCs should be used whenever procedures with potential for generating aerosols are conducted. All work involving inoculation of virus-containing samples into rodent species permissive for chronic infection should be conducted at ABSL-4 [38].

3.4.4 Treatment and Prevention

The pathogenesis of both HFRS and HPS is complex and is associated with an intense immune activation that leads to changes in vascular permeability. Aggressive supportive care and careful clinical management of fluid levels are essential to the successful treatment of critically ill patients. HPS cases may quickly progress to respiratory failure and shock that requires extracorporeal membrane oxygenation (ECMO) and careful monitoring of cardiac output and respiratory function [155]. Beneficial effect of treatment with the antiviral drug ribavirin has been reported for HFRS patients in China when administered early in the course of illness [110]; however, due to the rapid progression of HPS and the fact that the diseases are rarely recognized until after the onset of cardiopulmonary involvement, ribavirin has not had a beneficial effect on the treatment of HPS patients [156]. A role for antiviral drugs in the prophylaxis of intimate contacts of patients infected with Andes virus could be beneficial, but has yet to be demonstrated. A number of hantavirus vaccines have been tested in humans, including both rodent brain-derived and cell culture-derived formalin-inactivated vaccines. Most have targeted Hantaan or Seoul virus, either individually or in combination, although at least one candidate DNA vaccine has included Puumala virus. In addition, vaccinia-vectored vaccines expressing Gn, Gc, and N viral proteins and plasmid DNA delivered by gene gun have been tested (reviewed in [216]). Hantavax® is a commercially available suckling mouse brain-derived formalin-inactivated vaccine marketed in Korea; however, its efficacy has not been demonstrated [180]. Inactivated vaccines for Hantaan and Seoul viruses have also been produced in China with neutralizing antibody produced in over 90 % of those receiving three doses [63].

4 Unresolved Problems

The bunyaviruses exist in nature through silent transmission cycles that typically involve an animal vector (arthropod or rodent) and only occasionally result in human infection, often occurring in rural environments where human contact with potentially infectious vectors is more likely to occur. Those exposed are frequently residents of less developed regions of the world who may lack access to modern medical facilities and resources leading to a definitive diagnosis or effective treatment. Populations at greatest risk are often those least able to afford preventive vaccines, diagnostic tests, or effective therapeutics should they be available for these low incidence diseases, and as a result few economic incentives exist for commercial investment in the development of the critical tools needed to address the diseases caused by bunyaviruses. Inexpensive, accurate diagnostic tests are needed to better define the incidence of human infection. With the availability of better diagnostics, a greater appreciation of the full impact of bunyavirus infections on human and animal health will become apparent and may lead to investments in vaccine production or discovery of therapeutic interventions.

Much remains to be learned regarding the natural history of the bunyaviruses, including how they exist in nature and interact with their vector; the molecular mechanisms developed by the vector to limit virus spread; and why some vectors efficiently maintain and transmit these viruses, while

other closely related species do not. Critical information is still needed to better understand how these viruses survive adverse environmental conditions and the key role played by vectors in maintaining these pathogens in nature in some cases over a long period of time. Further, a better understanding of the behavior of vector species could lead to improved prevention, development of intervention strategies, and control of human and animal disease.

Bunyaviruses cause a wide range of clinical illness from febrile disease to frank hemorrhagic fever, encephalitis, and death, yet the pathogenesis of human bunyavirus infection is not well understood. This is due in part to the relatively rare access to acutely ill patients, thus limiting the opportunity for in-depth clinical evaluations and interventions, and also to the absence of robust animal models that faithfully replicate human infection. Furthermore, core questions (e.g., through genomic or proteomic studies) will need to focus on the identification of pathogenic determinants and virulence markers, defining host and tissue specificity.

The bunyaviruses represent one of the most geographically diverse families of viruses known, existing under virtually all climatic conditions and being maintained in nature through a wide variety of vectors. As such, they are often seen as the cause of "new" or emerging infectious diseases, often encountered as humans occupy previously uninhabited regions of the world or become exposed through the modification of the environment that allows the introduction of potentially infectious vectors. Comprehensive determination of bunyavirus genome sequences will be fundamental for a better understanding of their relationship and help developing taxonomic schemes, ultimately supporting diagnostics. We can expect greater interaction between humans and the bunyaviruses in the future as populations increase, our environment changes, and we experience more rapid and frequent travels over greater distances, bringing these "exotic" infections to our doorsteps.

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Coronaviruses

10

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1 Introduction

Coronaviruses of humans have been classified as a subfamily of the Coronaviridae family. The viruses are roughly spherical, enveloped particles 120–160 nm in diameter. Their name derives from the characteristic "crown"-like projections on their surface, approximately 20 nm long. They are positive-sense, single-stranded RNA viruses, are sensitive to heat and lipid solvents, and have a distinct replication strategy common to other viruses in the order Nidovirales [1–3]. Coronaviruses have in the past been divided into three groups. In part, because of increasing work on coronavirus discovery in the wake of the outbreak of severe acute respiratory syndrome (SARS) in 2003, a number of new coronaviruses of humans (hCoVs) have been identified. This has resulted in the three groups being reclassified as genera of the Coronavirinae subfamily. Before the SARS outbreak, while it was recognized that the coronavirus infected many different species, particularly domestic and laboratory animals, their extreme diversity in nature was not fully appreciated. Although severe diseases of differing characteristics were known to occur in animals, in humans the viruses were thought to cause only acute respiratory infection which were generally mild. All this changed with the recognition that SARS was caused by a coronavirus (SARS-CoV). This signaled that coronaviruses could produce lethal disease and encouraged more broadly based research on the agent and its prevention and control. In the process, two new respiratory or endemic coronaviruses have been identified as causing human infections, which appear to resemble in epidemiologic characteristics those previously known. Very recently, MERS (Middle East respiratory syndrome) coronavirus, a novel human betacoronavirus lineage C virus, has been discovered zoonotically infecting humans in Middle Eastern countries.

In light of the differences between the endemic respiratory and the SARS coronaviruses in terms of epidemiology and clinical expression, they will be covered separately in much of this chapter.

2 Historical Background

Coronaviruses were first identified from domestic and laboratory animals before they were identified in humans. Infectious bronchitis virus of chickens was actually isolated in embryonated eggs in the 1940s. The late recognition of these viruses was, in large part, because of difficulty in recovering the human viruses using standard cell culture techniques [4]. The first human coronaviruses were identified by different techniques in the United Kingdom and the United States at approximately the same time. The British Medical Research Council's Common Cold Research Unit had been studying fluids collected from persons with natural respiratory infections by standard cell culture isolation methods and by inoculating them into human volunteers. Rhinoviruses or other cytopathogenic agents could be recovered from a portion of the fluids [5]. There was an additional, substantial portion from which no agents could be isolated but that could still cause colds in the volunteers. Organ cultures of human embryonic trachea or nasal epithelium were then used in an effort to detect the recalcitrant viruses present. A specimen, B814, that had been collected in 1960 from a boy with a common cold had not yielded a virus on inoculation into cell culture. After the specimen had been passaged serially three times in human tracheal organ culture, it could still cause colds on inoculation into volun-

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teers, which indicated that replication had taken place [6]. In Chicago during the winter of 1962, five agents were isolated in primary human kidney cell cultures from specimens collected from medical students with common colds. The viruses were ultimately adapted to WI-38 cultures and exhibited a type of cytopathic effect (CPE) not previously seen. A prototype strain, 229E, was selected for characterization and was found to be RNA containing, ether labile, and 89 nm in diameter but distinct serologically from any known ortho- or paramyxoviruses. Sera collected from the five medical students all exhibited a fourfold rise in neutralization antibody titer against 229E [7]. It became clear that these "novel" viruses were of more than passing significance when organ culture methods were added to standard cell culture techniques in a study of acute respiratory infections of adults conducted at the National Institutes of Health (NIH). Six viruses were found that grew in organ but not cell culture and were ether labile; on electron microscopy, the agents were shown to resemble avian infectious bronchitis virus (IBV) in structure, and these represented a distinct family of viruses [8]. The B814 and 229E strains were soon also demonstrated to have a similar structure on electron microscopy and to develop in infected cells by budding into cytoplasmic vesicles [9, 10]. As a result of the similarity of the human agents to IBV and also to mouse hepatitis virus (MHV), they were collectively considered to represent a group of vertebrate viruses distinct from the myxoviruses antigenically and structurally [11]. The name coronavirus was eventually adopted for the group to describe the fringe of projections seen around them on electron microscopy [1]. Except for 229E, none of the human coronaviruses had been successfully propagated in a system other than organ culture. McIntosh et al. reported successful adaptation of two of the NIH isolates, OC (organ culture) 38 and OC43, to the brains of suckling mice [12]. These strains were shown to be essentially identical antigenically but quite distinct from MHV. Only OC38 and OC43 could be so adapted; the other four OC strains resisted such attempts. The IBV was known to exhibit hemagglutination under certain conditions, but no such phenomenon had been demonstrated for the human strains until OC38 and OC43 were adapted to mice. Kaye and Dowdle found that the infected brain preparations would directly and specifically agglutinate red cells obtained from chickens, rats, and mice [13]. This technique greatly expanded the ability to do sero-epidemiologic studies, since it was simple and reproducible.

Subsequent developments included adaption of OC38 and OC43 to growth in cell monolayers; either mouse brain or organ culture material could be used as sources of virus [9]. Not only was CPE available for reading of neutralization tests, but also the OC38 and OC43 viruses were found to hemadsorb red cells of rats and mice, making available a more precise means of evaluating endpoints in tests involving these organ culture-derived strains [14]. The other OC strains and B814 that could not be adapted to mouse brain resisted adaptation to cell culture as well; these distinct viruses have since been lost and may actually have been rediscovered recently.

Work on the coronaviruses of humans proceeded slowly, with debate about how frequently the viruses caused lower rather than upper respiratory disease. The methodological problems of working with them were a major impediment, as was their apparent involvement only in a relatively mild disease. All this changed when in 2002 SARS appeared first in China and then in other countries of the world. The near panic resulted because of its transmission characteristics, case fatality, and the fact that the agent was initially unknown. That last aspect was solved quickly with the identification of the causative agent as a new coronavirus. The knowledge that the virus emerged from a zoonotic reservoir spurred investigation of its possible source. Small mammals (civet cats) in live game-animal markets in Guangdong were identified carrying closely related viruses. This led to the identification of these animals as amplifier hosts and to the game-animal wet markets as an interface where zoonotic infection of humans was being initiated [15]. The natural reservoir was later identified to be Chinese horseshoe bats [16, 17]. The SARS epidemic ended following the use of various population control measures and unexpectedly has not recurred. However, increased attention to the coronaviruses continued globally. New coronaviruses were identified in various animal hosts, and two additional coronaviruses were identified in humans, the first since 229E and OC43. NL63 was identified in Amsterdam in 2004 from a 7-month-old child with febrile bronchiolitis [18]. The same virus was also isolated at almost the same time by the group in Rotterdam from an 8-month-old child with pneumonia [19]. The second virus, HKU1, was detected in a specimen collected from a 71-year-old man from China with pneumonia and then from another adult with the same diagnosis [20]. With the development of real-time PCR techniques for all four human respiratory coronaviruses, it has now become possible to identify them in many situations. Such identification is now typically done not only for these viruses but also for a variety of other respiratory agents. This has meant that coronaviruses are now detected as much in clinical settings as in epidemiologic studies.

3 Methodology

3.1 Sources of Mortality Data

Until the occurrence of SARS, coronaviruses of humans were not thought to cause death, except, possibly, in those with underlying conditions. This was in contrast to the situation in animals, where infections were sometimes fatal, depending on the particular virus. Since the SARS episode, the major change which has affected data on the respiratory coronaviruses has been more ready detection using the polymerase chain reaction (PCR) technique, so that infections are

now recognized in those who might previously have not been studied. This has especially been the case in hospitalized individuals, particularly those who will likely experience severe outcomes, such as the immunocompromised. It is also known from earlier studies that coronavirus frequently infects small children and reinfects adults, including persons with chronic respiratory disease [21]. It would be logical to assume that deaths could occur in these most susceptible segments of the population, but they are probably not very frequent.

A problem during SARS was in defining the specificity of infection, whether inapparent, mild, or fatal. This was related to the lack of a readily available diagnostic test in many areas where outbreaks occurred. To a large extent, cases were classified using a clinical case definition. In some cases that survived, there was an attempt at serological confirmation.

3.2 Sources of Morbidity Data

Since the coronaviruses of humans, other than the SARS virus, usually produce illnesses indistinguishable from those caused by other respiratory viruses, it is not possible to define morbidity in the absence of laboratory identification. Before PCR became available, it was difficult directly to identify the infecting virus; thus, besides anecdotal reports, most of the epidemiologic studies were based on identifying rises in antibody titer. In contrast, since development of the PCR technique, direct identification has become relatively simple. However, this seeming advance has often been accompanied by the use of the method to determine the incidence of infection in population groups and to define characteristics of associated disease or even seasonality. The major problem is the short duration of many of the available studies and the concentration on hospitalized individuals. As a result, while it is possible to say these viruses can sometimes cause hospitalization and to infer the particular clinical diagnoses they produce, it is difficult to estimate what proportion of overall illnesses are severe. A small number of studies have been population based and have produced the only data available for determining the overall impact of illness [22–24]. While the older population-based studies were limited to 229E, OC43, or both, they are useful since there are few recent investigations that give the same information. They were conducted in different settings but in some cases contemporaneously which allows direct comparison. In fact, almost no studies include all four recognized coronaviruses and cover more than a single year. This makes it difficult to discuss year-to-year variation in frequency of activity as well as seasonality, as was possible with the older data. Such studies are therefore of continuing interest, at least as background, in determining the long-term occurrence of the viruses. These original investigations were typically broadly based, with coronavirus infection forming part of overall evaluations of the role of viruses in general in respiratory illnesses. As indicated in the selected listing in Table 10.1, a variety of different open and closed populations were used for these studies. The 229E strain was originally isolated from medical students in Chicago as part of a long-term study of respiratory illnesses in young adults [7, 25]. Employee groups were the source of specimens in the NIH [26, 27] and in the studies at Charlottesville, Virginia [30]. Infection was also evaluated in children's homes [28] and boarding schools [5], among military recruits [34], and among children hospitalized for severe respiratory illnesses in various parts of the world [26]. Serological methods were used to detect occurrence in persons with acute exacerbations of asthma [33] or chronic obstructive respiratory disease [21]. Patterns of coronavirus infection were identified among the general population residing in the Tecumseh, Michigan, community as part of a longitudinal study of respiratory illness [31, 32]. Also included in Table 10.1 are more recent studies using PCR to identify infection. The previous studies, based on serology, often did not characterize infections identified clinically; in fact, challenge studies of volunteers were employed early, to determine characteristics of illnesses, because of problems associated with direct isolation [35, 36]. The more recent studies were able to characterize the illnesses clinically but have other limitations as indicated above.

Table 10.1 Selected longitudinal studies on the epidemiology of coronavirus infection in humans

Location	Population	Virus studied	Years
Chicago, IL [25]	Medical students	229E	1961–1967
Washington, DC [26, 27]	Hospitalized children	229E, OC43	1962–1967
Bethesda, MD [26, 27]	Adult employees	229E, OC43	1962–1967
Atlanta, GA [28, 29]	Institutionalized children	229E, OC43	1960–1967
Charlottesville, VA [30]	Working adults	229E, OC43	1962–1970
Tecumseh, MI [31, 32]	General community	229E, OC43	1965–1970
Denver, CO [33]	Hospitalized asthmatic children	229E, OC43	1967–1969
N. and S. Carolina [34]	Military	229E, OC43	1970–1972
Nashville, TN, and Rochester, NY	Hospitalized children <5 years of age	4 viruses	2001–2003
Melbourne, Australia [23]	Preschool children	NL63	2003–2004
Edinburgh, Scotland [22]	Medically attended illnesses—general population	4 viruses	2006–2009

3.3 Serological Surveys

Relatively simple serological technique was available for the first two coronaviruses identified (229E and OC43), and surveys of antibody prevalence were carried out in various parts of the world. Many surveys formed a part of studies directed mainly toward determination of incidence of infection. Information on the prevalence of antibody was available for populations in the United States [27, 30, 31], the United Kingdom [36], Brazil [37], and other parts of the world. A special situation was determining the meaning of the presence in man of antibody against coronaviruses of animals. The finding of mouse hepatitis antibodies in military recruits and in children and adults from the general population was surprising when first described in 1964 [38]. It is now recognized that this did not indicate past experience with MHV but rather with human coronavirus strains known to cross-react with it. Similarly, antibodies in human sera against the hemagglutinating encephalomyelitis virus of swine and the coronavirus of calf diarrhea also appear to represent cross-reactions with OC43 or related strains [29, 39]. In contrast, in a survey of antibodies to avian IBV, none could be found in a military population. Low-level antibodies were detected only in a portion of subjects who had close contact with poultry [40]. The virus is not known to cross-react with the human strains. More recently, following a gap in active work on the epidemiology of coronaviruses, ELISA methods have been developed for at least some of the viruses [41, 42]. These have not been as much used as the older techniques, given the availability of PCR, and may not be as specific to the particular strain.

3.4 Laboratory Methods

3.4.1 Viral Identification

Laboratory diagnosis can be achieved by identification of the virus in clinical specimens, using virus isolation or antigen or molecular detection techniques. Identification of infection can also be accomplished by detecting a host antibody response. Relevant specimens for virus detection are typically respiratory specimens, such as nasopharyngeal aspirates, washes or swabs, nose or throat swabs, and, when available, endotracheal aspirates or bronchoalveolar lavages. In the case of SARS, in which disseminated infection may occur, viral RNA could also readily be detected in the feces and in the serum or plasma.

Although PCR-based methods are becoming the "gold standard" in diagnosis, virus isolation is indispensable for characterizing virus, studying pathogenesis, determining susceptibility to antivirals, and developing novel antivirals and vaccines. The human coronavirus 229E was originally isolated in cell culture and later adapted to roller culture monolayers of human embryonic lung fibroblast cells such as WI-38 and MRC-5. A cytopathic effect of small, granular, round cells appears at the periphery of the cell monolayer [7]. Although these cells can be used for the cell-adapted prototype 229E virus strain, primary isolation of new 229E-like agents remains difficult. The human embryonic intestine (MA177) semicontinuous cell line has been used for the primary isolation of other 229E-like viruses [26]. Human coronaviruses OC38 and OC43, not related to 229E, were originally isolated in organ cultures of human trachea or lung [6, 8, 43, 44]. These two strains are similar and have been further adapted to replicate in suckling mouse brain and to primary monkey kidney and BS-C-1 cell cultures [9, 12, 14]. Another cell system, LI32, a heteroploid human lung line, has been reported to be suitable for primary isolation of 229E, a related virus (LP), and the B814, the first-described organ culture agent [45, 46]. This last finding has not been confirmed by other workers [9]. Similarly, MRC-C cells have been used for 229E-like viruses and human rhabdomyosarcoma cells for propagating 229E and OC43 [47, 48].

Human coronavirus NL63 was initially isolated in African green monkey kidney cells (LLC-MK2) [18]. Human colon carcinoma cells (CaCo-2) have recently been shown to be more susceptible to NL63 infection and show more prominent cytopathic effect [49]. HKU1 was initially discovered in 2005 by "broad-range" (primers designed to detect all known coronaviruses, rather than being specific for known coronavirus types) reverse transcriptase PCR (RT-PCR) [20], and new isolates remain difficult to grow reproducibly in the laboratory. However, it has been successfully cultured in human ciliated airway epithelial cells (HAE) [50].

SARS-CoV was initially isolated in 2003 on African green monkey kidney epithelial cells (VeroE6) and in fetal rhesus kidney cells [51, 52] during the SARS epidemic in 2003. VeroE6 cells, which are deficient in interferon induction, continue to be the cells of choice for culturing SARS-CoV at present. Following isolation of this novel agent, electron microscopy and molecular methods (random primer PCR and virus detection arrays) followed by partial genetic sequencing led to its identification as novel coronavirus.

Recently, MERS, a novel human coronavirus of the betacoronavirus lineage C, has been detected in patients from Middle Eastern countries with severe pneumonia and renal dysfunction. Vero and VeroE6 cells are suitable for primary isolation of this virus from clinical specimens [53].

Continuous transformed cell lines do not mimic the physiological state of cells in tissues in vivo, and this may be the reason why many coronaviruses are difficult to culture in vitro. The use of primary cells from the relevant species, cells differentiated in vitro in air–liquid interface cultures and ex vivo organ cultures (as was used in the early days of virology), may be needed for the isolation of more fastidious viruses, since some animal viruses are more readily isolated

in culture although they are species-specific in their in vitro growth characteristics, especially on primary isolation [54–57]. Embryonated egg culture has been used as a host system for avian coronaviruses [58]. However, none of the plethora of bat coronaviruses detected by RT-PCR have been readily cultured in vitro to date, even in primary bat epithelial cell lines [59].

3.4.2 Antigen Detection

Immunofluorescence tests on cells from the respiratory tract (e.g., nasopharyngeal aspirates or swab) using commercially available reagents [60] or polyclonal [61] or monoclonal antibodies to 229E and HKU1 [62, 63] have been reported, but these are not widely used. Such antigen detection tests can also be used for the diagnosis of SARS-CoV infection [62]. Several ELISA systems have been developed to detect coronaviruses including coronavirus 229E [64] and the nucleocapsid (N) or spike (S) proteins of SARS-CoV in respiratory samples [65, 66].

3.4.3 Molecular Diagnostic Methods

Since the majority of human coronaviruses cannot be readily cultured in vitro, reverse transcriptase PCR (RT-PCR) and real-time quantitative RT-PCR have become the methods of choice for detecting and quantifying coronaviruses in clinical samples and for discovering novel viruses. RT-PCR methods were used for the detection of 229E and OC43 viruses in clinical specimens [67]. There are now a number of commercial assays that detect a range of respiratory pathogens (including coronaviruses) by the use of a combination of PCR amplification together with nucleic acid hybridization in Luminex bead assay formats. These methods provide the opportunity for the rapid detection of a panel of over 15 respiratory viruses including a number of coronaviruses in a clinical specimen. However, the sensitivity is generally less than that provided by individual RT-PCR methods [68, 69].

Apart from detection of known coronaviruses, RT-PCR is useful in virus discovery and further characterization. This includes a wide range of coronaviruses in bats detected solely by such broad-range RT-PCR methods because these viruses cannot at present be cultured [70]. For example, the first identification of HKU-1 as a cause of human disease was initially based on detection of viral RNA in clinical specimens by broad-range RT-PCR with primers designed to detect all viruses within the coronavirus family [20]. NL63 was discovered using the VIDISCA (virus-discovery-cDNA-amplified restriction fragment length polymorphism) method [18]. Amplified sequences from RT-PCR permit viral genome sequence analysis, which sheds light on virus structure, characteristics, biological properties, phylogeny, host and tissue tropism, epidemiology, cross-species transmission, and drug design [53, 71–75].

3.4.4 Serological Tests

The demonstration of rising antibody titers between acute and convalescent sera to a specific viral antigen provides evidence of recent infection, while the detection of antibody in seroepidemiologic surveys provides evidence of past infection. Methods that can be used for serodiagnosis have included the complement fixation test, neutralization test, indirect immunofluorescent (IF) assay, and enzyme-linked immunosorbent assay (ELISA). Hemagglutination-inhibition (HI) and gel-diffusion tests are less frequently used nowadays.

Neutralization test can also be used on coronaviruses that only grow in organ cultures [8]. Neutralizing antibodies could be detected as early as 5–10 days after symptom onset during SARS infection [76]. The seroprevalence for SARS-CoV in asymptomatic children and adults living in high- and low-risk regions in Hong Kong in 2003 showed that subclinical infection was rare [77, 78]. Pseudotyped virus expressing the SARS spike protein can also be used for detecting neutralizing antibody to SARS-CoV without the need for handling the live SARS-CoV, which has to be carried out in BSL-3 containment [79].

Indirect immunofluorescence tests use virus-infected cells fixed to inactivate virus infectivity as the solid-phase antigen to bind antibody in serum specimens. Anti-SARS-CoV antibodies present in the serum would bind to the viral antigens expressed on the infected VeroE6 cells; these primary antibodies could then be detected by adding secondary antihuman antibodies labeled with FITC [80]. IgM subclass antibodies to SARS-CoV, though declining in titer, can be detectable for more than 6 months after onset of disease. There is less of a decline in titers of IgG antibodies and neutralizing antibodies to the virus. Such assays can also be adapted to detect low- and high-avidity IgG antibodies both for discriminating early from late antibody responses and for distinguishing anamnestic cross-reactive antibody responses from primary specific responses. This may be useful in some clinical situations [81].

ELISA assays have been also developed for detecting antibodies to coronaviruses [82, 83] and have been used to investigate the epidemiology of coronavirus infections [84]. The elicited antibodies detected by ELISA predominantly react with the viral surface proteins rather than the ribonucleoprotein [85], and infections of 229E- and OC43-like viruses can be distinguished in ELISA assays [86]. ELISA has been used to study the seroprevalence of HKU1, showing an increase from 0 % in age <10 years old to a plateau of 21.6 % in the age group of 31–40 years old [87]. Recombinant protein-based ELISA assays for detecting antibody to SARS-CoV have been developed [88, 89]. The duration of antibodies by ELISA to the SARS-CoV spike protein was long-lived and paralleled neutralizing antibody responses, while those to the nucleoprotein was less long-lived [90].

A protein-based line immunoassay which individually detects antibody to HCoV 229E, NL63, OC43, HKU-1, and SARS-CoV nucleocapsid protein has been evaluated [91]. Paired sera from confirmed OC43 or 229E infections and 49 convalescent sera from SARS patients showed that there was considerable cross-reactivity between the two betacoronaviruses OC43 and HKU1 and between the two alphacoronaviruses 229E and NL63. However, 229E- or OC43-infected patients did not develop cross-reactive antibody to SARS-CoV. It is important to keep in mind such cross-reactions when evaluating the results of serological assays. It is also relevant that immunofluorescent assays appear to manifest the greatest cross-reactivity. Neutralization tests are likely to be the most specific although this has not been systematically studied with the recently discovered coronaviruses.

Historically CF or HI tests were used in epidemiologic studies of coronaviruses [7]. By this method, the CF test detected antibody in low titer and for only a short time after infection. However, if the antigen was highly concentrated, antibody could be detected at a higher titer, and this antibody persisted in the population so that the CF method could be employed in surveys of prevalence [92]. An indirect HI test for 229E virus using tanned sheep erythrocytes has been described which appears to be highly sensitive with no cross-reactions with OC43 virus [93]. CF tests can be satisfactorily performed with OC43 virus using infected suckling mouse brain as antigen [27]. The same mouse brain material can also be used in the HI test for OC43 antibody. In this test, the hemagglutination titer has been higher for rat than for chicken erythrocytes but sufficient with the chicken cells so that HI testing could generally be employed; this is of particular importance in view of the wider availability of chicken erythrocytes and the spontaneous agglutination that often complicates working with rat erythrocytes. Serum to be tested did not require treatment with receptor-destroying enzyme but rather standard heat inactivation at 56 °C. The agglutination took place equally at various temperatures including room temperature [94]. It has also been possible to demonstrate precipitin lines on gel-diffusion tests with coronavirus antigens concentrated 10- to 50-fold. Two or three precipitin lines were observed by Bradburne [92] in tests with hyperimmune animal or human serum, but others have identified only one such line [94].

The neutralization, CF, HI, gel-diffusion, and immunofluorescent techniques have been used in the antigenic analyses of the older strains of 229E and OC43 [36, 95]. Cross-reactive antibody responses among hCoVs have been reported. When sera from individuals experimentally infected with 229E- and OC43-like coronaviruses, including organ culture viruses, were tested, they are found to cross-react within each group but not across groups. Thus, it is possible that the ELISA test with 229E and OC43 antigens may be able to detect infection with most, if not all, human 229E and OC43 coronaviruses [86]. However, persons with antibody to 229E and OC43 (most of the adult population) did not have cross-reacting antibody to SARS-CoV even in immunofluorescent tests, allowing these serological tests to be reliably used for diagnosis and seroepidemiology of SARS. Patients who had OC43 infections without prior exposure to SARS-CoV had increases of antibodies specific for the infecting OC43 virus but not to SARS-CoV. However, antibody responses to SARS-CoV antibody were sometimes associated with an increase in preexisting IgG antibody titers for human coronaviruses OC43, 229E, and NL63 by immunofluorescent assays. This probably reflects anamnestic cross-reactive antibody responses to coronaviruses to which the patient has had prior exposure (i.e., similar to the concept of "original antigenic sin") [96, 97]. The cross-reactivity is less when purifying nucleocapsid proteins are used in ELISA or Western blot assays.

4 Biological Characteristics

4.1 Classification

All human coronaviruses (hCoVs) are enveloped, positive-strand RNA viruses and belong to the subfamily *Coronavirinae* in the family *Coronaviridae*, order *Nidovirales*. The subfamily *Coronavirinae* is further divided into three genera, *Alpha-*, *Beta-*, and *Gammacoronaviruses*, corresponding to the previous informal classification groups I, II, and III, respectively; there is also a recently recognized *Deltacoronavirus* genus [98, 99] (Fig. 10.1). The genus *Betacoronavirus* consists of four separate lineages, designated from A to D, which correspond to the former subgroup 2A to D. As viruses sharing more than 90 % amino acid (aa) sequence identity in the conserved replicase pp1ab domain are treated as the same species, *OC43* and *human enteric coronavirus* (*HECV*) are thus now regarded as one species (*Betacoronavirus 1*).

At present, only members of alpha- and betacoronaviruses are known to infect humans. They differ from each other in nsp1 protein, which is distinct in size and sequence (gammacoronaviruses have no nsp1). In addition, alphacoronaviruses commonly share an accessory gene designated as ORF3. The type species for alpha- and betacoronaviruses are *Alphacoronavirus 1* (equivalent to *porcine transmissible gastroenteritis coronavirus*, *TGEV*, in older literature) and *murine coronavirus* (equivalent to *mouse hepatitis virus*, *MHV*), respectively. The viruses that are human pathogens are the alphacoronaviruses *229E* and *NL63*, the betacoronavirus lineage A viruses betacoronavirus 1 (which comprises *OC43* and human enteric coronavirus which are now regarded as variants of the same species) and HKU1, and the betacoronavirus

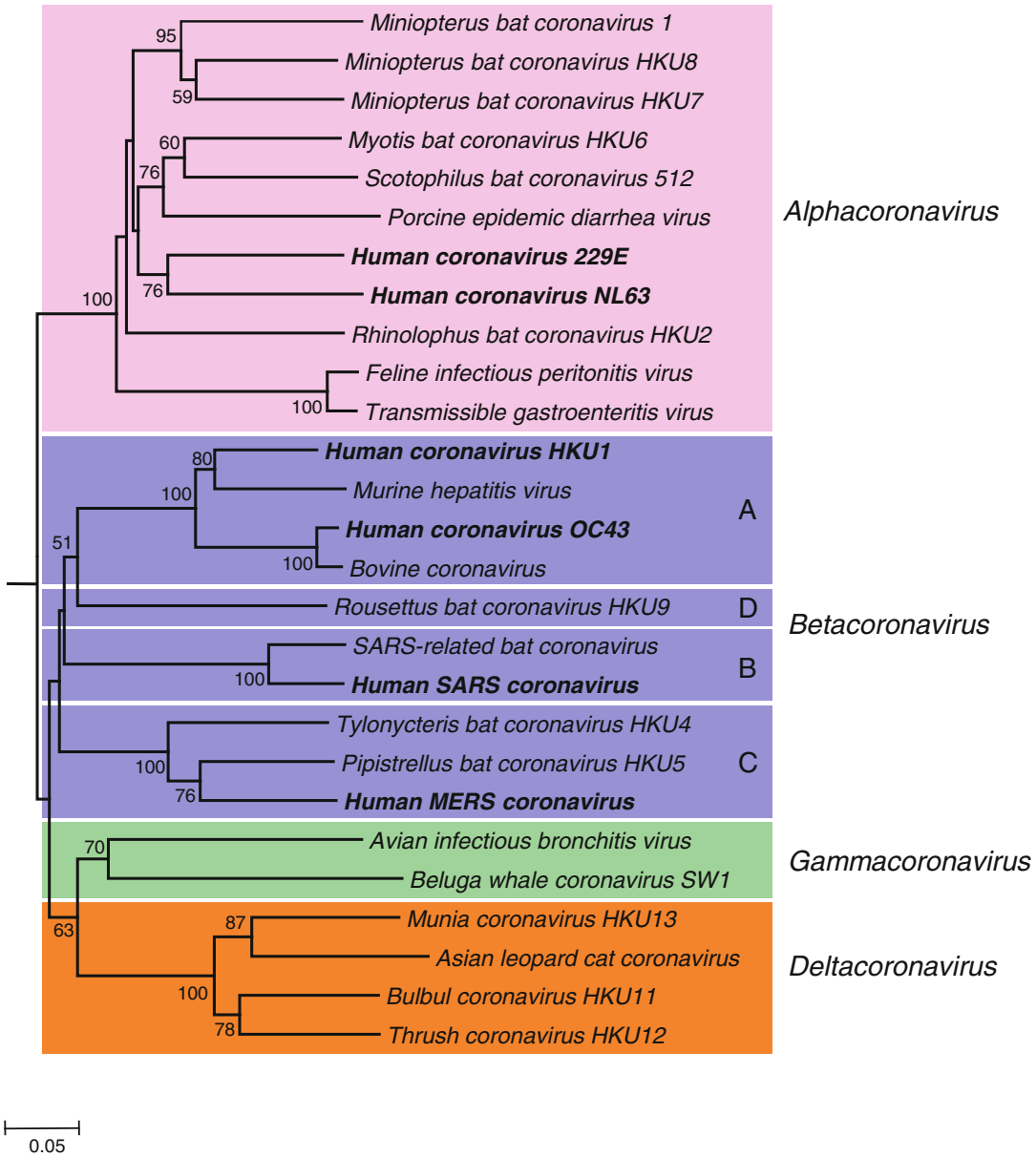

Fig. 10.1 Phylogenetic relationships of *Coronavirinae*. A rooted phylogenetic tree generated from nucleotide alignments of RdRp gene by neighbor-joining method with equine torovirus Berne as an out-group. Four genera of coronaviruses are indicated by different colors, *Alpha-* (*pink*), *Beta-* (*blue*), *Gamma-* (*green*), and *Deltacoronavirus* (*orange*). The distinct betacoronavirus lineages A through D were denoted. Human coronaviruses are denoted in *bold* (Figure is based on Refs. [1–3] and includes novel human betacoronavirus coronavirus 2c)

lineage B virus SARS-*related coronavirus* [98]. Recently, MERS (Middle East respiratory syndrome) coronavirus, a novel human coronavirus in lineage C, has been isolated. Phylogenetically, it is closely related to bat viruses previously detected in China and in Europe [53].

4.2 Genome and Structure

The name coronavirus comes from its appearance under the electron microscope with large 20 nm petal-shaped surface projections ("spikes") on a 120–160 nm spherical or

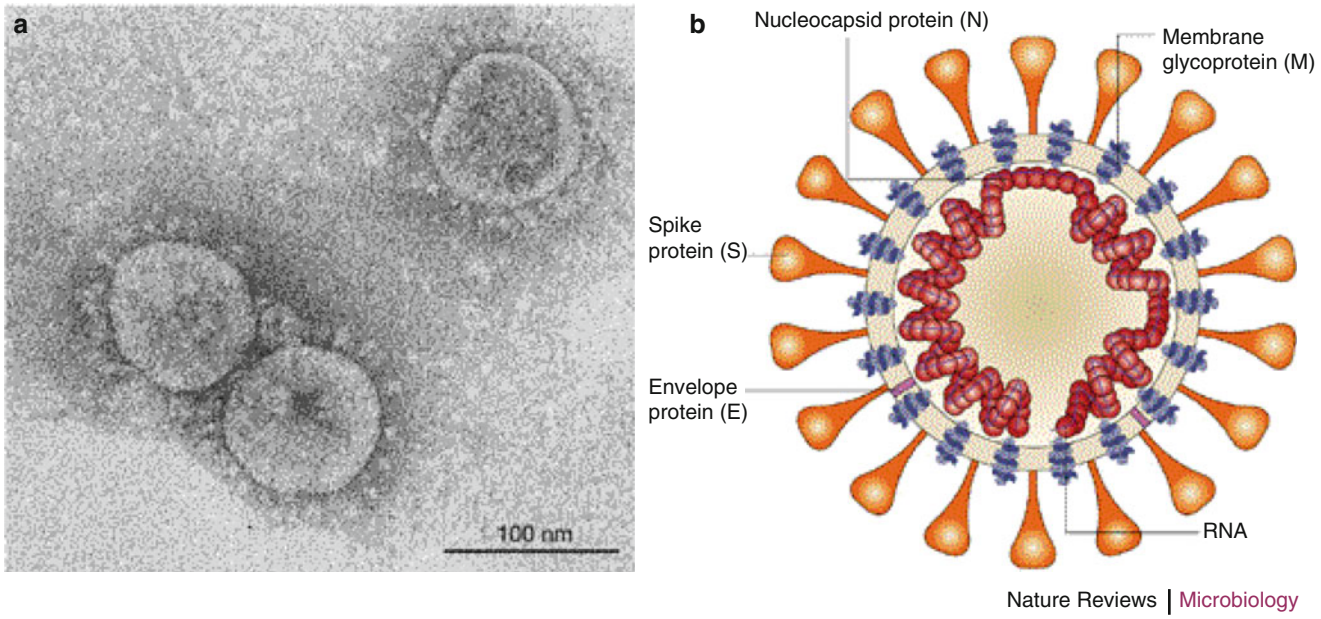

Fig. 10.2 Morphology of coronaviruses. (**a**) Electron micrograph of *severe acute respiratory syndrome-related coronavirus* (*SARS-related CoV*) cultivated in Vero cells. Large, club-shaped protrusions (*spike protein*) form a crown-like corona that gives the virus its name (Image courtesy of Dr L. Kolesnikova, Institute of Virology, Marburg, Germany). (**b**) Schematic representation of the virus. A lipid bilayer comprising the spike (*S*), membrane (*M*), and envelope (*E*) protein cloaks the helical nucleocapsid, which consists of the nucleocapsid (*N*) protein that is associated with the viral linear positive-stranded RNA. The lipid envelope is derived from intracellular membranes of the host cells [100]

pleomorphic body resembling a solar corona [100] (Fig. 10.2). Lineage A betacoronaviruses display an additional surface projection, the 5–7 nm homodimeric hemagglutinin-esterase (HE) glycoprotein. The interior ribonucleoprotein looks like either a long strand with 1–2 nm diameter or a helix condensed into coiled structures with 10–20 nm diameter. The virions are sensitive to heat, lipid solvents, nonionic detergents, formaldehyde, oxidizing agents, and UV irradiation [98].

The genome of coronaviruses consists of a linear positive single-stranded RNA between 26 and 32 kilobases and is the largest RNA virus genome to cause infection in humans [101]. The infectious genome has multiple open reading frames (ORFs), six of which are conserved across the subfamily and are arranged (in 5′–3′order) as ORF1a and 1b (which encode for two huge polyproteins, pp1a and pp1ab) and the ORFs for structural proteins, spike (S), membrane (M), envelope (E), and nucleocapsid (N). The two polyproteins pp1a and pp1ab from ORF1a/b are autoproteolytically cleaved into about 16 nonstructural proteins (nsp) which subsequently form into the replicase. Between the structural proteins S, M, E, and N lies the ORFs coding for the accessory proteins whose function is not essential for virus replication in vitro and the functions of many of them in vivo are still unknown.

As a positive-sense RNA genome, genome of coronaviruses serves as template for both replication and viral protein synthesis. Following entry into the cell by receptor-mediated endocytosis and uncoating of the virus genome, ORF1a/b of the genome is first translated to generate replicase proteins. These replicases use the positive viral genome as the template to generate full-length negative-sense RNAs, which in turn serve as templates for generating additional full-length genomes (i.e., replication). In viral protein synthesis, the 3′ proximal genes of the viral genome are first transcribed to segmented subgenomic negative-sense RNAs by discontinuous minus-strand RNA synthesis. The process is initiated at the 3′ end of the genome and proceeds until they encounter one of the transcriptional regulatory sequences (TRS) that reside upstream (5′) of most ORFs. Through base-pairing interactions, the nascent transcript is transferred to the complementary leader TRS, and transcription continues through the 5′ end of the genome. Therefore, all mRNAs of a coronavirus characteristically contain a common 5′ leader sequence fused to a downstream gene sequence. These subgenomic RNAs then serve as templates for positive-sense mRNA production and subsequent translation into viral proteins [101].

Three structural proteins S, E, and M are found on the viral lipid-membrane envelope; these are acquired as the virus buds into the endoplasmic reticulum, intermediate compartment, and/or Golgi complex of the host cell [98]. The spike protein (S) is a large homotrimeric type I membrane glycoprotein (1,128–1,472 aa). The S protein carries the receptor binding domain and is a class I fusion protein that triggers fusion between viral and host cell membranes

within the endocytic vesicle, thereby releasing the genome into the cytoplasm. The envelope protein (E) is a pentameric integral membrane protein (74–109 aa) that acts as ion channel for preferential transport of different ions into the virion. Although present in low copy number in a virion, E is significantly involved in virus budding, morphogenesis, and intracellular trafficking [102]. The membrane protein (M) is an integral type III triple-spanning membrane protein (218–263 aa). Being the most abundant protein in the viral envelope, it is essential in virus assembly within the infected cell. It could also interfere with host immune responses by inhibiting type I interferon production [103, 104]. The nucleocapsid protein (N) is a phosphoprotein that encapsidates the RNA viral genome to form ribonucleoprotein complex and regulates viral replication and translation (349–470 aa). It has RNA chaperone activity and also functions as an interferon antagonist [105]. In addition to these, the group A betacoronaviruses (includes OC43/HECV and HKU1) express an extra accessory homodimeric type I envelope glycoprotein, hemagglutinin esterase (HE), on its surface. It is related to subunit 1 of the influenza C virus hemagglutinin-esterase fusion protein (HEF) and mediates reversible virion attachment to *O*-acetylated sialic acids [106].

5 Descriptive Epidemiology

5.1 Incidence and Prevalence of Respiratory Coronaviruses

Coronaviruses are of major importance in common respiratory infections of all age groups. Before the identification of the newer endemic human coronaviruses, NL63 and HKU-1, it was recognized from the earlier studies that other unidentified human coronaviruses existed. It is still unknown if additional unidentified viruses exist, so that it is still useful to discuss burden in terms of both the individual virus and coronaviruses in general. Incidence of infection with 229E and OC43 exhibited a marked cyclical pattern, and reported rates can be expected to vary based on the number of seasons of high viral activity included in a particular study, again indicating the need for long-term evaluation. Table 10.2 presents a summary of results obtained in four studies which established the patterns of infection and illness [25, 28, 30, 31].

Another approach toward developing a minimal estimate of the total role of coronaviruses in respiratory illnesses comes from a study involving exhaustive laboratory examination, including organ culture, of specimens from 38 common colds. Coronaviruses were isolated from 18 % of the specimens, but an additional 13 %, which were negative in the laboratory, produced colds when given to volunteers [108]. Based on these results, which came from a limited age group, it has been estimated that coronaviruses could be responsible for at least 14 % of all respiratory illnesses in a general population [109].

Table 10.2 Reported frequency of infection or respiratory illness with 229E and OC43 in four locations

	Mean incidence of infection with	
Study	229E	OC43
Chicago medical students [25]	15/100/year	—
Tecumseh, MI [31, 32]	7.7/100/year	17.1/100/year
	Proportion of illnesses associated with	
	229E	OC43
Charlottesville, VA, employees [10]	1.7 % of illnesses	2.4 % of illnesses
Atlanta, GA, children [28, 108]	4.3 % of illnesses	3.3 % of illnesses

5.1.1 Incidence and Prevalence of 229E Virus

The frequency of 229E illness and infection was determined in several long-term investigations. The activity of 229E was found to be high in three out of 6 years of a study among Chicago medical students. The mean annual incidence of infection during the total period was 15 % where the criterion for identification was a reproducible twofold seroconversion determined by CF. There was marked year-to-year variation in infection frequency, ranging from a high of 35 % of those tested in 1966–1967 to a low of 1 % in 1964–1965. However, nearly 97 % of the infections occurred during the months from January to May, often at a time when isolation of rhinoviruses was at a low and seroconversions for 229E were only rarely accompanied by a rise in titer against another respiratory agent [25].

The serological study of 229E activity in the community of Tecumseh, Michigan, initially covered 2 years, which included one period of high prevalence. As with the study in Chicago, routine blood specimens were collected so that infection rates could be determined; however, the study group was composed of individuals of all ages living in their homes. Over the 2 years, infections were detected in 7.7 % of individuals tested by CF, as shown in the curve in Fig. 10.3. However, this appeared to be an underestimate of the actual activity of the virus. Serum specimens had been collected on a regular basis, 6 months apart; rises in titer by CF occurred most frequently in those pairs in which the second specimen was collected in April 1967, clearly indicating the peak period of viral dissemination. Both CF and the more sensitive N test results were combined to give an overall infection rate for the population studied; this rate, 34 %, was remarkably similar to the 35 % observed in Chicago at the same time. Because of the limited period of viral activity, it was possible to compare illness rates of those infected with persons not infected matched by age and sex; it was estimated that 45 % of the infections had

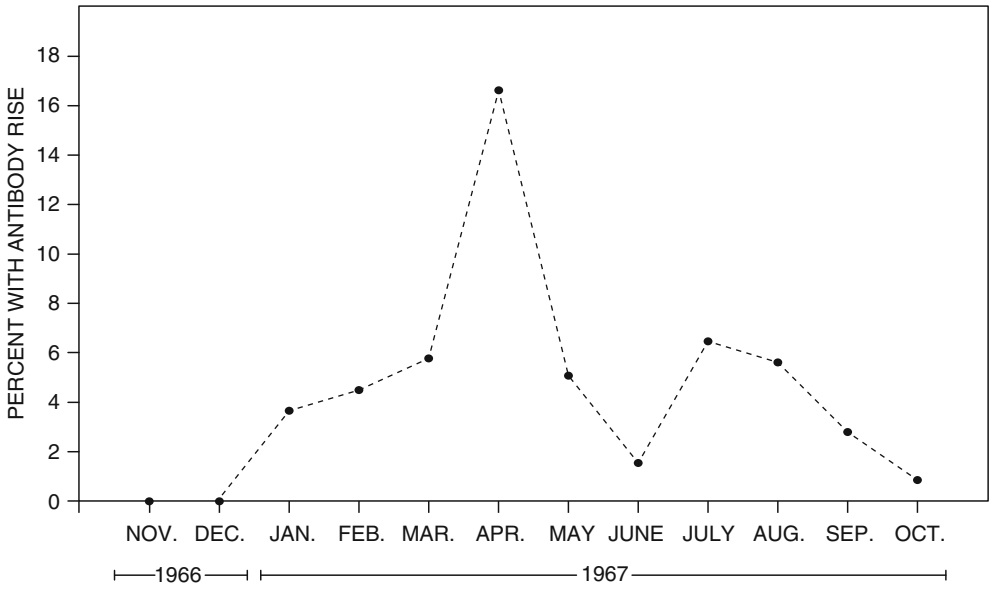

Fig. 10.3 Serological incidence by CF of infection with 229E virus in Tecumseh, Michigan, 1966–1967

produced clinical disease. Thus, the rate of 229E-associated illnesses during the outbreak was 15 per 100 persons studied. Activity in all age groups was apparent, including those under 25 years of age [31].

In other investigations of 229E activity, attention has been directed mainly toward study of associated illnesses; in such studies, sera have been collected before and after the illness rather than continually on a routine basis as done to determine infection rates. Employees at State Farm Insurance Company, in Charlottesville, Virginia, were studied during an 8-year period for rises in titer for both 229E and OC43. By CF, 229E infection could be related to 3 % of the colds that occurred in the winter–spring and to 0.4 % of colds that occurred in the summer–fall. There was some year-to-year variation in activity, but differences in the number of specimens tested from various years did not permit complete identification of cyclical patterns [30]. Employees of the NIH with respiratory illness were studied by both isolation and serology for 229E infection over a 6-year period. Again, attention was specifically directed toward certain segments of the 6 years, and no specimens were tested during other segments. Of particular interest once more is the segment from December 1966 to April 1967. Isolation of rhinoviruses and myxoviruses was uncommon at this time, but respiratory illness continued to occur. During this period, 24 % of those persons with colds studied had rises in titer for 229E. As part of the same investigation, paired blood specimens collected from infants and children admitted to the hospital with acute lower respiratory disease during the 1967 period of 229E activity were tested for rise in antibody against the virus, but none was found [26, 27]. Healthy children institutionalized in Atlanta, Georgia, were studied from 1960 to 1968; antibody response to 229E was determined by the indirect hemagglutination test. The investigation involved collection of serum specimens related to illness and also routine collection of sera from some non-ill individuals. Frequency of infection showed marked variation from year to year. Overall, 4 % of colds could be associated with 229E infection, with greatest association in autumn, winter, and spring [28]. A more recent study took advantage of specimens sent from medical facilities in Edinburgh, Scotland, for laboratory identification of infection to study the incidence of a number of respiratory viruses identified by PCR. Overall, 229E was found in only 0.3 % of those sampled, lowest of any of the four coronaviruses studied. This may reflect that the source of the specimens was from illnesses seen in hospitals and primary care facilities, and coronaviruses are mainly involved in milder illnesses [22].

Surveys of prevalence of 229E antibody have been carried out to document past history of infection, often as parts of longitudinal studies. A general finding is that antibody is present in a significant portion of adults who, despite possessing this antibody, can subsequently experience reinfection and illness. Reports of antibody prevalence in adults in the United States have varied from 19 to 41 %, depending on the type of test used to determine antibody and the time of collection of serum [27, 30, 110]. Children under 10 years of age exhibited lower mean antibody titers than older children or adults [27, 31]. Individual sera from normal healthy adults collected serially in Britain from 1965 to 1970 were tested by Bradburne and Somerset. It is of interest that the proportion of sera positive by CF increased from approximately 17 % in specimens collected in October–December 1966 to 62 % in those collected in July–September 1967. This suggests that the spring 1967 outbreak that occurred in several parts of the United States may have taken place in Britain as well.

5.1.2 Incidence and Prevalence of OC43 Virus

Populations employed to study infection and illness caused by OC43 virus were generally been the same ones employed to study the occurrence of 229E virus. Kaye et al. [107] used the group of institutionalized children in Atlanta, Georgia, to identify infection by means of their HI test. Infections with the agent were detected in all years of the study, but with definite cyclical variation. Seasons most involved were the winter and spring. Overall, 3 % of the illnesses recorded in the 7-year period could be associated with OC43 infection, with a high of 7 % in 1960–1961. Interestingly, testing of the sera collected routinely from non-ill individuals indicated that an additional equal number of OC43 infections were occurring without the production of symptoms [107]. The Charlottesville study of adult employees was of both OC43 and 229E infections. Here, too, the emphasis was on illness, and in all years studied OC43 was associated with 5 % of colds in the winter–spring and with no illnesses in the summer–fall. Again, there was cyclical variation from year to year in the number of rises in titer detected [30].

The original isolations of OC38 and OC43 were made in December and January 1965–1966 as part of the study carried out among NIH employees with colds. Testing of sera collected from these employees indicated that during this period, up to 29 % of the colds studied were accompanied by rise in titer for OC43. In the children hospitalized with lower respiratory disease, up to 10 % of illnesses during this period were associated with such a titer rise. However, it was impossible to show that the relationship to disease was truly etiologic. This finding was in contrast to that seen with 229E, in which no rises in titer were detected in such cases [27, 111]. In the Tecumseh study, occurrence of OC43 infection was determined in the community population over a 4-year period: CF and HI tests were used on all specimens, and N tests were used as an aid in evaluating these results in selected specimens. During the total period, OC43-related infection was detected in 17.1 % of the 910 persons studied for 1 year. Most of the infections took place in the winter–spring months of 1965–1966, 1967–1968, and 1968–1969. The only winter–spring period without such activity was in 1966–1967, when the 229E outbreak had taken place. The 1968–1969 outbreak of OC43 infection was nearly as widespread as the prior 229E outbreak, with 25.6 % of the population studied showing evidence of infection. Of special note was the fact that children under 5 years of age had the highest infection rates [32, 112]. More recently, in the Edinburgh study involving medical care, OC43 was the most commonly identified coronavirus but only was identified by PCR in 0.85 % of specimens. This again may be a reflection of the source of the specimens. Surveys of antibody prevalence have been conducted in several settings using OC43 antigens. McIntosh et al. [27] found that children began to acquire antibody to this virus in the first year of life. By the third year of life, more than 50 % had antibody present. Among adults, 69 % could be demonstrated to have antibody; this indicates, in view of the high incidence of infection with the agents in all age groups, the frequency with which such infections must represent reinfection. The high prevalence of antibody has been confirmed in other studies [28, 30, 112]. In Britain, Bradburne and Somerset followed prevalence of antibody for OC43 over time, as they also had done with 229E [36]. Each year, the greatest prevalence of antibody was found in the winter–spring period. The single highest point in antibody prevalence was in January–March 1969, at the same time the OC43 outbreak was occurring in some parts of the United States [30, 38].

5.1.3 Geographic Distribution

Occurrence of coronavirus infection has now been documented, by isolation, PCR, or serology, throughout the world. In earlier studies, in the United States, in addition to the studies listed in the first part of Table 10.1, a 229E-like virus was isolated in California, and OC43 and 229E have been demonstrated to be present in many regions of the country [21, 113]. Extensive studies have been carried out by the Common Cold Research Unit, which have demonstrated the presence of the agents in Britain. The activity of 229E virus has been documented in Brazil in an early study of children and adults with and without respiratory illness. Significant rises in antibody titer accompanied respiratory infection in the nonhospitalized children. Prevalence of antibody was determined by CF, and like the situation in some studies in the north temperate zone, children had little antibody, whereas 26 % of adults were antibody positive [37]. Later investigations have confirmed the worldwide distribution of these agents [112, 114]. In particular, the widespread use of PCR has now allowed easy documentation of the activity of all the coronaviruses. In fact, one of the four viruses now recognized, HKU1, was first identified in the subtropical city of Hong Kong [20]. These findings suggest that coronaviruses are worldwide in distribution and cause similar types of illness in different localities [115], as has been noted with many other respiratory viruses [116, 117].

The newly recognized human MERS coronavirus infections have only occurred in Middle Eastern countries (Jordan, Qatar, Saudi Arabia, the United Arab Emirates) with limited secondary transmission being reported in France, Italy, Tunisia, and the United Kingdom. The infection is probably of zoonotic origin although other scenarios cannot be completely excluded at present. Persons with immunosuppressive conditions and other underlying diseases appear to be particularly susceptible to infection. While there have been significant clusters of infection in some health-care facilities, MERS coronavirus appears to have limited capacity for human-to-human spread at present [118–120].

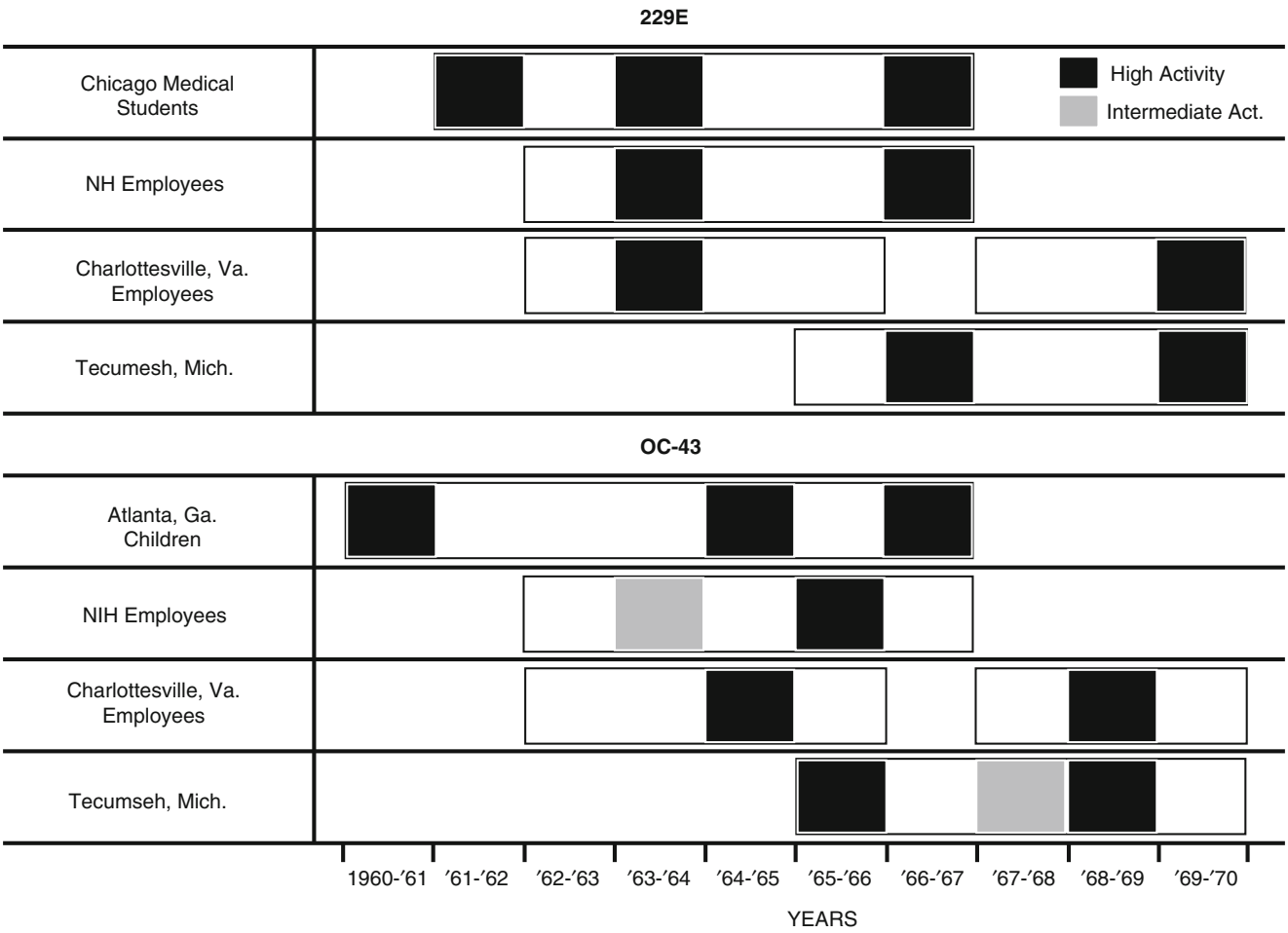

Fig. 10.4 Cyclic behavior of 229E and OC43 viruses observed in five longitudinal studies

5.1.4 Temporal Distribution

Because most illnesses caused by coronaviruses are similar to those caused by other respiratory viruses, it is impossible to identify epidemic behavior of the viruses clinically. In early epidemiologic studies, there was, however, evidence of variation in the frequency of infection on both a seasonal and a cyclical basis. In these investigations, isolation and rises in antibody titer for all types of coronaviruses were rare events outside the period from December through May. This is the portion of the year in which isolation rates for rhinoviruses often reach their lowest level. An exception to this rule was a study in which frequent rises in titer were detected by ELISA in summer as well [84]. More recent studies identifying the viruses by PCR have largely confirmed the winter seasonality of the viruses in the north temperate zone; one study observed that the timing of coronavirus identifications was similar to that of influenza in winter–early spring [22]. As expected, the seasonality appears to differ in places like Hong Kong, based on accumulating data [121].

In the earlier multiyear, population-based studies, a cyclical pattern could also be seen in the occurrence of individual virus types. In Fig. 10.4, data are summarized from five longitudinal studies of coronavirus activity carried out in different parts of the United States. In all studies, some sporadic activity did occur in nearly all years studied, but rises in antibody titers were concentrated in certain years in which they far exceeded the means for the entire studies. Those periods are indicated as solid black boxes in the figure. The times during which specimens were collected in each investigation are indicated in the figure by the white boxes. Activity of 229E was detected in all four studies at the same time, even though two were in the Midwest and two in the eastern United States. It seems possible, on the basis of these data, to postulate a 2- to 3-year cycle for this agent. The greatest number of infections in Chicago was seen in 1967, after the absence of the agent for 3 years; that pattern suggests a role of herd immunity in determining the time of reappearance of the agent.

With OC43, the situation is quite different. As with 229E, in no investigation did 2 years with high rates of infection or illness follow one another. A possible exception was in the Tecumseh study. However, the agent that caused the rises in titer in 1967–1968 did not appear as closely related

serologically to OC43 as the agent involved in the other two outbreaks. This observation indicates a problem in identifying cycling of OC43 using the serological test employed.

More recent studies using PCR have mainly not been population based or have focused on a limited number of the four known respiratory coronaviruses, so that comparable observations across all of the viruses are not possible. However, it is possible to conclude that, in any winter season, all four viruses may be identified in a single geographic area [122, 123]. There are likely to be increases of one or more in specific years, but it is unlikely that any coronavirus disappears completely; this is somewhat similar to our growing realization, with better surveillance, of the long-term occurrence of influenza types and subtypes [22].

5.1.5 Age

There is little available evidence that the respiratory coronaviruses behave differently than other respiratory viruses: infections are most common in children and decrease with increasing age. However, it is unclear whether the drop-off is modest or more extreme, as is the case with respiratory syncytial virus [124]. In the Tecumseh study, a total population group was followed. During the 1968–1969 OC43 outbreak, infection rates were relatively uniform for all age groups, varying from a high of 29.2 per 100 person-years in the 0–4 age group to 22.2 in those over 40 years of age [32]. The reversal of the pattern of age-specific infection rates customarily associated with the respiratory viruses becomes complete with 229E. Infection with this virus has been more difficult to demonstrate in small children than in adults. In Tecumseh, during the 1966–1967 outbreak, highest age-specific infection rates by CF were found among those 15–29 years of age, following a steady increase in infection frequency from the 0- to 4-year-olds. However, when neutralization tests were used to detect infection, the 15- to 19-year-olds still had high infection rates, but the serial increase to that point among younger age groups was much less steep [31]. This suggests that the apparent sparing of small children with 229E may be an artifact resulting from the relative insensitivity of the young to the serological procedures commonly employed. It would be surprising if two different coronavirus serotypes behaved so differently [125].

5.1.6 Other Factors

There is little evidence for or against a sex differential in infections with the coronaviruses. In Tecumseh, adult females experienced higher infection rates with OC43 than adult males, which is in conformity with the usual patterns of all respiratory illnesses [126]. Similarly, female volunteers appeared to be more susceptible to infection with 229E-like strains than males in artificial challenge studies [127]. In the study by Candeias et al. of antibody prevalence, the results were examined by sex, but no significant differences could be observed [43]. There are no data available on occupational or racial susceptibility to infection or on the role of socioeconomic status in influencing rates. Occurrence of infection in closed or special populations, such as military recruits or residents of children's institutions, has been reported [5, 26, 34]. The role of the school-age child in dissemination of coronavirus has not yet been clearly defined, but it would be surprising if these infections differed in their transmission pattern so markedly from that documented with the other agents. Because of the high frequency of infection in older children and adults, other sites of dissemination may also be of significance. It has been possible to show that the family unit is of importance in transmission, since clustering of 229E and OC43 infections in families was observed in the Tecumseh and Seattle studies [31, 128].

Although nutritional and genetic factors have not been associated with susceptibility to coronavirus infections, there are clear indications that the viruses are associated with exacerbations of chronic obstructive respiratory disease. Such a finding is hardly surprising in view of the high infection rates that have been observed in unselected older adults [129]. It has not yet been demonstrated whether this represents true increased susceptibility to infection or simply a more severe form of expression of the infection when it occurs in an already compromised host. In addition to the situation in older individuals, there is evidence that both OC43 and 229E may trigger acute attacks of wheezing in young asthmatics; in fact, in one study, coronaviruses were the most common agent involved in episodes of wheezy bronchitis [21, 33, 117, 130]. Recent studies using the PCR technique also associate the viruses with illnesses including pneumonia in immunocompromised patients [63, 115, 131]. One study identified all the viruses over the course of a year but the newer viruses, NL63 and HKU1, most commonly. Again, this may be a reflection of these viruses being most common at that point in time; it should be noted that shortly after the first identification of NL63 in one city in the Netherlands, it was again identified in another, which may indicate increased circulation at the time [18, 132].

5.2 Epidemiology of Severe Acute Respiratory Syndrome

In late 2002, the SARS coronavirus emerged in humans in southern China as a zoonotic pathogen [133]. Infection spread in Guangdong province for approximately 3 months before an infected individual visited Hong Kong in mid-February 2003. That case infected a number of tourists, sparking a global outbreak, and also went on to initiate a large outbreak in Hong Kong [73]. The subsequent global outbreak lasted around 4 months and had a substantial impact on global travel, trade, and economy [134]. Sustained epidemics have not occurred since 2003, although there have been a few sporadic events or minor outbreaks in Singapore,

Taiwan, and mainland China in 2003–2004, with most of them linked to laboratory releases and only four cases from mainland China perhaps of animal origin [135].

SARS patients initially developed influenza-like prodromal nonspecific symptoms including fever in the first week and usually presented cough, dyspnea, and diarrhea within 14 days. Severe illness developed rapidly progressing to respiratory distress and oxygen desaturation requiring intensive care and potentially resulting in death [136]. The World Health Organization (WHO) defined a suspected SARS case as a person with high fever (>38 °C) and cough/breathing difficulty, who either had close contact with a suspect or probable case of SARS or resided in or traveled to an area with recent local transmission of SARS in the 10 days prior to onset of symptoms. A suspected case became a probable case when (1) the patient's chest X-ray (CXR) presented infiltrates consistent with pneumonia or respiratory distress syndrome (RDS), (2) he/she was positive for SARS-CoV by laboratory assays, or (3) his/her autopsy findings were consistent with the pathology of RDS without an identifiable cause [137].

5.2.1 SARS Epidemiology in Time, Place, and Person

In total, 8,096 "probable" SARS cases were reported to the World Health Organization by August 2003 [138]. The most affected areas were Hong Kong, with 1,755 "probable" cases among a population of 6.8 million, and mainland China, with 5,327 "probable" cases among a population of 1.3 billion. Taiwan, Canada, and Singapore also experienced notable epidemics, with 346, 251, and 238 probable cases, respectively, while altogether cases were reported in more than 25 different countries and administrative regions. Reported cases of SARS globally and in Hong Kong by time of symptom onset are shown in Fig. 10.5.

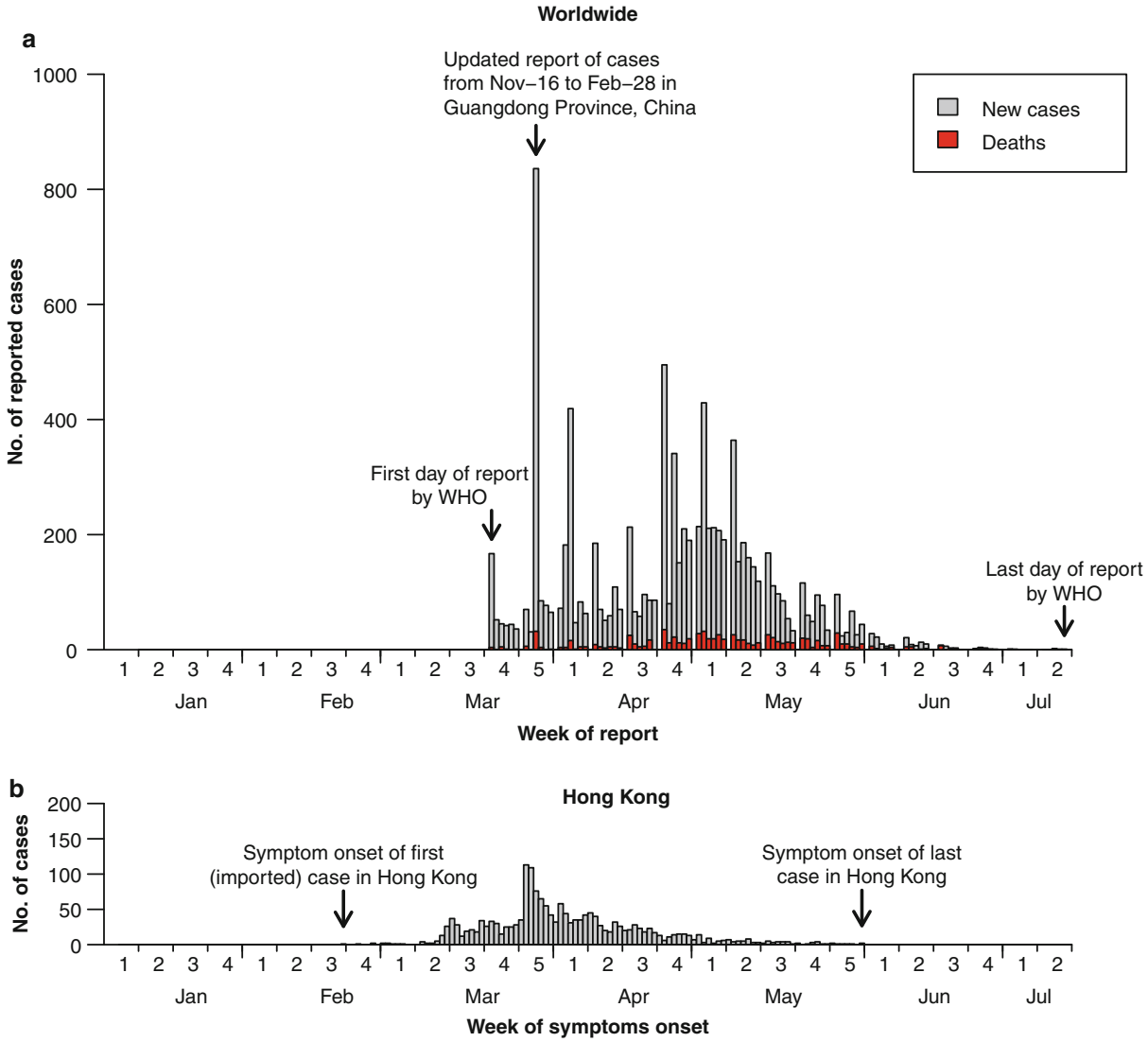

Fig. 10.5 Probable cases of SARS by week of onset (Source: [138]). (**a**) Cases worldwide, (**b**) Cases in Hong Kong

A common feature of SARS outbreaks in different regions was the central role of hospitals and transmission among patients and health-care workers [139–143]. A peak in infectiousness was thought to occur around 10 days after illness onset [144], by which time cases would have been hospitalized, and certain medical procedures were particularly prone to generating transmission [145]. Hospital transmission played a prominent role in the initial epidemic in Hong Kong, with more than 250 cases attributed to an outbreak at the Prince of Wales Hospital in early March 2003 [146, 147]. The Canadian outbreak began when a case returning from Hong Kong was admitted to hospital, and 72 % of cases were subsequently attributed to nosocomial transmission [143, 148].

Old age and the presence of comorbidities including diabetes mellitus, hypertension, coronary artery disease, and chronic obstructive pulmonary disease increased the risk of death or adverse outcomes, such as admission to an ICU requiring mechanical ventilation and development of ARDS [149, 150]. Sex (male), high lactate dehydrogenase concentration at presentation, and higher SARS-CoV viral load have been found contributing to higher case fatality rate as well [151]. Genetic factors may contribute to host susceptibility to SARS infection [78, 152, 153]. To date, there have been no studies on the relationship of race, socioeconomic status, occupation, or nutrition to susceptibility to SARS infection.

5.2.2 SARS Transmission Dynamics

Concerted efforts were made during the SARS epidemic to determine the transmission dynamics and thereby support control [154, 155]. Contact tracing exercises provided information to estimate the incubation period at around 5 days, with around 95 % of infections leading to illness onset within 10–14 days [156–158]. Early in the epidemic, delays between illness onset and admission to hospital were typically 5–7 days, and a measure of the success of public health control measures was the reduction in onset-admission intervals to just 1–2 days by the end of the epidemic [150, 157]. On average, patients remained in hospital for around 3–4 weeks [157].

The basic reproductive number, R_0, an estimate of the average number of secondary cases resulting from one infected case in a completely susceptible population, was estimated to be in the range 2–3 [154, 155, 159]. The average time between successive cases was around 8.4 days [155]. Due to these features taken together, in the early stages of outbreaks, the number of cases approximately doubled every week.

One issue of early controversy was the case fatality risk. Early in the epidemic, technical errors led to underestimation [160]. For example, on March 25, the World Health Organization reported the case fatality risk to be around 4 %, based on 49 deaths among more than 1,000 cases at that time [161]. This estimate was erroneously low because cases had not yet recovered, and some cases would subsequently succumb to the disease [160]. After the epidemic, the case fatality risk was estimated to be 9.6 % with 744 deaths among the 8,096 probable cases [135]. However, this masks substantial variability between affected regions, from around 7 % in Beijing to around 17 % in Hong Kong [150]. Reasons for variation remain unclear but could partly be attributed to case definitions, partly to case mix including age and underlying heath conditions [150] and partly to case management [162].

Few cases are thought to have been asymptomatic or subclinical. Serological studies were conducted in various groups including health-care workers, close contacts of cases, other patients, and the general community in affected regions, and a review of these studies found that the average seropositivity rate was just 0.1 % among more than 20,000 individuals [163].

5.2.3 Successful Control of the Global SARS Epidemic

Despite a basic reproductive number in the range 2–3, higher than influenza, the global epidemic of SARS was effectively controlled by appropriate nosocomial infection control measures. A range of interventions contributed to containment [164], including the use of engineering controls such as negative-pressure isolation rooms [143]; improved adherence to the use of personal protective equipment such as gowns, gloves, and masks [165]; as well as administrative measures including patient triaging and isolation, visitor restrictions, and establishment of dedicated SARS teams of staff [166]. The importance of strict infection control was illustrated particularly well by the experiences in Taiwan and Toronto, where control of the initial outbreaks was followed by complacency and subsequent second waves [167, 168]. Despite the importance of infection control strategies, there are also examples of individuals with SARS who were hospitalized where infection control practices were lax and yet their infection did not result in outbreaks [169]. Patient factors may also have had a role in the risk of transmission [170].

6 Mechanism and Route of Transmission

The four endemic respiratory coronaviruses are presumably transmitted by the respiratory route. It has been possible to induce infection experimentally in volunteers by inoculating virus into the nose [35, 44]. The virus is most stable at pH 6.0, and low temperature appears to protect it against varied relative humidity [171, 172]. No other route of transmission for coronaviruses seems involved in man, although animal coronaviruses are infectious by the fecal–oral route [57]. There is currently no direct evidence to aid in identifying the main mechanisms of transmission. However, it is possible to compare the epidemiologic behavior of the coronaviruses with that of other respiratory agents, the transmission mechanisms

of which have been more directly studied. Large-scale outbreaks of coronavirus infections have taken place, as in Tecumseh in 1967 [31]. This is much more analogous to the situation seen with influenza than to that with the rhinoviruses [173]. Rhinoviruses are thought to be transmitted by large droplet and may at times spread via fomites [174].

Unlike the situation with the SARS coronaviruses, there is no evidence that any animal reservoir or vector is involved in the maintenance of infection or transmission of the other respiratory human coronaviruses. There has been a report of antibody to avian IBV in the sera of poultry workers but not of controls, but no evidence of any further transmission [40].

The SARS coronavirus was thought to spread through a number of different modes, most commonly via direct close contact. Health-care workers involved in direct patient care duties often had the highest attack rates [175, 176], while contact precautions were effective in preventing transmission [165]. SARS coronavirus was capable of surviving on dried, inert surfaces and was found on some hospital surfaces [177], and indirect contact was implied in the infections of some nonmedical staff [178]. Although there is substantial evidence to support the role of transmission through droplet and direct contact and some evidence to support transmission by indirect contact, there is relatively little evidence for airborne transmission [179]. In one large community outbreak in Hong Kong, a computational fluid dynamics model was used to demonstrate that airborne transmission was consistent with the observed pattern of infections [180], although this hypothesis was not formally compared with other possible explanations. However, lacking other evidence of airborne transmission despite unprotected extended exposures in health-care settings [169, 181, 182], the World Health Organization classified SARS as a disease with "opportunistic airborne" transmission to indicate that the disease naturally spreads by non-airborne routes but under special environmental conditions may spread by the airborne route [183, 184]. Diseases that are spread by opportunistic airborne transmission do not require special airborne infection isolation measures, for example, negative-pressure isolation rooms, but special precautions are recommended for high-risk procedures.

7 Pathogenesis and Immunity

7.1 Etiology and Immunity

Data that demonstrate the etiologic role of coronaviruses in respiratory infections are derived from laboratory and field studies. Coronaviruses interfere with the action of cilia in tracheal organ culture, which suggests that they could have the same effect in vivo. Epidemiologic studies also have demonstrated association of 229E infection with disease. During the 1967 outbreak of 229E infection in Tecumseh, Michigan, illness was significantly more common among those with infection than among matched subjects without infection [31]. Similarly, 229E infection among Chicago medical students was statistically associated with illness when those with rises in titer were used as their own controls [25]. Furthermore, experimental inoculation of volunteers with strains of 229E and OC43 isolated in the laboratory has resulted in clinical illness, fulfilling Koch's postulates modified by Rivers [185] for attributing an etiologic role to a microbe as a cause of disease [35, 36, 47].

SARS-CoV has also fulfilled the Koch–Rivers postulates for association with the disease of SARS. The virus was detected in patients with SARS but not in those without disease, and the virus was detected at the site of the pathology, that is, lung [51]. The virus was isolated in pure culture from the lung biopsy of a patient with SARS, and experimental infection of cynomolgus macaques with this virus produced a comparable disease. A specific immune response to the virus was demonstrated, and the virus was successfully re-isolated from the site of pathology in the infected animal [186]. Fulfilling Koch–Rivers postulates for more recently discovered human coronaviruses has been more challenging because of the lack of suitable animal models that recapitulate the disease in humans [187], and their etiologic association with disease lies largely on epidemiologic grounds.

An important characteristic of the respiratory coronaviruses is their apparent high rate of reinfection, which in volunteers has now been documented to be possible within a year of prior infection [188]. In the Tecumseh study, 81.5 % of those infected with OC43 actually possessed prior N antibody [189]. Possession of circulating OC43 HI antibody among the Atlanta children did not appear to play a role in modifying severity of a subsequent illness [28]. With 229E virus, Hamre and Beem [25] demonstrated that the frequency of rise in titer detected by N was inversely proportional to preinfection levels of N antibody, which would indicate that this antibody exerted some protective effect. However, the importance of this N antibody could not be confirmed when infection was detected by CF. Thus, circulating N antibody as measured at present may bear a relationship to modification of infection, but this association is not a very strong one. Since coronavirus infections involve mainly the surface of the respiratory tract, it is likely that secretory IgA antibody plays a more direct role in protection; this had in fact been demonstrated with a swine coronavirus [190] and subsequently with 229E in humans experimentally infected [191].

7.2 Virus Tropism and Pathogenesis

Interaction between coronavirus spike proteins and host cell receptors determines specifically the host range, tissue

Table 10.3 Human coronaviruses and their major receptors

Human coronavirus	Major receptor	Receptor expression
229E	Aminopeptidase N (APN) [4]	Epithelial cells of kidney, intestine, and respiratory tract; granulocytes; fibroblasts; endothelial cells; cerebral pericytes at blood–brain barrier; synaptic junctions; macrophages; and dendritic cells [4, 193, 194]
Betacoronavirus 1 (OC43/ HECV)	9-*O*-Acetylated sialic acid-containing receptors [195]	Erythrocytes, neural gangliosides, gut mucins [193, 196]
SARS-related CoV	Angiotensin-converting enzyme 2 (ACE2) [197]	Lung alveolar epithelial cells, enterocytes of small intestine, arterial and venous endothelial cells, arterial smooth muscle cells [198]
NL63	ACE2 [199]	Same as above
HKU1	Unknown	Not known
MERS coronavirus	DPP4 (CD26)	Found in many tissues including the respiratory epithelium [200]

From the wide range of receptor expression, one could appreciate that coronaviruses could cause illness in many parts of the body, including the respiratory and gastroenteric systems [101, 201]

tropism, and pathogenesis [192]. Coronaviruses have a wide spectrum of susceptible host cell range, determined by the expression of the relevant receptors (Table 10.3). Related coronaviruses may use the same or similar receptors for entry, and major receptors for hCoVs include aminopeptidase (APN) and ACE2. Most alphacoronaviruses bind to APN with the exception of NL63 and SARS-CoV which binds to ACE2. Some of the betacoronaviruses (including OC43) attach to 4- or 9-O-acetylated sialic acids via the virus S and HE proteins. The HE protein also has enzymatic activity to cleave sialic acid linkages and thus serves to release virus from infected cells after replication is completed. Some human coronaviruses have other binding receptors that allow virus attachment to host cells, but these are not sufficient as functional receptors to mediate viral entry by themselves. These include calcium-dependent (C-type) lectins such as L-SIGN (**l**iver/lymph node-**s**pecific **i**ntercellular adhesion molecule-3-**g**rabbing **n**onintegrin), which may serve as a receptor for 229E and SARS [202, 203]. Recognition of a receptor from the same family in another host species is possible and may allow cross-species transmission. For example, 229E can use either human or feline APN but not porcine APN [201], and human and palm civet ACE2 serve as receptors for epidemic strains of SARS-CoV, but mouse and rat ACE2 do not [204, 205].

The receptor for MERS coronavirus has been identified to be DPP4 (also known as CD26), a protein that is widely conserved across mammalian species and found on the surface of several cell types, including the human upper airways. As with APN and ACE2, DPP4 is an ectopeptidase that cleaves amino acids from biologically active peptides [200].

The human coronavirus OC43 and bovine coronaviruses share close genetic similarity suggesting that they arose from a common ancestor less than 150 years ago [206]. Coronaviruses can undergo dramatic changes in tissue tropism and virulence within the same host. For example, porcine enteric transmissible gastroenteritis virus caused a severe enteric disease in pigs. A spontaneously occurring genetic mutation (deletion) occurring in the spike gene led to a change in virulence and was associated with a switch of virus tropism from the gastrointestinal tract to the respiratory tract [207].

7.3 Pathology and Pathogenesis

There is limited data on the pathology of coronaviruses other than SARS-CoV because these infections are generally mild. Electron micrographic changes from the nasal mucosa of a child with a coronavirus infection showed minimal pathological changes [208].

Although the clinically major pathology of SARS was that seen in the respiratory tract, SARS-CoV caused a disseminated infection with virus being found in the feces, urine, and plasma or serum [209]. Early disease was associated with desquamation of the alveolar epithelium and disseminated alveolar damage with hyaline membrane formation in the alveolar spaces. Viral antigen was demonstrated in alveolar and bronchial epithelial cells and alveolar macrophages [210]. In intestinal biopsy specimens of patients with SARS, virus infection of intestinal epithelium was demonstrated by electron microscopy with minimal cytopathic effect which is consistent with the watery diarrhea seen in these patients [211]. High serum levels of pro-inflammatory chemokines (CXCL10, IL-8) and cytokines (IL-1 and IL-6) suggested a role for immunopathology although it is uncertain whether these inflammatory responses are causally relevant or an epiphenomenon in the pathogenesis [212].

8 Patterns of Host Response

8.1 Disease Characteristics

The incubation period of coronavirus colds is relatively short. In studies involving volunteers, the mean period from inoculation of virus to development of symptoms was from

3.2 to 3.5 days, depending on the strain (range, 2–4 days) [35, 44]. Following exposure, the virus apparently multiplies superficially in the respiratory tract in a manner similar to that in which multiplication occurs in vitro. Nasal airway resistance and temperature of the nasal mucosa increase [213]. Virus excretion usually reaches a detectable level at the time symptoms begin and lasts for 1–4 days. The duration of the illness is from 6 to 7 days on the average but with some lasting up to 18 days. Serological response either to induced or to naturally acquired infection has been quite variable depending on the infecting strain and the serological test employed. For example, among those experimentally infected with OC38 or OC43 virus who had a cold produced, only 46 % had rises in titer by HI and 23 % by CF. Fewer than half of those infected with 229E showed a CF rise. It is not clear how the existence of titer or preinfection antibody affects the magnitude of the response detected by these tests. Rises in N antibody titer are easier to detect and have been found with sensitive techniques in all volunteers experimentally infected [36, 92]. The use of the ELISA test has given added sensitivity in antibody detection; it is not as yet clear if decreased specificity should be a concern.

The respiratory coronaviruses cause cold-like illness that on an individual basis is difficult to distinguish from illness caused by other respiratory viruses. They have also been reported to cause pneumonia and other severe respiratory infections, such as croup and bronchiolitis [115, 123, 214]. Most of the evidence of their involvement in severe disease comes from reports from hospitals of the identification of the viruses by PCR. It is thus impossible to say what proportion of infections, which appear to be common, that result in hospitalization are in fact caused by these viruses. Another problem recently encountered is the frequent identification by PCR of other viruses in those in whom a coronavirus is found [22]. This complicates determining the primary etiology. In induced infections in volunteers, the most prominent findings have been coryza and nasal discharge, with the discharge being more profuse than that customarily seen with rhinovirus colds [35]. Sore throat has been somewhat less common and in children has been associated with pharyngeal injection [215]. Experimental colds caused by B814 virus were about as severe as those caused by 229E; however, natural OC43 infections caused illnesses with considerably more cough and sore throat than did 229E infections [216]. The mean duration of coronavirus colds, at 6.5 days, was shorter than that seen in rhinovirus colds, at 9.5 days [35].

Clinical disease occurred in no more than 45 % of those infected with 229E in Tecumseh during the 1967 outbreak [31]. In Atlanta children, OC43 virus produced illness in about 50 % of those infected [28]. It is likely that with increase in age and concomitant experience with these agents, the ratio of clinically apparent to inapparent infection will decrease. As with other respiratory agents, a continuum of severity of symptoms exists among those in whom infection results in disease, and this may also be related to past experience with the viruses.

The mechanisms that lead to recovery from coronavirus infections have not been well defined. In volunteer studies, in persons infected with 229E-like strains, it is clear that symptomatic reinfection can occur after a period of about 1 year. It is not clear whether this is due to waning immunity or antigenic drift. Immunocompromised patients shed virus for a prolonged period and may sometimes be associated with a fatal outcome.

The novel MERS coronavirus initially presents with fever and myalgia, sometimes with gastrointestinal symptoms, rapidly progressing to severe viral pneumonia leading to respiratory failure, with renal dysfunction observed in some patients. Virus was detectable in the respiratory tract as well as the stool [119].

8.2 Viral Antigens Associated with Immunity

Virus-neutralizing antibodies are those that react with the virus S (and, where present, HE) protein although some antibodies against the virus M protein can also neutralize the virus in the presence of complement. Virus-neutralizing antibodies mainly bind to the N-terminal S1 part of the protein, which is also the part of the protein that manifests the greatest amino acid sequence variation. The removal of glycans from the S protein greatly reduces the binding of neutralizing antibodies. Antibodies to the S protein have also been associated with enhanced pathogenesis in feline infectious peritonitis virus, but such immunopathology has not so far been convincingly demonstrated with human coronaviruses. The nucleocapsid protein contributes to cell-mediated immune protection [209].

Neutralizing antibody responses to SARS-CoV appear in the second week of illness and peak at around 30 days of illness and antibodies remain detectable for many years. The major neutralizing epitope is in the region of the S protein amino acid residues 441–700 [98, 99, 209].

9 Control and Prevention

It is premature at present to think in terms of control of respiratory coronavirus infection by vaccination. Thus, preparation of vaccines using conventional types is impossible. The frequency of reinfection observed is so high that control by vaccination may not be practical, but it is possible that future studies may allow further characterization of truly protective antibodies. Work on vaccines for the animal viruses is in

progress, and these studies may help in understanding issues of protection. Chemoprophylaxis and related measures may be a more practical approach; it has been shown that recombinant α-interferon can prevent infections artificially produced in volunteers [217], and other approaches have been under investigation [25, 217–219]. There remains environmental control of infection; such efforts have rarely been useful for other respiratory agents, but they may be more efficacious if a practical barrier to transmission can be devised [220].

The situation is quite different in terms of the SARS-CoV, because of the severity of the disease produced. Here, the work on vaccines moved forward in the years immediately after 2003. Some of this activity of specific vaccine development continues but at a slower pace. Even when human cases were occurring, it was unclear how such a vaccine, if available, should be used, given the distribution of infection and occurrence of clinical disease. With disappearance of human cases, it has become even more difficult to decide on the appropriate vaccine target populations, except for those who might be exposed in a laboratory setting.

During the SARS episode, as no virus-specific antiviral agent was available, a variety of treatments were used, including the broad-acting antiviral agents ribavirin and interferon as well as corticosteroids. Because of the severity of the disease, many were used in combination, and it was difficult to say retrospectively whether any individually or in combination had a positive effect [162]. Corticosteroid therapy was associated with both short-term (secondary infections, increased viral load) and long-term (osteoporosis, avascular necrosis) adverse effects [221].

10 Unresolved Problems

The major problem in working with the respiratory coronaviruses has been solved with the development of RT-PCR. Previously, because of the difficulty in growing them in cell culture, epidemiologic and clinical studies had to rely on serology. While less limiting in epidemiologic studies, where regular blood collections can be scheduled, it sharply constrained the ability to identify the role of hCoVs in causing severe respiratory infections. Now we recognize the existence of four different coronaviruses, and there may be more that have not yet been identified. Paradoxically, the situation has now been reversed; there are many reports on the involvement of these agents in hospitalized cases, but epidemiologic studies involving all four hCoVs in different populations over time have been relatively scarce. It was previously thought that the viruses cycle in their appearance over a period of years, but recent evidence is lacking, especially pertaining to the newly identified viruses, NL63 and HKU1; however, there are still data suggesting that, in the temperate zones, the viruses are most active in late winter–spring. Ironically, with the PCR technique, it has become common to identify more than one virus from the same individual. This is not limited to the coronaviruses but includes many other respiratory viruses as well. There is a need to determine whether these are true coinfections or whether there may be asymptomatic or prolonged carriage involved. If they are real coinfections, there may be consequences of having more than one agent present during an illness.

The SARS-CoV emerged from a zoonotic reservoir and spread worldwide in a short period of time. We still do not know how and why this happened, and therefore we must be concerned that such an event could occur again. We do not have either vaccines or antivirals for the SARS-CoV; the need is probably greater for antivirals, given the severity of the illness and the question of how a vaccine would be used in the current situation. The recent identification of a novel coronavirus gives increased urgency to this need; it is likely that an anticoronavirus drug would be of use whatever the particular type involved. Overall, the epidemiologic lesson to be learned from SARS is the need for good surveillance at the animal–human interface. The virus was probably transmitting locally from human to human for months before it escaped to the rest of the world. If this had been recognized, there could have been earlier efforts to contain spread, which was effectively accomplished later, but only after much damage had been done.

The emergence of a novel MERS coronavirus with potential to cause severe human disease, though so far originating in the Middle East and manifesting limited human-to-human transmission including transmission within health-care facilities, is reminiscent of the emergence of SARS and is a concern for global public health [120].

The numbers of laboratory-confirmed patients with MERS continues to increase with 837 laboratory confirmed cases and 291 deaths being reported to WHO as of 23 July 2014. The median age of all cases is 52 years, but primary human cases (those who have no exposure to other confirmed cases) are older (median age 58 years) compared to secondary cases (median age 45 years). The majority of confirmed cases have underlying health conditions. All cases so far have a link to the Middle East with primary human infections reported from Jordan, Kuwait, Oman, Qatar, Saudi Arabia and the United Arab Emirates. Cases reported from outside the Middle East have either a history of travel to the Middle East or exposure to a patient who acquired infection from that region. Clusters of human cases and evidence of limited human-to-human transmission have been reported. To date, more than half of the secondary cases have been associated with health care settings, including health care workers. Health care workers appear to have less severe disease in general, although deaths have occasionally been reported [222].

MERS coronavirus (MERS-CoV) has been detected in nasal swabs of apparently healthy dromedary camels, and adult animals from the Middle East and Africa have high rates of sero-positivity [223, 224]. In some cases, infection of dromedary camel herds has preceded disease in humans in close contact with such animals [225]. However, in a large number of human cases, there appears to be no record of a history of contact with camels. The dromedary MERS-CoV virus appears genetically identical to those infecting humans, but abattoir workers with repeated exposure to potentially infected animals have little serological evidence of infection [223]. The geographic distribution of MERS-CoV infection in camels (including North East Africa) is wider than that reported in primary human cases reported so far. It remains to be seen whether this represents under-recognition of human cases in a wider geographic area. MERS-CoV antibodies have been detected in archived dromedary sera collected over two decades ago [226], and this may indicate that MERS is a recently recognised, rather than a newly emerging, disease. However, the possibility of a recent virus mutation that increased the risk of transmission to humans cannot be ruled out.

No specific validated therapeutic options or vaccines are available so far.

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Enteroviruses and Parechoviruses: Echoviruses, Coxsackieviruses, and Others

11

M. Steven Oberste and Susan I. Gerber

1 Introduction

The enteroviruses (genus *Enterovirus*, family *Picornaviridae*) are among the most common viruses infecting humans. In addition to the human enteroviruses (polioviruses, coxsackieviruses, echoviruses, and numbered enteroviruses), the genus *Enterovirus* also contains viruses that infect nonhuman primates and livestock, as well as the human rhinoviruses, a recent addition to the genus [117, 201] (Table 11.1). These viruses share a number of clinical, epidemiologic, and ecological characteristics as well as physical and biochemical properties. Originally, the enteroviruses were classified into the subgroups of polioviruses (PV), coxsackie A viruses (CVA) and coxsackie B viruses (CVB), and echoviruses, based on the empirical observations of their association with certain clinical syndromes or disease, tissue tropism, nature of disease in suckling mice, growth in certain specific cell cultures, and in some cases antigenic similarities [46–48, 203] (Table 11.2). The viruses now known as "parechoviruses" share a number of physical and biological properties with the enteroviruses and were originally classified in the genus *Enterovirus*. However, biological differences were apparent even 50 years ago, and, once molecular diagnostics and genome sequences became available, it was obvious that the parechoviruses were distinct from the enteroviruses and they have been reclassified into a separate genus, *Parechovirus* [45, 102]. Nevertheless, the enteroviruses and parechoviruses are very similar clinically, cause the same spectrum of illnesses, and can be detected in the same clinical specimens, so they are considered together here. The rhinoviruses are described in detail in Chap. 29.

The rate at which new enteroviruses and parechoviruses have been discovered has accelerated following the introduction of molecular typing methods [182, 184] and the ready availability of genome sequences for every known enterovirus type. In addition to the enteroviruses of humans, numerous enteroviruses of other animals are known, including those of nonhuman primates and of livestock, primarily cattle, swine, and sheep. In some cases, human enteroviruses and human parechoviruses have also been detected in nonhuman primates (Table 11.1) [89, 190].

Enteroviruses can cause a wide variety of illnesses in humans, ranging from minor undifferentiated febrile illness to severe and permanent paralysis. For all members of the group, however, subclinical infection is far more common than clinically manifest disease. Although certain enteroviruses have been more frequently responsible for epidemics involving a specific syndrome, the same serotypes may at other times and in other places be associated with infections having different clinical manifestations or producing no symptoms. On the other hand, different viruses may produce the same syndrome. For these reasons, clinical disease is not a satisfactory basis for classification or, as a rule, for diagnosis.

Poliomyelitis is an acute infectious disease that in its serious form affects the CNS. The destruction of motor neurons in the spinal cord results in flaccid paralysis. Because polioviruses can cause the most severe disease of any for which enteroviruses are responsible, these agents have received the most comprehensive study and have served as models in studies of other enteroviruses. The polioviruses are described in detail in Chap. 13.

The disease manifestations caused by the coxsackieviruses, echoviruses, and numbered enteroviruses largely overlap. The

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Table 11.1 Picornavirus genera, species[a], and (sero)types

Genera and species		# Types	Comments
Genus *Enterovirus*		**269**	
	Human enterovirus A[b]	22	Five have been found only in nonhuman primates
	Human enterovirus B[b]	60	Two have been found only in nonhuman primates
	Human enterovirus C[b]	21	
	Human enterovirus D[b]	4	
	Human rhinovirus A[b, c]	77	
	Human rhinovirus B[b, c]	25	
	Human rhinovirus C[b, c]	49	
	Simian enterovirus A	1	
	Bovine enterovirus	2	Possible 3rd type detected in sheep
	Porcine enterovirus B	3	
	Unclassified	5	Detected in nonhuman primates
Genus *Parechovirus*[d]		**20**	
	Human parechovirus[b]	16	Types 1 and 2 formerly classified in genus *Enterovirus*
	Ljungan virus	4	
Genus *Hepatovirus*[b, e]		**1**	
Genus *Cardiovirus*		**12**	
	Encephalomyocarditis virus[b]	1	
	Theilovirus[b]	11	
Genus *Kobuvirus*		**3**	
	Aichi virus[b]	1	
	Bovine kobuvirus	1	Also found in sheep and pigs
	Porcine kobuvirus	1	
	Unclassified	2	One virus detected in rodents and one in dogs
Genus *Teschovirus*		**11**	
Genus *Erbovirus*		**3**	
Genus *Aphthovirus*		**11**	
	Foot-and-mouth disease virus	7	
	Bovine rhinitis A virus	1	
Bovine rhinitis B virus		2	
	Equine rhinitis A virus	1	
Genus *Sapelovirus*		**8**	
	Simian sapelovirus	3	
	Porcine sapelovirus	1	Formerly porcine enterovirus A in genus *Enterovirus*
	Avian sapelovirus	1	
	Unclassified	3	One virus detected in rodents and two in sea lions
Genus *Senecavirus*		**1**	
Genus *Tremovirus*		**1**	
Genus *Avihepatovirus*		**3**	
Proposed genus *Aquamavirus*		**1**	
Proposed genus *Cosavirus*[b]		**4**	
Proposed genus *Megrivirus*		**1**	
Proposed genus *Salivirus*[b]		**1**	
Unclassified picornaviruses		**18**	Viruses detected in bats, cats, rodents, sheep, birds, fish, and reptiles

[a]The classification scheme shown is from the Picornavirus Study Group of the International Committee on the Taxonomy of Viruses [117, 118]. The types that comprise the human enterovirus species are listed in Tables 11.3, 11.4, 11.5, and 11.6
[b]At least one virus in the genus or species has been detected in humans
[c]See Chap. 30
[d]The human parechovirus types are listed in Table 11.7
[e]See Chap. 18

Table 11.2 Classification of human enteroviruses

Traditional taxonomy	Current taxonomy
Polioviruses	[*Human*] *enterovirus A* (*HEV-A*)
PV1–3	CAV2–8, 10, 12, 14, 16; EV71, EV76 EV89, EV90, EV91, EV114, EV119 (EV92, SV19, SV43, SV46, and BA13 are classified in HEV-A, but they have been detected only in nonhuman primates)
Coxsackie A viruses	[*Human*] *enterovirus B* (*HEV-B*)
CVA1–22, 24	CAV9; CBV1–6; E1–7, 9, 11–21, 24–27, 29–33; EV69, EV73–75, EV77–88, EV100–101, EV106–107 (EV110 and SA5 are classified in HEV-B, but they have been detected only in nonhuman primates)
Coxsackie B viruses	[*Human*] *enterovirus C* (*HEV-C*)
CVB1–6	PV1–3, CAV1, 11, 13, 17, 19–22, 24, EV95–96, EV99, EV102, EV104–105, EV109, EV113, EV116–118
Echoviruses	[*Human*] *enterovirus D* (*HEV-D*)
E1–7, 9, 11–21, 24–27, 29–33	EV68, 70, EV94, EV111 (EV120 is classified in HEV-D, but it has been detected only in nonhuman primates)
Numbered enteroviruses	*Unassigned*
EV68–71	EV122–123

In the new taxonomy, "Human" is in brackets because there is a pending taxonomy proposal to drop host species from picornavirus species names [117]. The gaps in numbering result from changes in classification. Since the time of their discovery and initial classification, some serotypes have been found to be identical to another enterovirus (i.e., coxsackievirus A15 is the same as coxsackievirus A11, coxsackievirus A18 is the same as coxsackievirus A13, coxsackievirus A23 is the same as echovirus 9, echovirus 8 is the same as echovirus 1, and echovirus 34 is a variant of CVA24). In addition, some serotypes have been reclassified as members of other picornavirus genera or other virus families. Echovirus 10 is reovirus 1 (genus *Orthoreovirus*, family *Reoviridae*), echovirus 28 is human rhinovirus 1A (genus *Enterovirus*, family *Picornaviridae*), enterovirus 72 is human hepatitis A virus (genus *Hepatovirus*, family *Picornaviridae*), and echoviruses 22 and 23 are now considered human parechoviruses 1 and 2, respectively (genus *Parechovirus*, family *Picornaviridae*). Additional types (SV6, EV103, EV108, EV112, EV115, and EV121) have been detected only in nonhuman primates and are classified in the proposed species, *Enterovirus J*. Modified from Khetsuriani et al. (2006)

coxsackieviruses produce a variety of illnesses, including aseptic meningitis, herpangina, epidemic myalgia (pleurodynia, Bornholm disease), hand, foot, and mouth disease, myocarditis, pericarditis, pneumonia, rashes, and common colds, with some differences between the group A and group B coxsackieviruses. They may also have a role in some congenital malformations and in the development of type 1 diabetes. Aseptic meningitis, febrile illnesses with or without rash, and common colds are among the diseases caused by echoviruses. Among the newer enterovirus (EV) types, EV68 has caused lower respiratory illness, EV70 is the agent of widespread epidemics of acute hemorrhagic conjunctivitis (as is a variant of CVA24), and EV71 has caused aseptic meningitis, brainstem encephalitis, and hand, foot, and mouth disease in a number of countries. EV71 is described in detail in Chap. 12. Most of the numbered enteroviruses are also associated with the same illnesses as the coxsackieviruses and echoviruses. (Further details of clinical manifestations of enterovirus infections are given in Sect. 8.)

2 Historical Background

Enteroviruses have been studied in detail for over a century. As a result, a great deal of information is available in earlier reviews and textbooks [55, 65, 149, 150, 157, 166, 200–202, 225, 238, 251]. Consequently, only a few key highlights will be addressed here (see also Chap. 14).

The history of the enteroviruses begins with the history of poliovirus. Paralytic poliomyelitis appears in records of early antiquity, but it was first recognized as a clinical entity only in the late eighteenth and early nineteenth centuries. Poliomyelitis became the subject of intense study after large epidemics began to appear in Europe and North America. In 1908, Landsteiner and Popper successfully transmitted the disease to nonhuman primates, marking identification of the first human "filterable agent" (virus) [123]. During the first half of the twentieth century, many details of poliovirus biology and pathogenesis were elucidated, primarily by using nonhuman primates as a model system, though some strains could be adapted to growth in laboratory rodents, allowing for studies that were not feasible in larger animals. Despite the primary pathology occurring in the anterior horn of the spinal cord, the virus was shown to be excreted in stools of patients, suggesting a role for fecal-oral transmission and enteric replication. Certain nonhuman primates could be infected by the alimentary route, further aiding in the understanding of pathogenesis and transmission. Significant antigenic differences among poliovirus strains were documented, and neutralization studies identified three antigenic types [24]. One of the key innovations—and, indeed, a key enabling technology for all of animal virology—was the discovery that polioviruses could be isolated and propagated in vitro, in cell cultures derived from primate nonneural tissues [67].

The first of the viruses in coxsackievirus group A was isolated by inoculation of infant mice with fecal material from two paralyzed children during an epidemic of poliomyelitis in 1948 in Coxsackie, New York [54]. Additional coxsackie A viruses, as well as the first coxsackieviruses of

group B [144], were discovered shortly thereafter by similar methods. Group B coxsackieviruses were associated with aseptic meningitis and with epidemic myalgia and pleurodynia [52]. Group A and B coxsackieviruses were distinguished by their differing pathological effects in baby mice as well as their antigenic properties (see Sect. 4).

Once human and monkey cell cultures were implemented in a number of virology laboratories, largely for poliovirus isolation from stool specimens of patients with acute flaccid paralysis [67], additional enteric viruses were discovered. While these new viruses readily produced cytopathology in cultured cells, they were not pathogenic for laboratory animals, unlike the polioviruses and coxsackieviruses [146, 219]. These viruses could be isolated from healthy children [84, 96, 146, 216] as well as from patients with aseptic meningitis [146, 147], and multiple serotypes were identified [147, 216]. Because they failed to produce illness in laboratory animals and were not yet clearly associated with human disease, they were called "orphan" viruses or human enteric viruses; later they became known as ECHO (enteric cytopathogenic human orphan) viruses [47], a name subsequently simplified to "echoviruses." "Orphan" viruses causing cytopathology in culture were also identified in nonhuman primates and livestock.

In addition to their characteristic mouse pathogenicity, certain of the coxsackieviruses were found to grow readily in tissue cultures; other strains, serologically identical with the mouse-pathogenic prototype, failed to produce disease in baby mice. Conversely, certain strains of echoviruses were found to be pathogenic for mice. As instances of such overlapping properties accumulated, blurring the initial distinction made between coxsackieviruses and echoviruses, it was recommended that subsequently, as new enterovirus types were discovered, they would simply be assigned sequential numbers, as enterovirus 68, enterovirus 69, and so on [222]. The currently accepted enterovirus types are listed in Tables 11.3, 11.4, 11.5, and 11.6, and the parechovirus types are listed in Table 11.7.

3 Methods for Epidemiologic Analysis

3.1 Sources of Mortality Data

In the United States, enterovirus infections are not generally notifiable in that they are not required to be reported to local, state, and national public health authorities. In addition, encephalitis and aseptic meningitis are not uniformly reportable, although some jurisdictions within the United States may collect information on these illnesses. Often, enteroviral infections may go unrecognized unless clinicians order appropriate testing, and because there are no recommended antivirals, virologic diagnosis does not lead to a specific antiviral therapy.

The most recent enterovirus mortality data for the United States is described in the Morbidity and Mortality Weekly Report published by the Centers for Disease Control and Prevention [113], with periodic updates [36]. The National Enterovirus Surveillance System (NESS) is a voluntary passive reporting system that monitors trends in circulating enteroviruses since 1961. The data in NESS are contributed by state and local public health laboratories and a few large clinical reference laboratories. Outcome was reported for 3,392 (15.9 %) of 24,654 cases during 1983–1998. During this period, 131 (3.3 %) fatal outcomes were reported. Of the 115 deaths with known age, 77 (67 %) occurred among children <1 year. In 1998, the NESS reporting form was simplified to encourage broader reporting by public health laboratories, and outcome data are no longer collected.

3.2 Sources of Morbidity Data

Sources of population morbidity data for enteroviruses are often not representative due to variable diagnostic capabilities, lack of uniform reporting, and lack of necessary correlation between enteroviral excretion and association with disease. Most morbidity data are obtained from outbreak investigations and case reports, with consequent extrapolations to the surrounding community.

Historically, the Virus Watch Program in the 1960s in the United States provided prospective longitudinal data from a defined population [83, 241]. Families were observed for acute illnesses for a period of years and periodically sampled for viruses. Although an expensive study design, data collected about illness incidence with accompanying virologic sampling yielded important information about the association of enterovirus identification with disease [49, 66, 83].

Trends in enterovirus types and human parechovirus (HPeV) types are reported through NESS in the United States [113]. Although only laboratory data is reported through NESS, descriptions of diseases associated with common serotypes may be apparent amidst outbreak reports where detailed clinical information is collected. For example, in 2007 and 2008 CVB1 was the predominant serotype reported, and in 2007 CVB1 was the cause of an outbreak of severe neonatal infections in the United States [34]. In addition to NESS, the Infectious Agents Surveillance Report from Japan's National Institute of Infectious Diseases describes viral detections associated with specific diseases such as herpangina and hand, foot, and mouth disease.

3.3 Serological and Clinical Surveys

Since there are many enterovirus and HPeV types, few serological surveys exist that fully characterize seroprevalence in vulnerable populations. However, understanding

susceptibility to EV71 infection has received more attention because of the potential for large outbreaks of hand, foot, and mouth disease (HFMD) including a subset of children with severe neurologic complications, particularly in Asia. In Shanghai, after a large outbreak of HFMD in 2010, a serosurvey revealed that loss of maternal antibodies later in infancy and lack of acquired anti-EV71 immunity were likely responsible for a large number of severe HFMD cases in the 1–2-year age group in 2011 [285]. Likewise in Singapore, high EV71 neutralizing antibody titers were identified in a group of children aged one to six years compared to older children, indicating that infections were acquired in early childhood [7].

The predominantly circulating enteroviral serotypes may change over time and may be associated with large outbreaks of clinically recognizable syndromes. Enterovirus surveillance systems vary greatly worldwide, but all contribute information regarding circulating serotypes over geography and time [201]. Molecular typing and phylogenetic surveys yield important information for detection of new viral strains and outbreaks of clinical disease [169, 270].

Although enteroviruses are commonly identified among children, especially in summer and autumn months, targeted epidemiologic surveillance may provide a snapshot of circulating seasonal serotypes. Recently, specimens obtained clinically from children were collected along with environmental samples in the Republic of Georgia from 2002 to 2005 [114]. A wide range of enteroviruses were identified, with notable genetic diversity, including some that have been rarely identified in the United States. The information obtained from environmental and clinical specimens created a better understanding of circulating serotypes, which is important in preparing for large-scale outbreaks associated with the emergence of new enterovirus strains. Another survey of clinical specimens obtained from hospitalized children in Cyprus from 2003 to 2007 demonstrated changes in predominant serotypes over time; these changes were consistent with those in other European countries [254]. In the United States, NESS has provided seasonal information on circulating serotypes (Fig. 11.1). Correlations between serotype detection and certain specimen types may reflect clinical presentations observed in the community [36, 113]. For example, echovirus 9 and echovirus 30, which are commonly associated with aseptic meningitis,

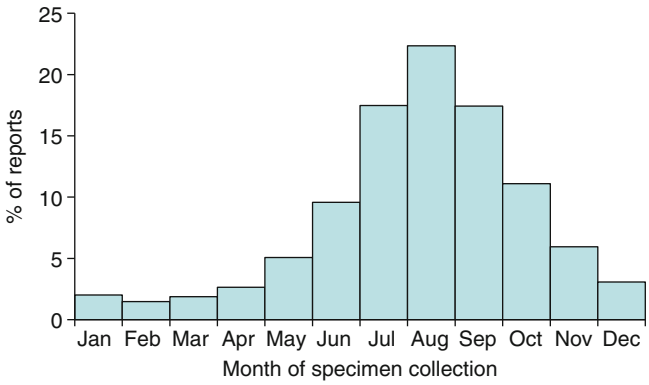

Fig. 11.1 Percentage of enterovirus reports by month, United States, by month of specimen collection, 1983–2005 (From Khetsuriani et al. [114], in the public domain)

Table 11.3 Enterovirus species A (EV-A)

Type	Prototype strain	Geographic origin	Illness or source	Accession number	Investigator
CVA2	Fleetwood	Delaware	Poliomyelitis	AY421760	Dalldorf
CVA3	Olson	New York	Meningitis	AY421761	Dalldorf
CVA4	High point	North Carolina	Sewage of community with polio	AY421762	Melnick
CVA5	Swartz	New York	Poliomyelitis	AY421763	Dalldorf
CVA6	Gdula	New York	Meningitis	AY421764	Dalldorf
CVA7	Parker	New York	Meningitis	AY421765	Dalldorf
CVA8	Donovan	New York	Poliomyelitis	AY421766	Dalldorf
CVA10	Kowalik	New York	Meningitis	AY421767	Dalldorf
CVA12	Texas-12	Texas	Files in community with polio	AY421768	Melnick
CVA14	G-14	South Africa	None	AY421769	Gear
CVA16	G-10	South Africa	None	U05876	Gear
EV71	BrCr	California	Meningitis[a]	U22521	Schmidt
EV76	10226	France	Gastroenteritis	AY697458	Oberste
EV89	10359	Bangladesh	Acute flaccid paralysis	AY697459	Oberste
EV90	10399	Bangladesh	Acute flaccid paralysis	AY697460	Oberste
EV91	10406	Bangladesh	Acute flaccid paralysis	AY697461	Oberste
EV114	11610	Bangladesh	Acute flaccid paralysis	NA	Oberste
EV119	C13	Cameroon		NA	Norder

NA information not available

[a]An identical strain was isolated from the brain of a fatal encephalitis case in the same local outbreak of central nervous system disease

Table 11.4 Enterovirus species B (EV-B)

Type	Prototype strain	Geographic origin	Illness yielding prototype virus	Accession number	Investigator
CVA9	Bozek	New York	Meningitis	D00627	Dalldorf
CVB1	Conn-5	Connecticut	Meningitis	M16560	Melnick
CVB2	Ohio-1	Ohio	Summer grippe	AF085363	Melnick
CVB3	Nancy	Connecticut	Minor febrile illness	M16572	Melnick
CVB4	JVB	New York	Chest and abdominal pain	X05690	Sickles
CVB5	Faulkner	Kentucky	Mild paralytic disease with atrophy	AF114383	Steigman
CVB6	Schmidt	Philippines	None	AF1 05342	Hammon
E1	Farouk	Egypt	None	AF029859	Melnick
E2	Cornelis	Connecticut	Meningitis	AY302545	Melnick
E3	Morrisey	Connecticut	Meningitis	AY302553	Melnick
E4	Pesascek	Connecticut	Meningitis	AY302557	Melnick
E5	Noyce	Maine	Meningitis	AF083069	Melnick
E6	D'Amori	Rhode Island	Meningitis	AY302558	Melnick
E7	Wallace	Ohio	None	AY302559	Ramos-Alvarez
E9	Hill	Ohio	None	X84981	Ramos-Alvarez
E11	Gregory	Ohio	None	X80059	Ramos-Alvarez
E12	Travis	Philippine Islands	None	X79047	Hammon
E13	Del Carmen	Philippine Islands	None	AY302539	Hammon
E14	Tow	Rhode Island	Meningitis	AY302540	Melnick
E15	CH 96-51	West Virginia	None	AY302541	Ormsbee
E16	Harrington	Massachusetts	Meningitis	AY302542	Kibrick
E17	CHHE-29	Mexico City	None	AY302543	Ramos-Alvarez
E18	Metcalf	Ohio	Diarrhea	AF317694	Ramos-Alvarez
E19	Burke	Ohio	Diarrhea	AY302544	Ramos-Alvarez
E20	JV-1	Washington, DC	Fever	AY302546	Rosen
E21	Farina	Massachusetts	Meningitis	AY302547	Enders
E24	DeCamp	Ohio	Diarrhea	AY302548	Sabin
E25	JV-4	Washington, DC	Diarrhea	AY302549	Rosen
E26	Coronel	Philippine Islands	None	AY302550	Hammon
E27	Bacon	Philippine Islands	None	AY302551	Hammon
E29	JV-10	Washington, DC	None	AY302552	Rosen
E30	Bastianni	New York	Meningitis	AF162711	Plager
E31	Caldwell	Kansas	Meningitis	AY302554	Wenner
E32	PR-10	Puerto Rico	Meningitis	AY302555	Branche
E33	Toluca-3	Mexico	None	AY302556	Rosen
EV69	Toluca-1	Mexico	None	AY302560	Rosen
EV73	CA55-1988	California	Unknown	AF241359	Oberste
EV74	10213	California	Unknown	AY556057	Norder
EV75	10219	Oklahoma	Unknown	AY556070	Oberste
EV77	CF496-99	France	Unknown	AJ493062	Norder
EV78	W137-126/99	France	Unknown	AY208120	Norder
EV79	10384	California	Unknown	AY843297	Oberste
EV80	10387	California	Unknown	AY843298	Oberste
EV81	10389	California	Unknown	AY843299	Oberste
EV82	10390	California	Unknown	AY843300	Oberste
EV83	10392	California	Unknown	AY843301	Oberste
EV84	10603	Côte d'Ivoire	None	DQ902712	Oberste
EV85	10353	Bangladesh	Acute flaccid paralysis	AY843303	Oberste

Table 11.4 (continued)

Type	Prototype strain	Geographic origin	Illness yielding prototype virus	Accession number	Investigator
EV86	10354	Bangladesh	Acute flaccid paralysis	AY843304	Oberste
EV87	10396	Bangladesh	Acute flaccid paralysis	AY843305	Oberste
EV88	10398	Bangladesh	Acute flaccid paralysis	AY843306	Oberste
EV93	38-03	Democratic Republic of the Congo		EF127244	Junttila
EV97	10355	Bangladesh	Acute flaccid paralysis	AY843307	Oberste
EV98	T92-1499			AB426608	Yamashita
EV100	10500	Bangladesh	Acute flaccid paralysis	DQ902713	Oberste
EV101	10361	Côte d'Ivoire	None	AY843308	Oberste
EV106	10634	Bangladesh	Acute flaccid paralysis	NA	Oberste
EV107	TN94-0349	Thailand	None	AB266609	Yamashita

NA information not available

Echovirus types 1 and 8 share antigens, type 1 having the broader spectrum. Type 10 was soon excluded from this group: it turned out to be a larger RNA virus and was reclassified as a prototypic reovirus. Type 28 was reclassified as rhinovirus type 1. Types 22 and 23 have been reclassified as members of the genus parechovirus and are named parechovirus 1 and 2. Type 34, DN-19, is now considered a prime strain of CVA24, rather than a distinct echovirus. Additional newer serotypes (EV73 and higher) are proposed new types defined on the basis of genetic sequence information

Table 11.5 Enterovirus species C (EV-C)

Type	Prototype strain	Geographic origin	Illness in person with prototype	Accession number	Investigator
CVA1	Tompkins	Coxsackie, NY	Poliomyelitis	AF499635	Dalldorf
CVA11	Belgium-1	Belgium	Epidemic myalgia	AF499636	Curnen
CVA13	Flores	Mexico	None	AF499637	Sickles
CVA17	G-12	South Africa	None	AF499639	Gear
CVA19	NIH-8663	Japan	Guillain-Barré syndrome	AF499641	Huebner
CVA20	IH-35	New York	Infectious hepatitis	AF499642	Sickles
CVA21	Kuykendall; Coe	California	Poliomyelitis, mild respiratory diseases	AF546702	Lennette
CVA22	Chulman	New York	Vomiting and diarrhea	AF499643	Sickles
CVA24	Joseph	South Africa	None	D90457	Gear
PV1	Brunhilde	Maryland	Paralytic poliomyelitis	AY560657	Howe
PV2	Lansing	Michigan	Fatal paralytic poliomyelitis	AY082680	Armstrong
PV3	Leon	California	Fatal paralytic poliomyelitis	K01392	Kessel
EV95	5-05			NA	Norder
EV96	10358	Bangladesh	Acute flaccid paralysis	EF015886	Oberste
EV99	10461	Bangladesh	Acute flaccid paralysis	EF555644	Oberste
EV102	10424	Bangladesh	Acute flaccid paralysis	EF555645	Oberste
EV104	CL-1231094	Switzerland	Acute respiratory illness	EU840733	Tapparel
EV105	TW/NTU07	NA	NA	NA	Chang
EV109	NICA08-4327	Nicaragua	Acute respiratory illness	GQ865517	Yozwiak
EV116	126/Russia/10	Russia	NA	JX514942	Lukashev
EV117	LIT22	Lithuania		JX262382	Daleno
EV118	ISR10	Israel		JX961708	Daleno

NA information not available

Table 11.6 Enterovirus species D (EV-D)

Type	Prototype strain	Geographic origin	Illness in person with prototype	Accession number	Investigator
EV68	Fermon	California	Lower respiratory illness	AY426531	Scheible
EV70	J670/71	Japan	Acute hemorrhagic conjunctivitis	D00820	Kono
EV94	E210	Egypt	Detected in sewage	DQ916376	Smura
EV111	KK2640	Cameroon	None[a]	JF416935	Harvala

[a]The prototype strain was detected in a chimpanzee, but another strain of EV111 was detected in a human with acute flaccid paralysis in Democratic Republic of the Congo

Table 11.7 Human parechoviruses

Type	Prototype strain	Geographic origin	Illness in person with prototype	Accession number	Investigator
HPeV1	Harris	Ohio		L02971	Sabin
HPeV2	Williamson	Ohio		AJ005695	Sabin
HPeV3	A308/99	Japan		AB084913	Ito
HPeV4	T75-4077	California		AM235750	Al-Sunaidi
HPeV5	6760	Connecticut		AF055846	Oberste
HPeV6	NII428-2000	Japan		AB252577	Watanabe
HPeV7	PAK5045	Pakistan		EU556224	Li
HPeV8	BR/217/2006	Brazil		EU716175	Drexler
HPeV9	10902	Bangladesh		NA	Oberste
HPeV10	10903	Bangladesh		NA	Oberste
HPeV11	10905	Bangladesh		NA	Oberste
HPeV12	10904	Bangladesh		NA	Oberste
HPeV13	10901	Bangladesh		NA	Oberste
HPeV14	451564	Netherlands		FJ373179	Benschop
HPeV15	11614	Bangladesh		NA	Oberste
HPeV16	11615	Bangladesh		NA	Oberste

were mostly detected from cerebrospinal fluid specimens. Epidemiologic assessments of enteroviral serotypes and clinical disease worldwide are important for understanding outbreaks of disease caused by emergent strains in new geographic areas. This type of surveillance is also important to identify modified clinical presentations of common diseases such as HFMD caused by coxsackievirus A6 during 2010–2012 [22, 37, 76, 267, 277]. Knowledge of circulating enteroviral serotypes in time and place may allow for better recognition of large-scale outbreaks and consequent approaches to control viral transmission.

3.4 Detection of Viruses in the Environment

Enteroviruses excreted in feces will end up in sewage in developed countries and in surface waters and waterways in developing countries that lack a closed sewage collection system. As a result, virus isolation from sewage, whether in a closed or open system, provides an opportunity for a relatively unbiased sampling of enteroviruses circulating in the population. Typical sewage treatment systems may not fully remove or inactivate viruses, including enteroviruses [72]. The primary disadvantage of sewage testing is the extra effort required to remove solids and other potential inhibitors of cell culture isolation or molecular detection methods. However, a number of methods have been developed to overcome this problem, including simple filtration, adsorption to charged filters, and concentration by phase separation [228]. In some cases, concentration systems can be deployed in the field, to reduce the amount of sample that must be transported to the laboratory [155, 158, 159, 161, 261]. A second disadvantage is that sewage can often be expected to contain a mixture of enteric viruses, especially in areas with high population density and poor sanitation. Separation of the virus mixtures may require passage on multiple cell lines, cloning by limiting dilution, or plaque purification, steps which may impact the feasibility of routine environmental surveillance.

Environmental sampling has been used to supplement patient-based virus surveillance [20, 23, 43, 81, 110, 122, 135, 148], but it has also been applied to outbreak investigations, to demonstrate the source of infection. For example, isolation of coxsackievirus B5 from an open lake swimming area identified the lake as a source of an epidemic at a summer camp [91].

3.5 Laboratory Methods

Detailed descriptions of the principles and procedures for diagnosis of enterovirus infections have been published [166, 187, 202, 224].

Understanding the epidemiology of enterovirus infections and disease depends largely on the ability of the laboratory

to identify the specific virus involved and to integrate that information in the context of other studies of the same serotype or the same disease. Traditionally, enterovirus identification was based on virus isolation in culture and typing by serotype-specific antisera, either individually or in specific intersecting pools, a system which depends on shared, standardized reagents [47, 154]. While this system was sufficient for most purposes, and was the standard for more than four decades, it became increasingly complicated as more enterovirus serotypes were discovered. It was recognized as early as 1962 that "the rate of making new [enterovirus] discoveries has slowed, probably only because the labors involved in establishing 'new' serotypes are now so very great." [269] Since the late 1990s, molecular typing methods have become the standard for identifying enterovirus serotype (often now referred to as simply "type"), and the methods are equally adept at identifying new enteroviruses, more than 50 of which have been discovered since 2000.

3.5.1 Virus Isolation and Identification

The specimens that most frequently yield enterovirus or parechovirus in culture are stools, rectal swabs, throat swabs, and naso- or oropharyngeal swabs [156, 202]. In addition, cerebrospinal fluid (CSF) may yield virus in cases of neurologic illness, such as aseptic meningitis. In children, even after development of symptoms has led to hospitalization, a variety of non-polio enteroviruses may be detected in the blood, either free in the serum or in mononuclear leukocytes [212]. Virus may also be isolated from vesicle fluids (e.g., from HFMD cases), urine, conjunctival swabs (EV70 and CVA24 variant), and nasal secretions. In general, virus may be recovered from throat swabs up to about 2 weeks after onset of symptoms and from stool for up to 8 weeks [6]. In fatal cases of suspected enteroviral etiology, virus may be detected in the organ system affected as well as in colon contents. Of course, other viruses may also be isolated from feces or respiratory secretions, including rotaviruses, reoviruses, adenoviruses, rhinoviruses, and the viruses of measles, mumps, rubella, and herpes simplex. Many of these agents produce distinctive cytopathic effects (CPE), which at once differentiate them from enteroviruses.

Traditionally, experienced laboratory personnel could make a presumptive diagnosis of enteroviral infection on the basis of the nature of the associated illness (if any), the time of year when the specimen was obtained, the culture (or mouse) system in which the virus isolate grew, and the characteristic CPE observed in the cultures or the characteristic pathology induced in the mice. There was some value in reporting a presumptive identification without waiting for specific typing as early recognition of probable enteroviral infection could provide information for the management of a patient or of a community outbreak and serve to contraindicate administration of unnecessary or undesirable antibiotic therapy.

The enteroviruses can generally be propagated in either cell culture or suckling mice [156, 201, 202], but some of the newer enterovirus and parechovirus types have been identified only by direct detection in stool specimens. Most types can be grown in at least one human or primate continuous cell culture [119, 125, 180, 196, 205, 206, 211, 229, 234]; however, no single cell line can be used to isolate or propagate all cultivable enteroviruses. Despite many years of work, a few types (e.g., CVA19) have been propagated only in suckling mice. The typical host range of human enterovirus in cell cultures or animals is not clearly associated with a given virus species.

Infection of target cells depends on virus binding to specific receptors on the cell surface. Collectively, the enteroviruses use at least eight different receptors, including two different integrins ($\alpha_v\beta_3$ and $\alpha_2\beta_1$), decay-accelerating factor (DAF; CD55), the coxsackievirus-adenovirus receptor (CAR), intracellular adhesion molecule 1 (ICAM-1), scavenger receptor B2 (SCARB2), P-selectin glycoprotein ligand-1 (PSGL-1), and the poliovirus receptor (CD155) [174, 223, 236, 279]. Some enteroviruses may use more than one receptor (e.g., EV71 uses both SCARB2 and PSGL-1; some group B coxsackieviruses can use both CAR and DAF), and other, unidentified receptors may also exist. The parechoviruses use two different integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_1$, as well as at least one additional, unknown receptor. The procedure for virus isolation involves inoculation of appropriate specimens onto susceptible cultured cells. In clinical laboratories, it is common to simultaneously inoculate several types of human and primate cells to increase the spectrum of viruses that can be detected [156, 240]. The coxsackieviruses, including those that do not grow in cell culture, can be isolated and propagated by intracerebral inoculation of suckling mice [234].

It is usually not clinically necessary to identify the specific serotype for every enterovirus isolate; simply knowing an enterovirus has been detected is usually sufficient. However, knowing the type can be important in outbreak investigations, to determine whether a case is part of the outbreak or part of the sporadic enterovirus background. Knowing the type can also be of interest in cases that are particularly severe or that have an unusual presentation.

Many clinical laboratories no longer perform cell culture isolation for suspected enterovirus cases in routine diagnostic testing. When isolates are obtained, they may be confirmed as a specific enterovirus type by neutralization with type-specific antisera. The most widely used reagent antisera were prepared in horses and can be obtained from the World Health Organization (WHO) [85, 86, 151–153]; however, supplies are now quite limited. Type-specific monoclonal antibodies may also be used for typing, usually in indirect immunofluorescence assays to identify viruses isolated in culture [130, 218]. Commercially available monoclonal antibodies can be used to detect relatively common serotypes, including PV1 to 3, CVA9, CVA24, CVB1 to 6, E4, E6, E9, E11, E30, EV70, and EV71. Additional monoclonal

antibodies have recently been developed for CVA2, CVA4 to 5, and CVA10 [130]. Indirect immunofluorescence is faster and easier to perform than neutralization, and the reagents can be produced in large quantity as needed, but the method still suffers from the same limitations as other antigenic typing methods, namely, the requirement for a virus isolate in culture prior to typing and the need for a large number of reagents to identify all serotypes. Despite these limitations, the method has been adopted as the standard typing method in a large number of clinical and reference laboratories.

3.5.2 Molecular Detection and Identification

Because of distinct advantages in speed, molecular detection techniques have already supplanted traditional methods of detection and characterization as the gold standard. Specifically, molecular procedures are now the methods of choice for enterovirus detection in CSF and are widely used for diagnosis of patients with a clinical presentation of meningitis. Several of these are licensed for clinical use in the United States or Europe.

By far the most common use of reverse transcription (RT) PCR for enterovirus diagnosis is the direct detection of virus in clinical specimens [189, 220, 231]. The individual details of the procedures may vary, but all methods that can generically detect enterovirus are similar in their key features. The most important property of these tests is that the primers target conserved sequences in the 5′ non-translated region (NTR) of the virus genome. Many different primers targeting this region have been published, recognizing different sequence motifs and slightly different sequences within those sites [187, 226]. Many of these assays, however, have not been completely evaluated on a large number of clinical isolates to confirm reactivity with all enterovirus types and/or with multiple strains within a given type. Therefore, they have not been validated sufficiently for diagnostic use [187]. The major advantage of these pan-enterovirus RT-PCR assays is that rapid detection is possible, even with very small amounts of clinical specimens such as CSF. Such assays also facilitate detection of enteroviruses that do not readily grow in cell culture. As with all RT-PCR, the sensitivity of amplification of RNA from biological specimens is highly variable, depending on the nature of the specimen. Many assays can be shown to give a positive result even from only a few copies of viral RNA in a "clean" specimen such as CSF, but it is not uncommon for the sensitivity to be many orders of magnitude lower in other specimen types (e.g., stool). The introduction of "real-time" PCR methods has greatly improved sensitivity, as the fluorescence detection systems generally increase the sensitivity significantly.

A common goal in virus identification is knowledge of the sequence of the viral genome. Encoded within this sequence are determinants for all the biological properties that are attributable to a given virus. In theory, therefore, the nucleic acid sequence of a virus represents its ultimate characterization. All important information about a virus could potentially be obtained directly by PCR in conjunction with nucleic acid sequencing if all the molecular correlates of viral phenotypic determinants were understood. The genetic correlates for many enterovirus properties remain uncertain, but it is possible to use sequence information to assign a sequence to a particular type [30, 31, 175, 179, 182, 183, 187]. The most common molecular typing system is based on RT-PCR and nucleotide sequencing of a portion of the genomic region encoding VP1 [175, 182, 184]. The type is inferred by comparison of the partial VP1 sequence with a database containing VP1 sequences for the prototype and variant strains of all human enterovirus types [187]. Using this approach, strains of homologous types can be easily discriminated from heterologous types, and new types can be identified. This method can greatly reduce the time required to type an enterovirus and can be used to type samples that are difficult or impossible to type using standard immunologic reagents or that fail to grow in culture [175, 184]. The technique is also useful to rapidly determine whether viruses isolated during an outbreak are epidemiologically related.

3.5.3 Tests for Antibody

Testing for the presence of type-specific antibody against enteroviruses is appropriate only when (i) a known enterovirus isolate from the patient is available and confirmation of the infecting serotype is necessary or (ii) a seroepidemiologic survey is being conducted to determine the community or study-group history of experience with a particular serotype or group (e.g., polioviruses). For routine diagnosis in a single patient or a locality, virus detection by RT-PCR is far simpler and faster and is the recommended approach, with virus isolation and/or molecular typing as options depending on the individual circumstances. For any purpose except a serological survey, paired serum specimens are required; the first sample must be taken as early as possible in the course of the illness or infection, the second 3–4 weeks later.

The neutralization test [156, 160] is accurate and type specific; complement fixation and agglutination (or agglutination inhibition) tests are no longer commonly used and are not recommended. Acute and convalescent sera are usually tested simultaneously, using various dilutions of serum against a constant amount (usually 100 $CCID_{50}$) of the specific virus [266]. A fourfold or greater rise in type-specific neutralizing antibody titer is considered diagnostic of recent infection. Antibody, however, may already be present at the time the original specimen is obtained because of the extended incubation period and prodromal period of many enteroviral illnesses, which complicates interpretation of results. If neutralizing antibody titers are found to be equally high in both acute and convalescent specimens, the infection might have taken place either recently or many years

before, since neutralizing antibody to any of the enteroviruses persists for years if not for life. In addition to the homologous antibody, antibodies against other enterovirus types may appear transiently and at low levels.

For diagnosis and study of acute hemorrhagic conjunctivitis caused by enterovirus type 70, isolation of the virus has been difficult, and most of the recent outbreaks have been identified solely by serological means, though RT-PCR can also be used to detect and identify virus directly in conjunctival swab samples.

Many serological studies rely on the detection of IgM antibody as evidence for recent enterovirus infection, and this is sometimes used as an alternative to the neutralization test, especially in epidemiologic studies. Several groups have developed an enzyme-linked immunosorbent assay (ELISA) for enterovirus-specific IgM [14, 61, 78, 94, 248]. These tests have been found positive for nearly 90 % of culture-confirmed group B coxsackievirus infections and can be performed rapidly. The ELISA has been successfully applied for epidemiologic investigations of outbreaks [79] as well as for specific diagnostic use [57, 264]. Depending on the configuration and sensitivity of the test, from 10 % to nearly 70 % of serum samples show a heterotypic response caused by other enterovirus infections. This heterotypic response has been exploited to measure broadly reactive antibody, and the assay has been used to detect enterovirus infection generically [26, 245]. It is clear that the human immune response to enterovirus infection includes antibodies that react with both serotype-specific epitopes and shared epitopes [74]. Despite this problem, there is a reasonably high concordance of results between assays of different configurations [94]. The IgM assays that are used in epidemiologic studies have very good sensitivity and appear to be very specific for enterovirus infection; however, these assays detect heterotypic antibodies resulting from other EV infections and, therefore, cannot be considered strictly serotype specific. A positive result with either the neutralization test or IgM ELISA indicates a recent viral infection; however, the infecting serotype found with the IgM assay may not be the same one determined by the neutralization test, and because the duration of IgM is relatively long and variable among individuals, the presence of enteroviral IgM is not a definitive test for current infection. Therefore, IgM data must be interpreted with some caution.

3.5.4 Parechovirus Diagnostics

Despite their original classification, sequencing and PCR studies demonstrated that echoviruses 22 and 23 are distinct from the enteroviruses, resulting in their reclassification as members of a new picornavirus genus, *Parechovirus* [45, 102, 117, 242]. Since then, 14 additional human parechovirus types have been identified (Oberste MS, unpublished data, 2008) [3, 11, 17, 18, 62, 105, 126, 265]. Like the enteroviruses, human parechoviruses were traditionally detected and identified by virus isolation and antigenic typing [127]. By these methods, HPeV1 (formerly echovirus 22) consistently accounted for 2–4 % of "enteroviruses" reported to the CDC from 1975 to 2005 [113]. RT-PCR began to supplant virus culture as the method of choice for enterovirus detection in clinical diagnostic laboratories in the mid-1990s. Since that time, the number of HPeV1 reports has declined to under 1 %, probably because HPeV-containing specimens are usually reported as enterovirus PCR-negative and not further characterized.

More recently, a number of investigators have developed real-time RT-PCR assays to detect HPeVs [11, 16, 17, 51, 60, 108, 176, 178]. These methods target conserved sites in the parechovirus 5′ NTR that are analogous to those targeted by enterovirus-specific RT-PCR assays. Like the enterovirus assays, the HPeV RT-PCR assays vary in level of validation; in particular, several have not been shown to detect the more recently identified types. Despite this possible limitation, a number of recent studies have applied molecular methods to the detection of human parechoviruses in patients with enteritis, respiratory illness, and neonatal sepsis-like syndrome [3, 11, 19, 105, 257, 265]. As these methods are increasingly integrated into the diagnostic routine of clinical and reference laboratories, a better estimate will emerge of the burden of disease attributable to this group of picornaviruses.

4 Biological Characteristics

4.1 General Properties

Enteroviruses share the basic properties of picornaviruses, including a ~7,500 nt genome of single-stranded, positive-sense RNA, small size (diameter 22–30 nm), lack of an envelope (i.e., a "naked" nucleocapsid), and insensitivity to ether and other lipid solvents, indicating lack of essential lipids (Fig. 11.2) [117, 201]. The genome encodes a single long open reading frame, flanked by non-translated regions at the 5′ and 3′ ends. The virus replicates in the cytoplasm of infected cells.

The enterovirus infectious particle is an icosahedral virion consisting of 60 copies of each of four capsid proteins (VP1–4) and a molecule of single-stranded genomic RNA. The molecular biology of enterovirus replication is typical for that of other plus-strand RNA viruses: uptake into the host cell through attachment to a specific cellular receptor, release of genomic RNA, protein synthesis, genome replication, and encapsidation. The molecular mechanisms underlying each of these steps are under intensive investigation. Because genomic RNA is of positive polarity, infectious virus can be recovered by transfection of naked RNA into appropriate cell cultures. Full-length cDNA clones have been generated for

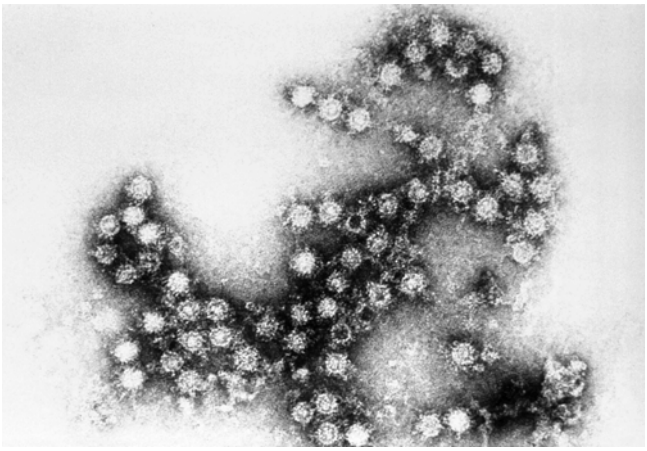

Fig. 11.2 Transmission electron micrograph of coxsackievirus B4 particles (From the Public Health Image Library, Centers for Disease Control and Prevention, 1981, in the public domain)

many enteroviruses, facilitating a reverse genetics approach to understanding the function of individual viral proteins and RNA elements.

Although much is known about the enteroviruses, still unresolved is the biochemical basis of virus stability associated with its portal of entry through the enteric tract, the determinants of virus spread in the host, and knowledge of how the virus penetrates its target tissue, thereby causing disease [167]. The key to the early events of infection is determined by unique cell surface receptors. The receptor plays a key role in the binding, penetration, and uncoating of the virus [223].

The human enteroviruses use at least eight different receptors to bind and gain entry into the host cell (CD155, DAF [CD55], CAR, ICAM-1, SCARB2, PSGL-1, and two different integrins) [174, 223, 236, 279]; however, the receptors for many enteroviruses remain unknown. The receptors for the polioviruses and rhinoviruses are members of a large group of normal cellular proteins known as the immunoglobulin gene superfamily [87, 177, 217, 223]. The receptors for echovirus 1 receptor and CVA9 are integrins [21, 252], proteins known to play a role in the interactions between cells and the extracellular matrix.

4.2 Reactions to Chemical and Physical Treatment

Enteroviruses are resistant to most laboratory disinfectants and to lipid solvents (e.g., ether), but they are rapidly inactivated by treatment with 0.3 % formaldehyde, 0.1 N HCl, or free residual chlorine at a level of 0.3–0.5 ppm. However, the presence of organic matter (e.g., in stool or sewage) is protective, so caution should be exercised when attempting to extrapolate from laboratory sensitivity data, often generated using purified virus, to real-world situations. The viruses lose infectivity rapidly when heated to 50 °C, but 1 M magnesium chloride can make the virions resistant to treatment at 50 °C for 1 h [259]. Enteroviruses are stable at freezing temperatures for decades, at 4 °C for weeks, and at room temperature for days. Virus in stool is stable at room temperature for at least 4 weeks and is also resistant to multiple freeze-thaw cycles. Desiccation quickly renders enteroviruses noninfectious. They are also inactivated by treatment with ultraviolet light, and vital dyes, such as neutral red, acridine orange, proflavine, can be incorporated into the structure of the viruses, making them susceptible to visible light [232, 260].

4.3 Antigenic Properties

The enterovirus serotypes were originally defined by neutralization assays, either in cell culture or in susceptible animal models [46–48, 203]. Antigenicity by neutralization in vitro correlates well with human immunity. Initially, enteroviruses were identified by neutralization with a standard set of antisera that recognized the "known" serotypes. When a virus was not neutralized by the standard panel, an antiserum was raised against the new virus and used in neutralization tests with standard reference strains. Strains which exhibited no cross-neutralization were considered putative new types. However, most of the newer types have been defined strictly on the basis of capsid sequences [185]; hence, their antigenic relationships to one another and to the older types is unknown. The extensive use of the neutralization assay in the 1950s and 1960s also revealed a number of minor cross-reactions and other antigenic relationships, both within and between the established types.

The neutralizing epitopes are located primarily at the edges of the "canyon" on the capsid surface, an area of virus-receptor interaction. These epitopes tend to be "conformational," i.e., they are not contiguous amino acids but, rather, are composed of residues on adjacent loops of one or more of the capsid proteins, VP1–3 (the VP4 protein is internal to the capsid and does not contribute to antigenicity). In the polioviruses, where the neutralization sites have been studied most extensively, sites on VP1 appear to be a major component of the key epitopes, with additional contributions by VP2 and VP3. Enterovirus capsids also contain a number of non-neutralizing epitopes, some of which are shared widely among the different types [74]. These can be readily detected by ELISA.

Despite the high error rate inherent in the enterovirus RNA-dependent RNA polymerases [1], the antigenic identity of enteroviruses is relatively stable (e.g., unlike influenza virus). Indeed, polio vaccines, developed using strains isolated over 50 years ago, are still effective against strains circulating today, even with considerable amino acid variation in the capsid (up to about 12 % difference in VP1).

4.4 Host Range

Almost by definition, humans are the natural host for the human enteroviruses. There is no evidence for zoonotic transmission, though at least some human enteroviruses can naturally infect certain nonhuman primate species [190]. In areas where humans and nonhuman primates are in frequent and close contact, such as in South Asia, infected primates presumably may participate in transmission, forming a single human and primate reservoir. Swine vesicular disease virus, an important livestock pathogen primarily because it is part of the differential diagnosis for foot-and-mouth disease virus, is genetically and antigenically related to coxsackievirus B5, presumably through infection of swine with the human virus at some time in the recent past [115, 287].

Many enteroviruses can infect or be adapted to infect common laboratory animal species, including rodents and monkeys, though blind passages may be needed in some cases. Indeed, the polioviruses and coxsackieviruses were first discovered by inoculation of laboratory animals. In newborn mice, the group A coxsackieviruses induce flaccid paralysis and extensive degeneration of skeletal muscle, with no involvement of the tongue, heart, and CNS; the animals usually succumb to infection within a week. By contrast, infection with group B coxsackieviruses proceeds more slowly and is characterized by spastic paralysis and tremors associated with encephalomyelitis, focal myositis, necrosis of brown fat pads, myocarditis, hepatitis, and acinar cell pancreatitis. With few exceptions, the echoviruses do not generally cause disease in mice. Most enteroviruses can be isolated and propagated in a wide range of cell cultures, usually of human or monkey origin, including cells derived from kidney, lung, and other tissues. However, some enteroviruses have been grown in only one or a few cell lines (e.g., some of the coxsackie A viruses have been propagated only in human rhabdomyosarcoma cells) [234]. The range of cells that can be infected is probably related to receptor usage, though differentially expressed intracellular factors necessary for virus replication could also play a role.

4.5 Replication of Enteroviruses

Most of the details of enterovirus replication have been elucidated through the use of poliovirus as a model [214]. When a virus particle binds to its cellular receptor, a conformational change is induced in the virion, resulting in extrusion of the genomic RNA into the cell cytoplasm [27, 95]. The viral RNA is translated through a cap-independent mechanism facilitated by direct interaction of the viral internal ribosome entry site ("IRES") in the 5′ NTR with cellular ribosomes [10]. Picornaviruses encode a single polyprotein, which is subsequently processed by viral proteinases to yield the mature viral proteins. A number of viral proteins interact with cellular proteins and structures to effectively inhibit cellular transcription and translation, shifting the cellular machinery to produce virus [53]. Once sufficient viral proteins are produced, viral synthesis shifts from an emphasis on translation to RNA replication, catalyzed by the virally encoded RNA-dependent RNA polymerase and using intracellular membranes derived from the rough endoplasmic reticulum and Golgi as a scaffold for the replication complexes [15, 170]. RNA synthesis is primed by the genome-linked viral protein ("VPg") which remains covalently linked to the genomic RNA [243]. Unlike cellular organisms and most DNA viruses, viral RNA-dependent RNA polymerases do not have proofreading and error-editing functions capable of correcting viral polymerase errors, and a large number of faulty nucleotide incorporations accumulate during RNA replication [272]. Mature virions are assembled in the cytoplasm through a mechanism that is not yet fully understood.

The time required from initiation of infection to completion of virus assembly ranges from 5 to 10 h, depending on pH, temperature, host cell, and number of particles to which the cell is exposed. Yields may be up to 100,000 particles per cell, but only 0.1–1 % of particles may be infectious.

Complete genome sequences have been determined for at least one strain of every enterovirus serotype, except for the absolute newest—even those can be expected soon, given the ease with which viral sequences can be generated [116]. Full-length cDNA clones are available for many serotypes, often downstream of a bacterial promoter than can be used to synthesize infectious RNA. Such clones have been invaluable in elucidating the function of individual viral proteins, as well as RNA structures that regulate viral translation and replication [192].

5 Descriptive Epidemiology

5.1 Key Features of Epidemiology of Enteroviruses

Over 100 enterovirus types have been identified, and many cause overlapping spectra of illness presentations, from subclinical or asymptomatic infections to central nervous system manifestations and sepsis. In particular, neonates and very young children are susceptible to more severe illness due to antigenic inexperience, and most children are exposed to enteroviruses early in life. However, some enterovirus infections may preferentially infect older age groups, and prognoses may vary. Parechoviruses are known to cause more severe disease in neonates and young children.

Enteroviruses are common and are primarily transmitted via the fecal-oral route. They may also be spread via respiratory droplets and from mother to infant prenatally and during the peripartum period. Enteroviruses may survive on surfaces

long enough that fomites may play a role in transmission. In addition, vesicle fluid may be another important route of transmission for children who have HFMD or other exanthems.

Outbreaks of enterovirus infections may be identified in communities and group settings. Families may spread enteroviruses among each other, and outbreaks often occur in day-care and school settings. Hygiene practices play an important role in transmission so viruses are often spread from child to child. Fecal shedding of enteroviruses may persist for several weeks, thus allowing children to potentially remain infectious for long periods.

In temperate climates, enteroviral infections are more often identified during summer and fall months, usually June–October (Fig. 11.1). However, in tropical climates, where sanitation may be poor and population density high, children may become infected with multiple enterovirus types and strains simultaneously and year-round. Because of the opportunity for increased transmission of enteroviruses in this setting, most neonates are born with maternal antibodies to a broader range of enterovirus types and are thus protected from illness. However, these children will acquire multiple enteroviral infections when maternal antibodies wane, usually in infancy and preschool ages.

5.2 General Epidemiology of Enteroviruses

5.2.1 Incidence and Prevalence

Seasonality of enteroviruses may be an indicator of disease incidence for common disease presentations such as HFMD. In summer and fall months, the percentage of specimens testing positive for enterovirus by NESS is elevated as compared to other seasons [36, 113]. Occurrences of clinical syndromes associated with specific enteroviral serotypes may serve as a marker of incidence of a particular enterovirus type in the community. Because surveillance is passive and dependent on case finding, the true incidence and prevalence of enteroviruses is unknown, and estimates of circulating enteroviruses are often obtained from outbreak investigations. For example, an epidemic of encephalitis in Uttar Pradesh, India, in 2008 was found to be associated with echovirus 19 and coxsackievirus B5 [121]. In 2007 in the United States, coxsackievirus B1 was the predominant type at the same time an outbreak of serious neonatal infections was reported [36, 270]. Outbreak investigations of clinical syndromes and associated enterovirus types will often correlate with predominant strains circulating in the community. Understanding trends of circulating serotypes may help predict increases in incidence and prevalence.

5.2.2 Epidemic and Endemic Behavior

Outbreaks of enterovirus infection/illness occur throughout the world. One set of types may be predominant for a time in a given geographic area, only to be succeeded by other types in subsequent years. There are two general patterns of circulation that describe enteroviruses in a population, endemic and epidemic activity. Endemic circulation within a population is best described as sporadic activity each year over a long period of time such that children acquire infection early in life. Most individuals have acquired antibodies within the first few years of life. Because of constant sporadic activity, a large population of nonimmune children will not occur; thus, it is unlikely a wide epidemic will follow. For example, in the United States between 1970 and 2005, coxsackieviruses A9, B2, B4, and enterovirus 71 were identified consistently at low levels of circulation with few distinct peaks during this time period [113]. The epidemic pattern is best described as large waves of circulation with longer periods of time between waves such that a nonimmune and therefore susceptible population accumulates, potentiating an outbreak when the virus is later introduced. Epidemic patterns of circulation in the United States from 1970 to 2005 were best exemplified by echoviruses 9, 13, and 30 and CVB5. Each of these viruses peaked and was among the most prevalent enteroviruses reported in a given year [113]. Interestingly, enteroviruses associated with endemic and epidemic patterns of circulation in one geographic area or country may exhibit a different pattern of circulation in another part of the world. Thus, enterovirus circulation patterns and surveillance priorities are unique to different areas in the world, and trends in enterovirus activity and identification of outbreaks should be an important priority such that information can be shared with other countries where a specific type may not yet be circulating.

Outbreaks of infections caused by enteroviruses and parechoviruses have occurred frequently in a wide variety of geographic regions at different times. Enterovirus 71, first isolated in California in 1969 from stools from an infant with encephalitis, is known to cause large outbreaks of HFMD, most frequently affecting young children. Over the next 3 years, 19 patients were identified with EV71 [5]. Notably, EV71 activity has been identified to some degree in many countries of the world, but the largest outbreaks have occurred more recently in the Asia-Pacific region. Large outbreaks of EV71 were identified in this region in the late 1990s, with the first report from Malaysia in 1997, followed by several outbreaks in other countries, including Singapore, Taiwan, and China [38, 141, 263, 288]. Although most affected children will have mild illnesses consistent with HFMD, a subset of children will have more serious clinical presentations with neurologic manifestations, including aseptic meningitis, acute flaccid paralysis, and brainstem encephalitis. The World Health Organization has drafted guidelines for clinical management and public health response to HFMD [275]. In Hong Kong, enterovirus 71 infection has been a notifiable disease since March 2009 [136]. These large outbreaks with associated severe neurologic disease appear to be restricted to the

Asia-Pacific region, while in other parts of the world EV71 is generally associated with uncomplicated HFMD with few serious cases. The reasons for this geographic disparity are unclear but could include genetic differences or cultural differences in treatment. Enterovirus 71 is discussed in more detail in Chap. 13.

Enteroviruses that cause acute hemorrhagic conjunctivitis (AHC) may cause large epidemics in specific regions of the world. AHC, primarily caused by EV70 and CVA24 variant, is a rapidly progressive and highly contagious disease. AHC was first recognized in 1969 in Ghana [40] and, at the time, was sometimes termed "Apollo eye disease" as its emergence coincided with the first moon landing. During a 4-month period, 13,664 cases were seen at one clinic, and all had subconjunctival hemorrhages associated with conjunctival inflammation. A subsequent pandemic occurred from 1969 to 1971, when hundreds of millions of people were estimated to have developed AHC. Since then, epidemics have been periodically identified, mostly in tropical regions of the world. Outbreaks of AHC caused by CVA24v have occurred in Singapore, Brazil, Uganda, Southern Sudan, China, and Cuba [35, 70, 246, 280, 284].

5.2.3 Geographic Distribution and Climate

Enteroviruses are most often identified during the summer to fall months in temperate climates. In the United States, 77.9 % of enterovirus cases were reported from June to October [113]. Likewise, NESS reported the majority of enterovirus detections during July–October in 2008 [36] (Fig. 11.1). However, seasonality is less pronounced in tropical climates, with the peak incidence generally occurring during the rainy season. Prevalence tends to be highest in the tropics and lowest in cold regions. Children who live in tropical climates and who may be exposed to poor hygienic circumstances may transmit enteroviruses year-round.

5.2.4 Age and Sex

Enteroviruses may cause disease in any age group, although young children are the primary reservoir for these infections. In the United States during 1970–2005, among reports where age was known, 44.2 % of cases were aged <1 year [113]. Similarly, from 2006 to 2008 from reports where age was known, 47 % of laboratory detections of enteroviruses and parechoviruses were from children aged ≤1 year [36]. In a recent study performed in Spain, 76.8 % of all meningitis cases where a pathogen was isolated were positive for enteroviruses and 65 % of the meningitis cases identified were in children ≤15 years [58].

Children may be most susceptible to enteroviral infections, but epidemiologic and clinical features of disease can vary across age groups. EV71 causes more severe neurologic manifestations in young children, particularly in Asia-Pacific countries. For example, in 2008 in Taiwan, a younger age was associated with severe central nervous system complications [263]. During an investigation of enterovirus-associated acute respiratory infections in older children and adults in China from 2006 to 2010, the two most frequently detected types were identified in two distinct age groups. The median age of patients with CVA21 was 22 (15–67) years, while the median age for EV68 detection was 34 (18–67) years [278].

Male preponderance has been documented among surveillance data and descriptions of outbreaks of enteroviral illnesses. In the United States from 1970 to 2005, males predominated in the <20-year age group, but no gender preponderance was seen in the ≥20-year age group (male/female ratios, 1.4 and 0.9, respectively) [113]. Surveillance data for echovirus 6 and echovirus 9 infections in Taiwan from 2000 to 2008 revealed a male preponderance for both types; 57 and 69 % of echovirus 6 and echovirus 9 cases, respectively, were male [124]. Among pediatric HFMD cases in Shanghai from 2009 to 2010, 61 % were male [286].

5.2.5 Occurrence in Families and Closed Ecological Units

Transmission of enteroviruses is a frequent event in families and contained group settings. The New York Virus Watch Program in the 1960s demonstrated that spread of coxsackieviruses within families was relatively high, 76 %, as compared to spread of echoviruses at 43 % [118]. The reason for this discordance is unknown. Outbreaks of enteroviruses may occur in day-care settings, preschools, and summer camps; in particular, clusters of illnesses will be more likely identified in group settings of younger children. Outbreaks have been described at summer camps [91, 140], where children were involved with multiple group activities and lived in cabins or crowded dwellings. Enterovirus outbreaks characterized by HFMD or aseptic meningitis have occurred in schools for younger children [2, 276]. Huang et al. reported an outbreak of EV71 infection among infants in a newborn nursery [99]. Seven of 19 infants were documented with clinical illnesses that included fever, poor activity, and drowsiness. Another nosocomial outbreak involved transmission of echovirus 30 from an index patient to five babies within a neonatal nursery [9]. In addition, a nosocomial outbreak of HPeV1 was described in a neonatal postintensive care unit in Croatia [132]. Seven patients, six of whom were born prematurely, were documented to have had HPeV1. Transmission of enteroviruses among older children and adolescents in a defined group has also been documented, including an outbreak of CVB2 infection identified among members of a high school football team [4].

5.2.6 Socioeconomic Setting

Low socioeconomic status has been associated with increased transmission of enteroviruses, most probably due to poor hygiene and high population density. Low socioeconomic

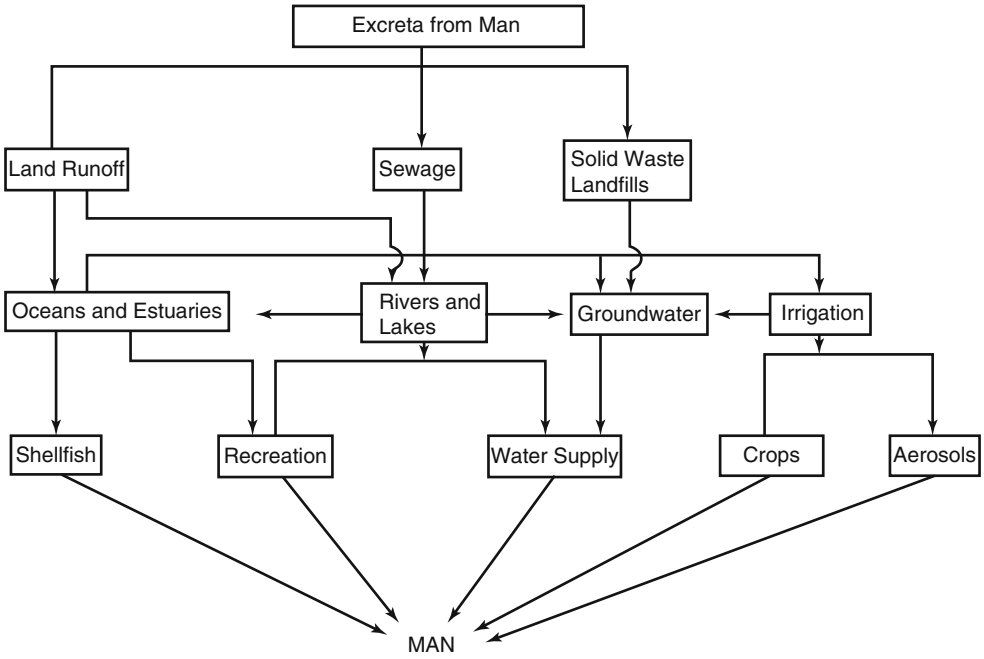

Fig. 11.3 Routes of enteric virus transmission (From Melnick et al. [156], used with permission)

settings have played a role in increased acquisition of enteroviruses in both temperate and tropical climates. Neonatal acquisition of enteroviruses was found to be associated with lower socioeconomic status in an urban area in the United States [107]. In tropical climates, where enteroviruses are transmitted continuously throughout the year, poor sanitation has been shown to be a factor in frequency of virus infection among infants [195].

6 Mechanisms and Routes of Transmission

Enteroviruses are primarily spread by the fecal-oral and respiratory routes (Fig. 11.3). Lack of hygiene will facilitate spread of virus person to person especially in areas of poor sanitation. Because individuals may shed virus in the stool for several weeks, fecal contamination of fingers and hands may lead to efficient spread particularly in young children. Droplets or aerosols may play a role in transmission and will also contribute toward contamination of the hands of young children. Under optimal conditions, which include temperature and pH, enteroviruses may survive for weeks in the environment, so fomites may play a role in viral spread as well.

Select enterovirus types may be spread from person to person by other specific routes. EV70 and CVA24 variant are causes of acute hemorrhagic conjunctivitis. Transmission of these viruses from person to person is most likely through eye secretions, as virus has been isolated from conjunctival swabs in outbreak settings [280]. In addition, enteroviruses that cause rashes may be transmitted from person to person via vesicular fluid which contains infectious virus.

Transmission from mothers to babies may occur prenatally, during the peripartum period, and possibly through breastfeeding. Mothers who have symptomatic or subclinical infections may transmit virus to neonates which can result in aseptic meningitis or sepsis-like disease. A study performed in Taiwan among pregnant women who had herpangina demonstrated an increased risk of infection in low birth weight and small for gestational age babies, as well as preterm delivery [41]. Enteroviruses have been detected in breast milk, though the role of breastfeeding in transmission from mothers to babies is unclear [139].

Shedding of enteric viruses, such as enteroviruses, in the stool raises the possibility of contamination of recreational waters. Enteroviruses have been identified in clams or oysters, and in recreational hot spring waters, but the role of shellfish and water in direct transmission to humans remains unclear [42, 98, 134]. Outbreaks of viral meningitis have been associated with swimming in ponds and pools [33, 109]. In 2001 in Germany, an outbreak of echoviruses 13 and 30 was found to be associated with swimming in a pond [90]. An investigation of an echovirus 30 outbreak among children in Italy in 1997 showed the risk of meningitis-like illness to be higher among children attending a common school, among children who swam at any public pool, and those who swam at a specific pool [68]. In Mexico in 2004, an outbreak of echovirus 30 and coxsackievirus A1 infections among travelers was associated with swimming in sewage-contaminated seawater [12]. Enteroviruses have the potential to contaminate recreational waters and may pose a health risk to swimmers.

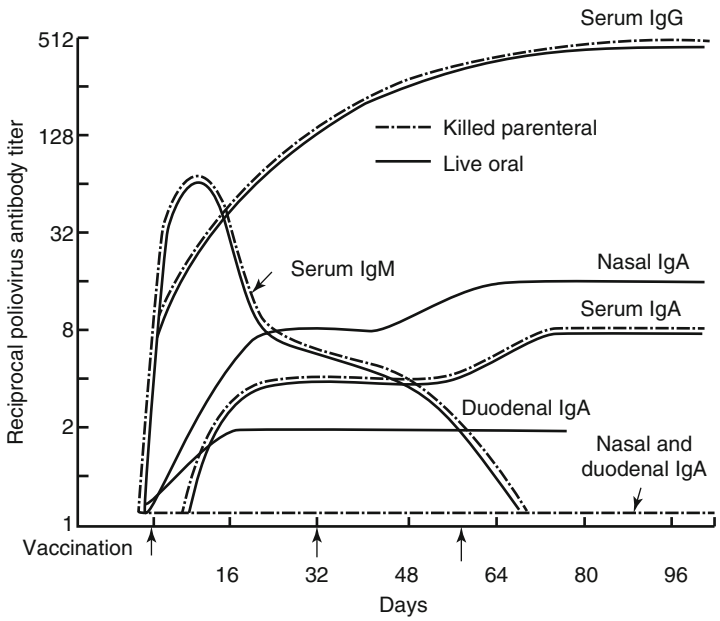

Fig. 11.4 Serum and secretory antibody response to oral administration of live attenuated polio vaccine and to intramuscular inoculation of killed polio vaccine (From Ogram et al. [192], used with permission)

7 Pathogenesis and Immunity

7.1 Pathogenesis

The enteroviruses and parechoviruses are transmitted primarily by the fecal-oral route, with entry through the alimentary tract, though respiratory transmission may also occur. The incubation period is generally 7–14 days, but it can range from as few as two to as long as 35 days. Initial replication occurs in the pharynx and gut, probably in lymphoid tissues such as Peyer's patches. In some cases, a brief viremia may be followed by replication at secondary sites in specific target organs such as the spinal cord and brain, meninges, myocardium, muscle, or skin, with some variability by strain or type. Virus is excreted in stool for 4–8 weeks postinfection and in respiratory secretions for 1–2 weeks [6]. Mixed infections and detection of multiple enteroviruses in a single stool sample are not uncommon, especially in areas with poor sanitation and high population density [188, 190, 204].

EV70 and certain strains of CVA24 are unique in that they can cause explosive outbreaks of acute hemorrhagic conjunctivitis, sometimes of pandemic proportions. These viruses are rarely detected in stool or respiratory specimens, but they can be readily detected in conjunctival swabs [199].

7.2 Immunity

Enterovirus infection elicits a strong humoral immune response. In young children, the response tends to be type specific, whereas older children and adults tend to mount a more heterotypic response, probably due to previous exposures to other enterovirus types and the presence of shared, cross-reactive epitopes [61, 208]. Enterovirus immunity in humans has been most easily studied using polio vaccine as a model system (Fig. 11.4) [191]. Antibody plays the major role in protection from disease. A type-specific poliovirus serum neutralization titer of 1:8 protects from paralytic disease; however, even very high titers do not protect from reinfection by the homologous virus. Maternal antibody passively transferred to the infant gradually wanes during the first 6 months of life, with a half-life of approximately 28 days. Type-specific immunity is also transferred from mother to infant via milk [145]. Upon exposure to virus, neutralizing antibody appears within a few days, often before the onset of symptoms; antibody may persist for life [209]. The tonsils and adenoids appear to play a role in development of immunity, as surgical removal decreases secretory antibody levels in the nasopharynx and resistance to poliovirus infection is also decreased.

Persons with primary immunodeficiency disorders, especially those with profound antibody deficits, are at increased risk for enterovirus disease, as they lack the ability to clear the infection [93, 142, 173, 181, 213, 235, 271, 290]. Such persons may become chronically infected and excrete virus over an extended period [93, 142]. There has been some suggestion that treatment with intravenous immune globulin is helpful in some cases, but, overall, results have been mixed. Patients treated for B-cell lymphoma with monoclonal antibody drugs such as rituximab appear to be at increased risk for persistent enteroviral infection and disease [8, 198], probably through depression of B-cell function [255].

8 Patterns of Host Response

8.1 Clinical Syndromes

Enteroviruses may cause a wide spectrum of clinical syndromes, with severity ranging from asymptomatic presentations to acute flaccid paralysis, encephalitis, and myocarditis. Specific enterovirus types may be associated with a particular clinical syndrome or multiple clinical presentations. In addition, a particular clinical syndrome may be caused by multiple enterovirus types. The ability of individual enterovirus and parechovirus types to cause overlapping clinical syndromes emphasizes the notion that typing in the laboratory is necessary for diagnosing a potential cause of an enterovirus illness.

8.1.1 Asymptomatic Infections

The vast majority of enterovirus infections are asymptomatic or result in only very minor symptoms such as lethargy and general malaise, transient low-grade fever, or minor upper respiratory tract illness [77, 171, 201, 225]. Despite the lack of discernible illness, persons with asymptomatic infections may excrete virus for long periods and participate in transmission.

8.1.2 Meningitis, Encephalitis, and Acute Flaccid Paralysis

Neurologic manifestations caused by non-polio enteroviruses and parechoviruses include aseptic meningitis, encephalitis, and acute flaccid paralysis. Aseptic meningitis is characterized by fever, myalgia, nuchal rigidity, nausea, and vomiting [227]. In small children <2 years of age, fever and irritability are the predominant symptoms [221]. In a prospective study of viral infections of the central nervous system in Spain from March 1, 2008 to February 28, 2009, the most common cause of meningitis was an enterovirus [58]. Aseptic meningitis may be caused by many enterovirus types including echoviruses 4, 6, 9, 11, 14, 16, 25, 30, 31, and 33 and coxsackieviruses B1–B6, A7, and A9. EV71 has frequently been associated with aseptic meningitis [193]. Ultimately, virtually all enteroviruses are capable of causing aseptic meningitis at some frequency.

Meningoencephalitis, aseptic meningitis with inflammation involving the brain parenchyma, and encephalitis without aseptic meningitis may also be caused by enteroviruses. Although an etiology is not associated with most cases of encephalitis, enteroviruses are consistently identified. From 1998 to 2005, the California Encephalitis Project reported nearly 1,600 encephalitis cases, of which 4.6 % were either confirmed as enterovirus etiology by detection in CNS samples or of possible enterovirus etiology, by detection in other specimen types [71]. Enterovirus encephalitis cases tended to be younger and had less severe symptoms than those with other identified etiologies. Similarly, the New York State Department of Health identified 21 cases of encephalitis due to enteroviruses from June 2004 to September 2007 [63].

EV71 is known to cause brainstem encephalitis and acute flaccid paralysis, particularly in young children in Asia-Pacific countries. Brainstem encephalitis is often associated with cardiorespiratory symptoms which may include neurogenic pulmonary edema. Severe cases of EV71 disease may also include acute flaccid paralysis as the presenting feature and anterior horn cell destruction may be identified as well [193].

8.1.3 Pleurodynia (Epidemic Myalgia, Bornholm Disease)

Pleurodynia is described as fever with accompanying chest and upper abdominal pain. It is characterized by abrupt onset of fever and lower chest pain, sometimes preceded by malaise, headache, and anorexia [13, 97, 268]. The chest pain may be located on either side or substernally, is intensified by movement, and may last from 2 days to 2 weeks. Abdominal pain resulting from involvement of the diaphragm occurs in approximately half the cases; in children, this often takes the place of chest pain and may be the chief complaint. The syndrome was first described after an outbreak on the island of Bornholm in Denmark. Epidemic pleurodynia or Bornholm syndrome is usually caused by group B coxsackieviruses. Less commonly, this syndrome may be caused by echoviruses and group A coxsackieviruses. A recent outbreak of epidemic pleurodynia caused by coxsackievirus B3 included clinical descriptions of pain with deep inspirations [100]. These findings may indicate inflammation of the pleura as part of this syndrome. The illness is self-limited, and recovery is complete with no long-term sequelae.

8.1.4 Herpangina

Herpangina is caused by a wide variety of different group A coxsackievirus types [82]. The illness is characterized by an abrupt onset of fever and sore throat. There may be anorexia, dysphagia, vomiting, and abdominal pain. The pharynx is usually hyperemic, and a few (not more than 10–12) characteristic tiny discrete vesicles with a red areola occur on the anterior pillars of the fauces, the posterior pharynx, the palate, the uvula, the tonsils, or the tongue. The illness is self-limited and occurs most frequently in small children.

8.1.5 Hand, Foot, and Mouth Disease

Hand, foot, and mouth disease (HFMD) is a common illness in young children. HFMD is characterized by almost universal occurrence of enanthem, usually on the buccal mucosa, generally followed by exanthem on the palms of the hands and soles of the feet. The oral lesions are ulcerative, while lesions on the hands and feet are usually vesicular. CVA16 and EV71 are the most common causes, though other group A coxsackieviruses are also frequently associated with HFMD, especially CVA6 and CVA10. The disease is usually mild and self-limiting, but more severe illness has accompanied large HFMD outbreaks due to EV71, primarily in Asia.

In 2010–2012, there were large outbreaks of hand, foot, and mouth disease due to coxsackievirus A6 in Asia, Europe, and North America [37, 69, 76, 80, 133, 162, 194]. These outbreaks were somewhat unusual in that illness was often more severe, with a more pronounced, often pustular-appearing rash that involved the face and trunk as well as hands and feet; in addition to young children, adults were also affected, often in the context of day-care centers or other exposures to very young children [37]. HFMD may sometimes be accompanied by onychomadesis (shedding of nails) sometime after resolution of initial symptoms [56, 194, 267]; however, the nails usually regrow without complication.

8.1.6 Respiratory Illness

Many different enteroviruses, from each of the traditional taxonomic groups and each of the current viral species, have been associated with mild upper respiratory tract illness [55, 165]. CVA21 has caused outbreaks of pharyngitis in military recruits and has induced respiratory tract disease in normal adult volunteers. For the most part, severe disease is rare, but EV68 was initially isolated from children with bronchiolitis and pneumonia [233]. More recently, EV68 has been associated with sporadic cases and outbreaks of bronchitis, bronchiolitis, and pneumonia [103, 104, 106, 143, 186, 215].

8.1.7 Eye Disease

Infection with a number of different enteroviruses has been occasionally accompanied by conjunctivitis. However, in 1970, an explosive epidemic of acute conjunctivitis occurred in Singapore, with some 60,000 cases reported. The causative agent was identified as an antigenic variant of CVA24 [128, 163]. Additional large outbreaks occurred elsewhere in Asia in subsequent years [39, 129, 282, 283], and CVA24 variant conjunctivitis has been reported globally since then [28, 29, 32, 64, 70, 111, 253]. Conjunctivitis associated with CVA24 variant is generally mild, with complete recovery in 1–2 weeks. The only potentially serious sequelae are due to secondary bacterial infections.

Beginning in 1969 and extending to 1971, a somewhat different form of conjunctivitis emerged in Africa, India, Southeast Asia, and Japan, reaching pandemic proportions, with tens of millions of cases. This pandemic of acute hemorrhagic conjunctivitis (AHC) was due to a new enterovirus, EV70 [120, 163, 281]. About 10 years later, AHC reappeared, causing outbreaks in several of the countries initially affected and spreading to the Caribbean and tropical Latin America, with a few cases reported in the continental United States [207], as well as several Pacific islands. AHC is characterized by severe eye pain, photophobia, and blurred vision, accompanied by subconjunctival hemorrhage which can vary in extent from discrete petechiae to large blotches. The incubation period is about 24 h, onset is sudden, and recovery is usually complete within less than 10 days.

For both forms of enteroviral conjunctivitis, virus can be readily detected in conjunctival swab and throat swab specimens by PCR. While CVA24 is excreted in feces, EV70 is only rarely detected in stool [163].

8.1.8 Cardiac Diseases

Acute Cardiac Disease

Group B coxsackieviruses are known to be associated with myocarditis. CVB3 is the most common cause of human viral myocarditis, although group A coxsackieviruses and echoviruses have been identified in association with cardiac disease [274]. Most cases of group B coxsackievirus myocarditis occur in young adults, though severe coxsackievirus B myocarditis can also occur among neonates. Although a number of serological studies have attempted to correlate myocarditis with enterovirus seropositivity or antibody titer, there has not been a clear association between viral antibody and causality of myocarditis. Endomyocardial biopsy is the diagnostic test of choice to determine etiology of viral myocarditis [137].

Chronic Cardiovascular Disease

The pathogenesis of chronic heart disease due to enterovirus-associated dilated cardiomyopathy has been thought to begin with acute myocarditis and progress over time. Although enteroviruses have not been isolated directly from these chronic cardiac disease patients, the presence of enteroviral RNA has been detected [138].

8.1.9 Neonatal Disease

Acquisition of enteroviruses is common during the first month of life [164]. In one study, 12.8 % of neonates were found to have enterovirus infections, although most of these infections were asymptomatic [107]. Of over 26,000 enterovirus detections reported to NESS during 1983–2003, 11.4 % of those with known age were from neonates (age $\leq$ 1 month) [112]. The 10 enterovirus types most frequently reported were echoviruses 11, 9, 6, and 30 as well as coxsackieviruses B1–B5 and CVA9. The risk of a fatal outcome was higher for neonates as compared to reports where age was noted to be $\geq$1 month. Infection with CVB1–4 and echoviruses 11 or 25 was significantly associated with increased risk for fatal outcome, with odds ratios of 1.4–2.3. Similarly, neonates comprised 9.5 % of patients from whom enterovirus was isolated during a 2-year study in the Netherlands [256].

Severe illnesses caused by enteroviruses in this age group include sepsis, meningoencephalitis, hepatitis, and myocarditis [247]. A study of neonatal enterovirus infections in a Taiwan children's hospital revealed 43 cases of nonspecific febrile illness, 61 cases of aseptic meningitis, and 42 cases of hepatic necrosis with coagulopathy [131]. Of these 42 cases, 10 were fatal; eight of the 10 fatalities were associated with group B coxsackievirus infections (four CVB1, three CVB3,

and 1 CVB2), with the remaining two cases due to echovirus 4 and an untypable enterovirus. In the United States, from 2007 to 2008, CDC received reports of six neonatal deaths associated with CVB1 infection, all of which were due to multisystem organ involvement [270]. During the outbreak, a cluster of eight neonates with severe myocarditis due to closely related strains of CVB1 was also described [258]. An additional two infants, one with a diagnosis of CVB1, were identified. Of these ten neonates, one patient died and one patient required cardiac transplantation. In the Netherlands, Freund et al. reported a case series of seven patients with severe neonatal myocarditis, of which two died and the remaining five infants had cardiac sequelae [73].

Human parechoviruses have been associated with severe disease in young infants [19, 25, 88, 257, 273]. In Spain, eight (3 %) of 264 CSF samples collected from children <2 months from January 2006 to September 2009 were positive for human parechoviruses [210]. Human parechovirus was identified in 7.4 % of CSF samples tested in 2006–2008 in a regional children's hospital in the Midwestern United States [237]. Associated clinical syndromes were mostly associated with sepsis and meningitis. Notably, CSF pleocytosis was rarely documented. Likewise, another case series identified ten children, aged 6–59 days, with parechovirus type 3 associated with workups for sepsis and meningitis [262]. HPeV3 was identified in 51 (13 %) of 388 CSF samples from children aged <6 months from a children's hospital in Kansas City in 2009 [239]. CSF pleocytosis is often absent [239, 257]. HPeV3 has been specifically associated with more severe disease in neonates, compared with HPeV1 [19].

8.1.10 Gastrointestinal Diseases

Group B coxsackieviruses have been associated with inflammation of the pancreas. Coxsackievirus-associated pancreatitis has been described in case reports [50, 197]. Mouse models of pancreatitis induced by group B coxsackieviruses have demonstrated pancreatic damage that may be similar to human disease [101].

8.1.11 Diabetes

While the connection is not yet conclusive, a number of studies have demonstrated an association between enterovirus infection and development of prediabetic autoimmunity or onset of type 1 diabetes in individuals at genetic risk for diabetes [44, 92, 168, 230]. Several ongoing longitudinal studies have also been designed to address the association between a variety of potential environmental triggers with diabetes onset, including enterovirus or other infections, foods, and other factors [reviewed in reference 244].

Helfand et al. tested sera from 128 children with new onset insulin-dependent diabetes mellitus and 120 matched controls [92]. The sera were tested for enterovirus-specific IgM using 14 different serotypes as antigen. Case children were significantly more likely than controls to have IgM to one or more of the enteroviruses tested, with the strongest association in the age group 13–18 years. A second study used RT-PCR to assess the presence of enterovirus RNA in three groups of children: 47 with newly diagnosed type 1 diabetes, 50 positive for at least one common islet autoantibody, and 50 negative for islet autoantibodies [168]. Enteroviral RNA was detected in 36 % of those with newly diagnosed diabetes, 20 % of the autoantibody-positive group, and only 4 % of the autoantibody-negative group, again suggesting an association between enterovirus infection and islet autoimmunity or diabetes. Similarly, another RT-PCR study detected enteroviral RNA in serum of 26 % of children with newly diagnosed type 1 diabetes versus 3 % of matched controls [230]. The latter study is significant in that it was conducted in Cuba, a country with high enterovirus prevalence and low rate of type 1 diabetes, a situation in which one might expect it to be difficult to demonstrate the association.

8.1.12 Summer Minor Illnesses with or Without Exanthems

Enteroviruses are often isolated from patients with undifferentiated acute febrile illnesses of short duration occurring during the summer or fall. In young children, the illness may be accompanied by a rubelliform, maculopapular rash on the face, neck, and chest. The incidence of rash tends to be higher in young children and decreases with age. Conjunctivitis may also be present. Echovirus 16 has been associated with outbreaks of "Boston exanthem disease," characterized by fever and maculopapular rash [171, 172].

9 Control and Prevention

There are currently no licensed antiviral therapies available to treat enterovirus or parechovirus infections or illness, but it is an area of active research and a number of compounds are in preclinical development [59, 249, 250]. Similarly, there are no vaccines available to prevent disease due to non-polio enteroviruses or parechoviruses. However, there is interest in developing a vaccine for EV71, particularly in Asia, where large outbreaks have occurred over the last 15 years, with a considerable burden of severe disease and hundreds of fatalities. One EV71 vaccine candidate has recently completed a phase II immunogenicity trial, with favorable results [289]. There has been apparent success with the use of immunoglobulin for prophylaxis and control of an outbreak of enterovirus infection in a nursery [75].

Prevention and control generally depend on stressing good personal hygiene, including hand washing and thorough cleaning of potentially contaminated items such as toys, etc., especially in settings such as day cares where very young children may be together. In a hospital setting, enteric

precautions are important, as nosocomial infections can be particularly serious in newborns and immunocompromised patients. Cohorting of patients and staff can also be useful to control transmission during an outbreak. In some cases, further virus spread has been limited by closing newborn nurseries to new admissions during an outbreak among newborns.

10 Unresolved Problems

Despite decades of clinical and basic research, there remain a number of key gaps in our knowledge of enterovirus and parechovirus disease, pathogenesis, and epidemiology. For example, "neonatal enteroviral sepsis" is well known as a potential outcome of perinatal infection, yet the burden of disease has not been described. One could imagine that having a reasonable estimate of the incidence of this life-threatening illness in such a highly vulnerable population might stimulate efforts to develop effective antiviral therapies. Enterovirus infection is generally within the differential diagnosis in cases of febrile or sepsis-like presentation in neonates; enterovirus RT-PCR is part of the standard of care, but the capacity to type, as well as detect, enteroviruses may be important to monitor trends in severe illness.

Similarly, we are just beginning to appreciate the role of human parechoviruses and their contribution to the overall burden of disease in young children. While enterovirus testing may be standard in most medium and large clinical centers, parechovirus RT-PCR is available in very few clinical laboratories. Several studies have detected enteroviruses and parechoviruses at similar rates in CSF in young children with aseptic meningitis. Given that enteroviruses are considered the major cause of aseptic meningitis in the United States and Europe, these data suggest that parechoviruses are a significant contributor to illness in this age group; if one considers antivirals against enteroviruses a worthwhile proposition, then one could also make a strong argument for antivirals targeting parechoviruses. It will be important to introduce parechovirus testing into the routine for diagnostic testing in cases of suspected enterovirus illness.

Enterovirus 71 remains an enigma—how can a virus that circulates more or less benignly in Europe and North America, causing HFMD and the occasional aseptic meningitis or encephalitis case with very few fatalities, become such a menace in Asia, with infections in the millions and deaths in the hundreds? Clearly there is a great deal to be learned about the epidemiology of EV71 in this setting. Is there a genetic component or some other Asia-specific factor that predisposes to severe disease? Development of an EV71 vaccine, probably based on the model of inactivated polio vaccine, is a high priority in the countries most affected by the large-scale EV71 outbreaks.

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Enteroviruses: Enterovirus 71

12

Mong How Ooi and Tom Solomon

1 Introduction and Historical Background

The global polio eradication campaign has eliminated this devastating enteroviral disease from Europe, the Americas, and most of Africa and Asia. However, in the past 15 years, a related virus, human enterovirus 71 (EV71), has emerged across Asia, where it threatens to become the "new polio" [120]. EV71 first appeared in California in the 1960s and subsequently caused sporadic cases or small outbreaks of hand-foot-and-mouth disease (HFMD) [120], neurological disease, or both. In 1997, the virus caused an unexpectedly large and severe outbreak with many fatalities in Sarawak, Malaysia. Since then, countries of the Asia-Pacific region have been hit by regular epidemics of EV71, including an epidemic in Taiwan in 1998, in which millions of people were thought to have been infected, and an outbreak of HFMD in China in 2009, during which nearly 500,000 cases were reported [13, 16, 33, 55, 56, 76, 98, 112, 146, 171, 177]. Although the virus circulates worldwide, the largest outbreaks of disease have so far been largely confined to the Asia-Pacific region, for reasons that are incompletely understood [12, 13, 16, 17, 33, 56, 70, 76, 98, 136, 146, 176, 178]. The neurological manifestations range from aseptic meningitis to acute flaccid paralysis and brainstem encephalitis. They are often accompanied by systemic complications such as severe pulmonary edema and shock, which are thought to be neurogenic in origin [22, 63].

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2 Descriptive Epidemiology

2.1 Initial Epidemiological Observations

Although EV71 was first isolated from the stool of a 9-month-old infant with encephalitis in California in 1969, a retrospective analysis of samples from the Netherlands showed that EV71 was circulating as early as 1963 [128, 147]. Before long small outbreaks of neurological infection including encephalitis and aseptic meningitis attributed to the newly identified neurotrophic virus were reported in New York, Melbourne, and Sweden in the early 1970s [9, 43, 74]. The dermotropic properties of EV71 were first recognized during epidemics of HFMD in Japan in 1973 [54, 67]. In the 1970s, two large and severe EV71 epidemics occurred in Europe. The first was in Bulgaria in 1975, initially attributed to polioviruses because its epidemiological, clinical, and pathological characteristics mimicked those of poliomyelitis [40, 137]. In fact, at the height of the epidemic, nationwide administration of live-attenuated poliovirus vaccine was instituted. Isolation of EV71 later confirmed this virus as the etiological agent in 347 (77 %) of 451 children who presented with nonspecific febrile illness or neurological disease, of whom 44 died. The second major European epidemic occurred in Hungary in 1978, with 1,550 cases (826 aseptic meningitis and 724 encephalitis) and 47 deaths. Unlike the Bulgarian epidemic, the Hungarian one included a small number of patients with HFMD [103].

2.2 EV71 Activity in the Asia-Pacific Region

After the Australian and Japanese EV71 epidemics of the 1970s, further small epidemics and sporadic clusters occurred in Hong Kong (1985) and Australia (1986) [51, 126]. Then in 1997, a large outbreak in Sarawak, Malaysia, heralded the beginning of a series of large outbreaks across the Asia-Pacific region. These continuing epidemics have established EV71 as a major public health threat across the region (Table 12.1) [11, 12, 27, 39, 64, 66, 71, 79, 82, 97, 136, 146, 177, 178].

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Table 12.1 EV71 genogroups circulating in Asia-Pacific region between 1973 and 2012 [11, 12, 27, 39, 64, 66, 71, 79, 82, 88, 97, 105, 121, 125, 136, 143, 146, 166, 177, 178]

Countries	1973	1980	1986	1988	1990	1993	1994	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Singapore	–	–	–	–	–	–	–	–	B3, B4	B3, C1	B3	B4	B4	C1, B4	B4	–	–	B5	–	B5, C2	–	–	–	–
Peninsular Malaysia	–	–	–	–	–	–	–	–	3, B4, C1[a], C2	C1	B4, C1	B4, C1	–	–	–	–	B5, C1	–	–	–	–	–	–	–
Sarawak, Malaysia	–	–	–	–	–	–	–	–	B3	C1	None	B4, C1	None	C1	B5, C1	None	B5	B5	–	B5	B5	–	–	B5
Perth, Australia	–	–	–	–	–	–	–	–	–	–	B3, C2	C1	None	None	–	–	–	–	–	–	–	–	–	–
Australia	–	–	–	–	–	–	–	–	–	C2	B3, C2	B4, C1	B4, C1	C1	C1	C4	–	–	–	–	–	–	–	–
Japan	B1	–	–	–	B2, C1	B2	C3		B3, B4, C2	C2	C2	B4	C2	B4, C2	C4, B5	C4	–	–	–	–	–	–	–	–
Taiwan	–	B1	B1	–	–	–	–	–	–	C2, B4[a]	B4	B4	B4	B4, C4[a]	B4, B5[a]	C4	C4, C5[a]	C5	B5, C5	B5, C2[a], C4[a], C5[a]	B5	C4	–	–
Korea	–	–	–	–	–	–	–	–	–	–	–	C3	None	None	C4	–	–	–	–	–	C4	–	–	–
Brunei	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	B5	–	–	–	–	–	–
Vietnam	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C1, C4, C5	–	–	–	C4	–	C4	–
Thailand	–	–	–	–	–	–	–	–	–	–	–	–	–	C1	C1	C1	–	B5, C1, C2, C4	B5, C1, C2, C4, C5	B5[a], C1, C2[a], C4	B5[a], C1[a], C2[a], C4	–	B5	–
China	–	–	–	–	–	–	–	C2	–	C4	–	C4	C4	C4	C4	C4	–	–	C4	C4	C4	C4	C4	–
Cambodia	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C4
Hong Kong	–	–	–	C1	–	–	C1	–	C2	C4	B3, C1, C4	C1, C4	B4, C4	C1, C2	C4	C4	C4	C4	C2, C4	B5, C2, C4	–	–	–	–

Subgenogroups underlined are those which caused large outbreaks
[a]Subgenogrous that were isolated in a smaller number
None: No HEV71 was detected despite active surveillance
– No data

Table 12.2 Clusters and outbreaks of EV71 infection occurring outside the Asia-Pacific region, 1988–2010

Country	Year	Ages	*N*	Clinical features and comments	Reference
Brazil	1988–1990	Children	90	IgM to EV71 in 20 blood samples of patients with flaccid paralysis	[42]
Finland	1994–2010	Children <11 years	928	Neutralizing antibodies in 1.6 % of serial serum samples	[58]
	1996–2008	Children <6 years	359	PCR positive in 0.3 % of serial stool samples	
Central African Republic	1997–2006	Children	93	EV71 in stool samples of patients with flaccid paralysis	[7]
Canada	1998	Children	20 EV71	Aseptic meningitis; respiratory symptoms	[100]
Brazil	1998–2001	Children ≤15 years	389	Neutralizing antibodies in 222 serum samples	[14]
Kenya	1999–2000	Children	–	–	[15]
France	2000–2009	Mostly children	81 EV71	Neurological disease, HFMD, fever rash, gastrointestinal symptoms, respiratory symptoms, and gingivostomatitis; 2 fatal: cardiorespiratory failure and encephalitis	[141]
Austria	2001–2004	Children	181 with aseptic meningitis	16 with EV71 in stool	[113]
United Kingdom	2001–2006	–	32 EV71	Most with neurological disease or HFMD	[8]
Norway	2002–2003	Children	19 EV71	Asymptomatic	[161]
Denver, USA	2003, 2005	Children: 4 weeks to 9 years	15 EV71	1 fatal	[116]
Denmark	2005–2008	Mostly infants	29 EV71	Aseptic meningitis, gastroenteritis, HFMD	[130]
Netherlands	2007	–	58 EV71	Fever, gastrointestinal, and neurological symptoms	[147]
Greece	2009–2010	Children	6 EV71	Fever, rash, or HFMD	[138]

During the Sarawak outbreak, between May and July 1997, a total of 2,618 HFMD cases and 34 deaths were recorded. EV71 also caused four deaths in peninsular Malaysia and a number of cases of severe neurological disease in Japan [13, 76, 92]. Another large epidemic occurred in Taiwan in 1998 when 405 children were hospitalized for serious neurological complications and 78 died. Epidemiological studies estimated that almost 1.5 million people were infected with the virus [56]. The largest epidemic to date occurred in China in 2008; approximately 490,000 children including 126 deaths were reported nationwide. At the epicenter in Anhui Province, more than 6,000 HFMD cases and 22 deaths were reported [178]. Surveillance improved following designation of HFMD as a notifiable disease in China in May 2008, and regular massive epidemics with alarming number of deaths were recorded. There were 1.1 million cases (353 deaths), 1.8 million cases (905 deaths), 1.6 million cases (509 deaths), and 2.2 million cases (567 deaths) reported in 2009, 2010, 2011, and 2012, respectively (http://www.chinacdc.cn/tjsj/fdcrbbg/index_1.html, accessed 15/7/2013). In addition to these very large outbreaks, many countries, including Sarawak, Taiwan, Singapore, Vietnam, and Japan, have experienced cyclical epidemics at 2- to 3-year intervals [59, 101, 119]. The most recent EV71 epidemic occurred in Cambodia in 2012. The outbreak, first labeled as a mysterious illness by local media, was associated with more than 50 sudden deaths within 24 h of hospitalization in children who were 3 years of age and below [129].

The clinico-epidemiological features of the EV71 epidemics in Asia in recent years differ considerably from earlier epidemics in that in addition to HFMD, aseptic meningitis, and flaccid paralysis, brainstem encephalitis associated with cardiopulmonary dysfunction also occurs, which has been the principal cause of death in most fatal cases in Asia [13, 63, 120, 146, 178]. The affected children have typically presented with a short febrile illness accompanied by subtle neurological signs, following which they develop signs of tachycardia, poor perfusion, and tachypnea, which rapidly develops into acute intractable cardiac impairment and fulminant, often fatal, pulmonary edema or hemorrhage [111]. Diagnostic imaging and autopsy examination indicate that this is associated with encephalitis in the brainstem, especially the medulla, and neurogenic pulmonary edema is thought to be the primary pathogenic process [22, 63, 91, 92]. Such rapidly fatal HFMD was not observed in epidemics caused by EV71 in the 1970s and 1980s, where aseptic meningitis was the predominant clinical manifestation [9, 74].

2.3 EV71 Circulation Outside the Asia-Pacific Region

Outside the Asia-Pacific region, EV71 has continued to circulate at low levels of activity in America, Europe, and Africa, producing sporadic cases or small outbreaks. The outbreaks involve mainly young children who develop aseptic meningitis, hand-foot-and-mouth disease, and other complications (Table 12.2).

Clearly, EV71 has spread across the world. Why the huge epidemics have been confined to the Asia-Pacific region, and whether they might be seen elsewhere in the future is not fully understood, though recent molecular epidemiological work on of the evolution of the different genotypes of virus has offered some key insights into this important question.

3 Molecular Epidemiology

3.1 Virus Genogroup, Evolution, and Geographical Distribution

Before 1999, data about the molecular epidemiology of EV71 were sparse. However, systematic laboratory surveillance established in several Asian countries following the first epidemics in the late 1990s has provided invaluable information on the geographical distribution, spread, and evolution of the virus.

The first complete phylogenetic analysis of EV71 based on the structural VP1 gene identified three independent lineages of EV71 and designated them as genogroups A, B, and C [11]. A sequence diversity of at least 15 % in the VP1 gene was used to distinguish genogroups. Genogroup A consists of a single member, the prototype BrCr strain, which was first identified in California in 1970 and until very recently had never been reported outside the USA (see below). The genogroup B viruses, subdivided into subgenogroups B1 and B2 with divergence of 12 % at the nucleotide level, were the predominant circulating strains in the 1970s and 1980s. The genogroup C viruses, also similarly subdivided into C1 and C2, were only identified later in the mid-1980s (Fig. 12.1). A recent phylogenetic analysis suggests that EV71 may have emerged from the genetically closely related coxsackie virus (CV) A16 as recently as 1940 [144].

A number of new subgenogroups within genogroups B and C have emerged in the Asia-Pacific region in recent years. Subgenogroup B3 and B4 viruses are thought to have co-circulated in the region since 1997 [12, 97, 136]. Subgenogroup B5 was first isolated in Sarawak and Japan in 2003 and was responsible for epidemics in Sarawak, Taiwan, and Brunei in 2006 [1, 66, 101, 119]. Except for the major community outbreak in Sydney in 1986, subgenogroup C1 viruses have mostly been isolated from sporadic cases since the mid-1980s, suggesting a low level of endemic circulation across the globe [11, 127]. Subgenogroup C2 viruses were responsible for the large EV71 epidemic in Taiwan in 1998 and the Perth outbreak in 1999 [12, 82, 96, 98]. Subgenogroup C3 was isolated in Japan in 1994 and Korea in 2000 [12, 68, 70]. Since 2000, subgenogroup C4 has been the predominant circulating genogroup in mainland China, through the most recent epidemic in 2008, and has occurred in Japan, Vietnam, and Taiwan [82, 136, 146, 178]. Subgenogroup C5 has been reported from Southern Vietnam and Taiwan [66, 146]. A genetically distinct EV71 strain (R13223, GenBank accession no. AY179600 to AY179602), with no genetic relationship to other strains recently isolated in the Asia-Pacific region, was isolated from a child with acute flaccid paralysis in India in 2001; it has been assigned as the only member of genogroup D [44]. In short, two major EV71 genogroups (B and C) have co-circulated and coevolved into subgenogroups in the Asia-Pacific region over the past 15 years. In Malaysia and Singapore, the genogroup B viruses have dominated, whereas in East Asia, particularly in mainland China and Vietnam, genogroup C viruses have dominated.

Interestingly, EV71 isolates of genogroup A were reported from 5 of 22 children presenting with HFMD in Anhui province of Central China during the 2008 outbreak that was the first ever reported detection of genogroup A viruses since the original prototype was isolated in America [174]. The nucleotide sequence of the complete VP1 gene of the isolates clusters very closely with that of the prototype genogroup A virus, with minimal divergence. This highly unexpected occurrence could indicate that the genogroup A virus has been circulating undetected in central China with very little evolutionary change for four decades or it could raise doubts about the source of the virus templates that were sequenced. Surveillance from the same outbreak by the Chinese Center for Disease Control does not appear to have identified any genogroup A viruses [178]. Hence, it is important to await confirmation by other laboratories before concluding that genogroup A viruses have reemerged. Another genetically distinct EV71 strain (NMA-03-008, GenBank accession no. JN255590) belonged to a previously unknown novel genogroup has been reported in Central African Republic; this indicates the existence of an additional African genogroup, which may have restricted geographical distribution so far [7]. Clearly, good surveillance programs are required in many different geographical regions in order to gather accurate and relevant information about EV71 transmission and evolution.

3.2 Transmission and Epidemic Potential of Genogroups

Following the initial outbreaks in Sarawak and Taiwan in the late 1990s, surveillance systems for EV71 were established in a number of countries in the Asia-Pacific region; while primarily aimed at monitoring transmission and spread, they have also provided invaluable insights into how the virus evolves in the community during outbreaks. For example, a virological surveillance system in Sarawak, Malaysia, has shown that increased EV71 circulation occurred every 3 years (1997, 2000, 2003, 2006, and 2008/09), closely paralleling increased reports of HFMD in the community [119]. The phenomenon of regular cyclical epidemics has also been observed in Fukushima Prefecture, Japan [59]. Such cyclical activity is presumed to relate to the availability of new birth cohorts of

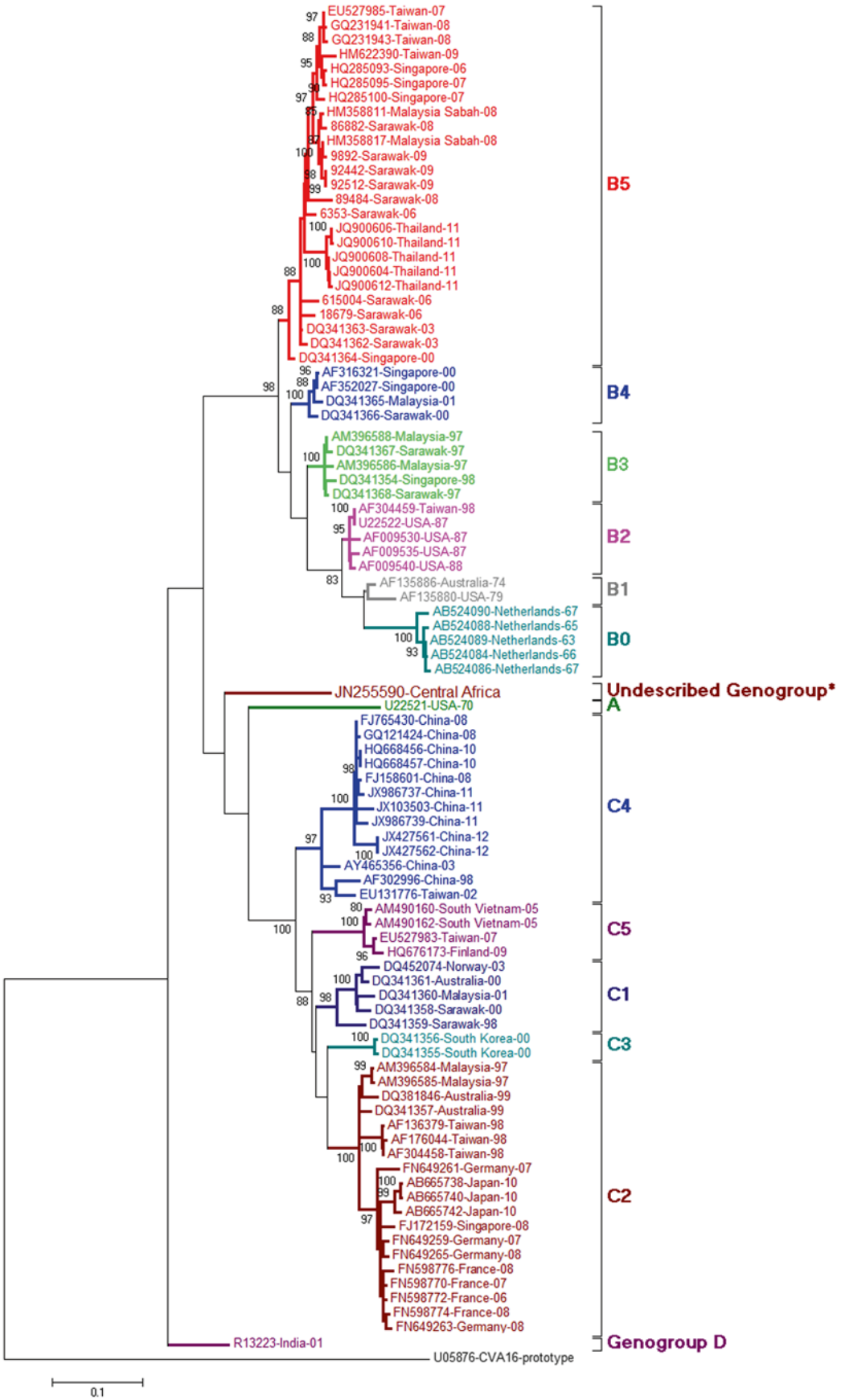

Fig. 12.1 Phylogenetic analysis of EV71 VP1 gene sequences. A neighbor-joining tree constructed using the Kimura-2 parameter as a model for nucleotide substitution. The robustness of the tree was determined by bootstrapping using 1,000 pseudoreplicates. Sequences are labeled according to the following convention: "GenBank accession number" – "Country of origin" – "Year of isolation." The scale bar represents nucleotide changes per site per year

susceptible children who have not been exposed to the virus during the earlier epidemics [23, 89]. Trying to make predictions about the epidemic potential of specific subgenogroups has proved difficult, beyond the observation that some subgenogroups, such as C1, appear to be less virulent and cause endemic disease rather than large epidemics, whereas other subgenogroups, particularly those that appear to have emerged in Asia in recent years, are associated with large epidemics.

More than one subgenogroup may co-circulate, and a shift from one dominant genogroup to another was described during outbreaks in Sarawak and Vietnam [12, 119, 146]. In Japan and Taiwan, subgenogroup B and C viruses have caused epidemics at different times (Table 12.1) [66, 82, 149]. On the contrary in the Netherlands, a permanent shift appears to have occurred, from genogroup B viruses prior to 1986 to genogroup C viruses since 1987; cross-neutralization among the genogroup B viruses, but not with genogroup C viruses, is postulated as one explanation for this, although other experimental cross-neutralization data did not support it [77, 102, 147, 148]. While the older subgenogroups of virus have been circulating and causing low levels of disease for many years, some of the more recently evolved subgenogroups such as genogroup B5, which possess distinctive antigenicity from other viruses may have the potential to cause massive epidemics [64, 148]. Although these have been confined to the Asia-Pacific region so far, the fact that humans are the natural reservoir for the virus and that international air travel is increasing means that every region is at risk of EV71 epidemic.

4 Biological Characteristics

EV71 is a small, non-enveloped virus with a positive-stranded RNA genome about 7.4 kb in size [169]. The genome has a single open reading frame encoding a polyprotein that is cleaved into 11 proteins: the four capsid proteins (P1 – VP1, VP2, VP3, VP4) and seven nonstructural proteins (P2 – 2A, 2B, 2C; P3 – 3A, 3B, 3C, 3D) [99]. The VP1 open reading frame has considerable genetic variability, which confers antigenic variability and enables investigators to differentiate EV71 strains [53, 100]. VP1 is also important for attachment to cellular receptors and for viral virulence [169]. Other details regarding the biological characteristics of EV71 were provided earlier in the molecular epidemiology section; additional general information regarding the characteristics of enteroviruses can be found in Chap. 11.

5 Clinical Features

EV71 infection may present with a wide spectrum of clinical features, although central nervous system (CNS) infection and HFMD are the two most commonly recognized disease manifestations [114].

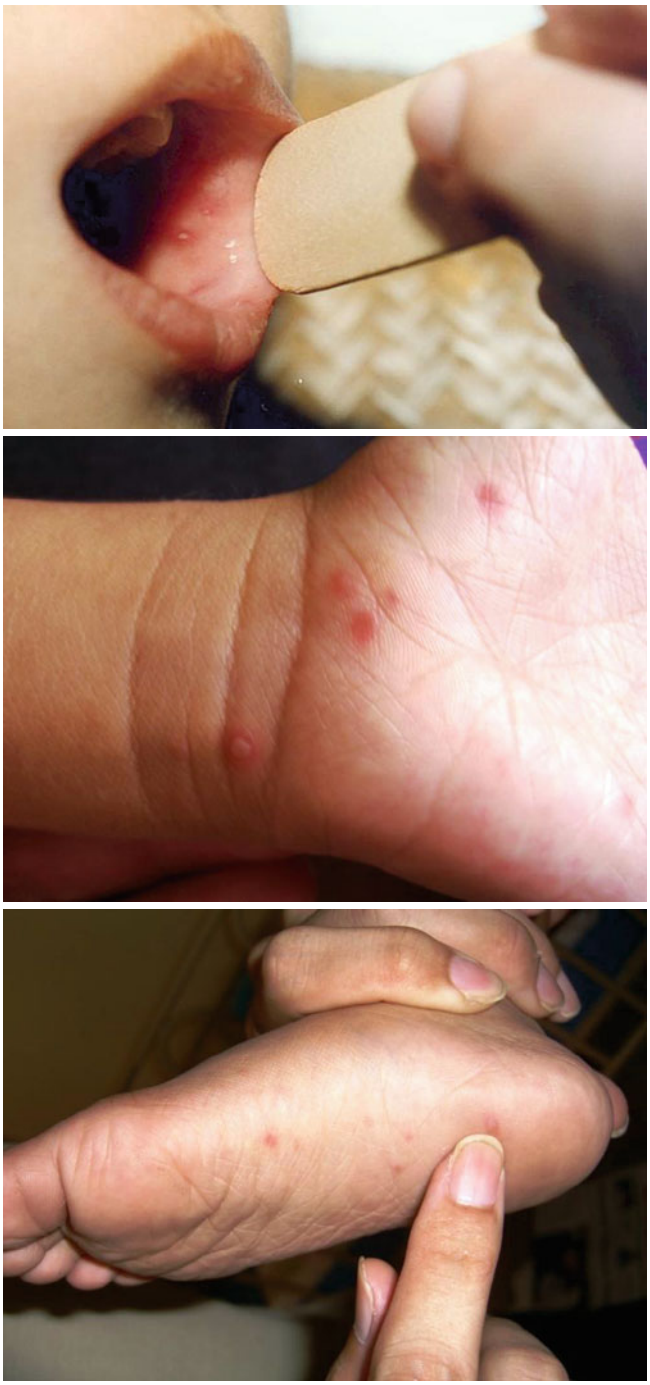

Fig. 12.2 Mucocutaneous lesions in hand-foot-and-mouth disease. This Malaysian child has ulcers that are seen inside the upper lip (*top*) and has vesicular and macular lesions on the wrists (*middle*) and soles (*bottom*) (Photos from T. Solomon) (Reproduced from Ooi et al. [111])

5.1 Mucocutaneous Manifestations

HFMD is a common childhood exanthema characterized by a short, usually mild, febrile illness with papulovesicular rashes over the palms and soles and multiple mouth ulcers (Fig. 12.2).

Table 12.3 Neurological syndromes associated with EV71 infection

Purely neurological manifestations	
Encephalitis, especially brainstem	Common
Acute flaccid paralysis (anterior myelitis)	Common
Encephalomyelitis	Common
Aseptic meningitis	Very common
Cerebellar ataxia	Uncommon
Transverse myelitis	Rare
Neurological plus systemic manifestations	
Brainstem encephalitis with cardiorespiratory failure	Common
Manifestations indicative of immune-mediated (para-infectious) mechanisms	
Guillain-Barré syndrome	Uncommon
Acute disseminated encephalomyelitis	Rare
Opsoclonus-myoclonus syndrome	Rare
Benign intracranial hypertension	Rare

Modified from McMinn [96]

Herpangina, a closely related childhood enanthema, is characterized by a febrile illness associated with multiple mouth ulcers at the anterior pharyngeal folds, uvula, tonsils, and soft palate. Although the classical HFMD picture is typically seen in older children with EV71, widespread and atypical rashes may occur in children aged 2 years and below. Besides EV71, another picornavirus, CVA16, is also a principal causative agent of HFMD in both sporadic and epidemic forms. The virus is not normally associated with neurological complication [114], but the rash it causes is indistinguishable from that caused by EV71. There may be other clues that HFMD is due to EV71 rather than CVA16; for example, studies from Sarawak and Taiwan show that children with EV71 are more likely to have a longer duration of fever (≥3 days), a higher peak temperature (≥38.5 °C), lethargy, and myoclonus [108, 152]. A household contact study in Taiwan during the 2001–2002 epidemic showed that, in addition to HFMD and herpangina, there is a broad range of mild clinical manifestations, including upper respiratory tract infection, gastroenteritis, and nonspecific rashes [24]. Other respiratory manifestations in young children include acute exacerbation of bronchial asthma, bronchiolitis, and pneumonia [100]. More than 20 % of adult contacts during one Taiwanese outbreak had symptoms of an upper respiratory tract infection, while asymptomatic infection occurred in more than 50 %, indicating that adults may be important source of infection for younger children [24].

5.2 Neurological, Respiratory, and Systemic Manifestations

Similar to other enteroviruses, EV71 can cause aseptic meningitis, acute flaccid paralysis, encephalitis, and other rarer manifestations (Table 12.3) [96].

Encephalitis typically affects the brainstem, and unlike most other enteroviruses, it is often accompanied by marked cardiorespiratory dysfunction. This feature is also seen in poliomyelitis and has been attributed to neurogenic pulmonary edema [6], although the mechanism of disease remains controversial.

During a 7-year prospective clinical study of HFMD in Sarawak that covered several epidemics, 10–30 % of hospitalized children with HFMD due to EV71 had CNS complications [108, 112]. Brainstem encephalitis was the most common presentation, accounting for about 60 % of neurological syndromes, followed by aseptic meningitis (36 %) and brainstem encephalitis with cardiorespiratory dysfunction (4 %). Most children with CNS disease will also have features of HFMD; however, a small proportion may present with neurological disease only. Myoclonic jerks are seen more often with EV71 than with other enterovirus infections. This sign may be an early indicator of neurological involvement, particularly in the brainstem [90]. However, it is not pathognomonic for enterovirus infection; myoclonus has also been reported in other virus infections of the CNS including Japanese B encephalitis, subacute sclerosing panencephalitis, and Nipah virus, herpes simplex virus, varicella-zoster virus, and HIV infection. Myoclonic jerks are also often seen in otherwise healthy young infants, particularly when they are asleep and may occur spontaneously or be provoked by a loud noise.

Seizures, if they occur at all in EV71 infection, tend to be seen in younger children and are short-lived with rapid recovery of consciousness, suggesting that they are febrile convulsions, rather than due to CNS infection itself. Unlike those seen in other viral encephalitides, recurrent and prolonged seizures are very rare with EV71 infection, a distinction probably reflecting predominantly brainstem rather than cortical involvement.

Brainstem encephalitis with associated pulmonary edema has been the hallmark of EV71 CNS infection in Asia since the late 1990s. This distinctive clinical syndrome has a stereotypic clinical course characterized by a

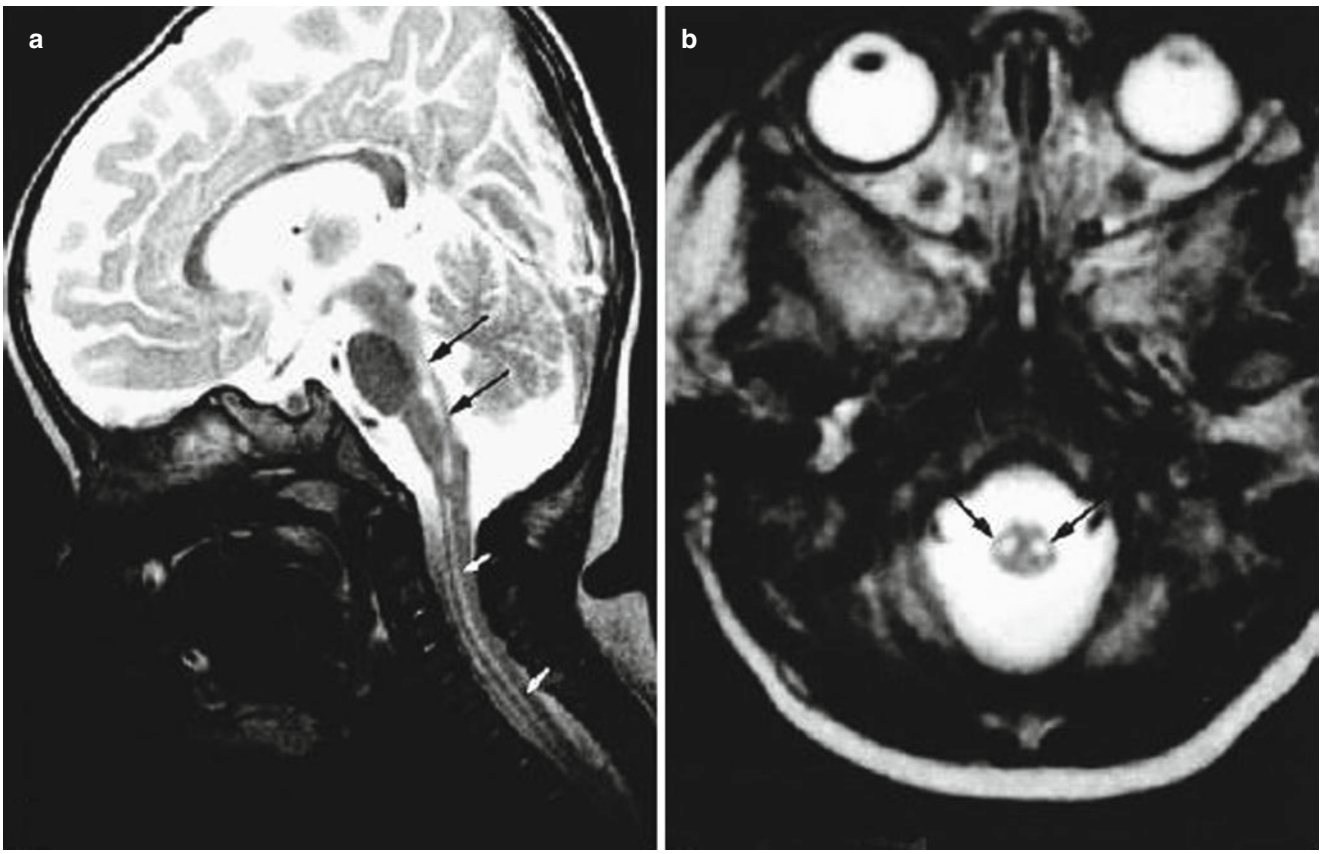

Fig. 12.3 MRI changes in EV71 encephalomyelitis (Modified from Shen et al. [131]). T2-weighted images of a 10-month-old female who presented 3 months earlier with somnolence tachycardia, tachypnea, and coma and who recovered consciousness but remained ventilator dependent. (**a**) Sagittal section showing high signal intensity in the posterior portion of the pons and medulla (*black arrows*) and anterior cervical cord (*white arrows*). (**b**) Axial section showing the high signal intensity in the two anterior horns of the cervical cords (*black arrows*)

prodromal illness of HFMD followed by a sudden deterioration that typically occurs after 3–5 days of fever. Children then develop acute rapidly progressing cardiorespiratory failure presenting as shock and pulmonary edema or hemorrhages. Without critical care support, most of these children die within 24 h of hospital admission and in some even before arriving at the hospital. A system of clinical staging (stage 1 through stage 4) has been used to help monitor the progress of the affected children during the clinical course of EV71 infection and to guide management, from uncomplicated febrile illness to CNS involvement to cardiorespiratory failure and development of neurological sequelae [20, 85, 86]. Such staging systems have not been adopted widely, possibly because they are not always easy to remember and they imply sequential progression through stages that do not always occur. In 2010, a WHO Informal Consultation on Hand Foot and Mouth Disease proposed the use of a simple clinical description of disease manifestation to assess the disease severity. It included uncomplicated HFMD/herpangina, HFMD with CNS involvement, HFMD with autonomic system dysregulation, and HFMD with cardiopulmonary failure [160].

Findings on magnetic resonance imaging (MRI) of children with brainstem encephalitis correlated well with those of autopsy examination; both procedures demonstrate frequent involvement of the medulla oblongata, reticular formation, pons, and midbrain in several studies (Fig. 12.3) [29, 131, 162].

Acute flaccid paralysis is the primary presenting feature of a number of neurological syndromes caused by EV71 including poliomyelitis-like paralysis, Guillain-Barré syndrome, and transverse myelitis. Poliomyelitis-like paralysis is probably the most common of these, though it may be less severe than that caused by polioviruses, with a higher recovery rate [96]. Other respiratory manifestations in young children include acute exacerbation of bronchial asthma, bronchiolitis, and pneumonia [100]. More than 20 % of adult contacts during one Taiwanese outbreak had symptoms of an upper respiratory tract infection, while asymptomatic infection occurred in more than 50 %, indicating that adults may be important source of infection for younger children [24].

6 Clinical Management

During outbreaks of EV71, tens of thousands of children develop symptoms, and while most of them have mild self-limiting illness, in a small proportion of apparently well children, the condition can rapidly deteriorate to severe and fatal neurological and systemic complications over days or even hours. Whereas in the past children with mild HFMD tended to be managed at home, with increasing parental awareness about the risk of fatal complications, many are now brought directly to hospital, and health services can easily become overwhelmed. The challenges faced by primary care clinicians are recognizing which patients are likely to deteriorate, knowing which investigations yield the best diagnostic information, and deciding which treatments might be appropriate, without the benefit of guidance from controlled clinical trials.

6.1 Laboratory Tests

In mild disease, the blood count is usually normal, but in severe disease, the white blood cell count is often high with a neutrophilia [25]. Blood urea and electrolytes are typically unaffected, but there may be hyperglycemia in severe disease [25]. Creatine kinase is sometimes elevated in patients with cardiac involvement [46] and elevated cardiac troponin I has been reported as a predictor of imminent cardiopulmonary failure in children with brainstem encephalitis [65]. Chest X-ray characteristically shows a normal heart size, even in the presence of marked pulmonary congestion, indicating that neither acute viral myocarditis nor congenital heart disease is causing the illness. There are often nonspecific ECG changes [46], and continuous monitoring may show abnormal beat-to-beat variability, which may predict imminent cardiovascular collapse [83]. Echocardiography shows generalized left ventricular hypokinesia, occasionally accompanied by mitral regurgitation, in children who are hemodynamically unstable with tachycardia, hypotension, or pulmonary edema [46]. Pericardial effusion is rare.

Lumbar puncture is essential in children who are unwell with suspected CNS involvement. In some patients, the clinical features, such as meningismus or myoclonic jerks, may clearly point to CNS involvement. However in other children particularly those younger than 2 years of age, there may just be high fever, vomiting or lethargy, but a lumbar puncture reveals CNS disease. There is typically a mild CSF lymphocytic pleocytosis of 10–100 cells per mm^3, but not always [116]. The CSF-to-plasma glucose ratio is usually normal but it can be low.

6.2 Virological Diagnosis

Laboratory diagnosis of EV71 is established primarily through virus isolation or molecular detection of the virus nucleic acid in appropriate clinical specimens.

6.2.1 Choice of Sample

A wide range of samples may be available, depending on the disease manifestations; these include throat and rectal swabs, serum, urine, and, when taken, cerebrospinal fluid (CSF), as well as fluid from vesicles and swabs from ulcers, if they are present. The sensitivity, specificity, and usefulness vary according to the sample [109]. In particular, virus detection in sterile sites such as vesicular fluid, CSF, serum, urine, serum, or autopsy material more reliably indicates a causative organism than does detection from non-sterile sites such as throat or rectum, which may indicate coincidental carriage. However, many of the sterile sites only occasionally yield virus. For example, virus is isolated from only 0–5 % of the CSF of patients with neurological disease [25, 40, 51, 67, 74, 103, 108, 112], because, as for poliomyelitis, the viral load in the CSF is very low [48]. The yield for serum is similarly low [112, 159]. Vesicular fluid, when present, is more useful. Although throat and rectal swabs are more likely to have an enterovirus detected, one study from Malaysia found that this was not always the same enterovirus as that isolated from a sterile site: using the isolate from vesicle swabs as a reference, 10 % of positive throat swabs gave a different isolate, and for rectal swabs the figure was 20 % [109]. Presumably, the isolate from the non-sterile site represented coincidental carriage, whereas that from the vesicles was actually pathogenic.

Prolonged viral shedding from the gastrointestinal tract (throat, rectum, or stool) may occur after complete resolution of EV71 infection, as it does for other enteroviruses; a study in Taiwan showed that EV71 may be detected in the throat up to 2 weeks after recovery from HFMD or herpangina; in the stool, it can be detected up to 11 weeks later [41]. During an outbreak, so many samples could potentially be positive that laboratories can soon become overwhelmed. In one study, the most efficient approach was to examine throat swabs for all patients plus swabs either from at least two vesicles when present or from the rectum when vesicles are absent [109].

6.2.2 Virus Isolation, Serotyping, and Nucleic Acid Detection

The gold standard for diagnosis of enterovirus infection is virus isolation. Several human and nonhuman primate cell lines may be used: rhabdomyosarcoma (RD), which is most efficient; human lung fibroblast cells (MRC5); and African green monkey kidney cells (Vero) [114]. In RD cells, a characteristic cytopathic effect is typically observed 7–10 days

after inoculation. However, to improve the yield, blind passage may be necessary before cytopathic effects become apparent. Once a cytopathic effect is observed, the virus is identified by neutralization tests using intersecting pools of type-specific antisera, by EV71-specific antisera, or by an indirect immunofluorescence assay using EV71-specific monoclonal antibodies [114]. More recently, a molecular "serotyping" approach has been devised. It involves amplifying part of the VP1 gene of the cultured virus, using polymerase chain reaction (PCR) and pan-enterovirus EV71-specific primers, and then sequencing the product [104]. To this end, several sets of primers directed at different regions of the VP1 gene of human enterovirus have been developed [10, 104, 115].

EV71-specific primers are now also being used to perform PCR directly on clinical samples. The advantage of this approach over virus culture is that it can provide rapid diagnosis in the midst of explosive EV71 outbreaks where urgent public health intervention is needed. Several sets of real-time RT-PCR protocols directed to detect EV71 and CVA16 in primary clinical samples have been published recently; however, their disadvantage is that the technique detects only the suspected virus for which primers are available, but they will miss any agents that are unexpected or for which no primers have been generated [34, 115, 142, 167]. DNA microarray technology is a powerful, though expensive, new tool designed to detect multiple pathogen targets by hybridization of pathogen-specific probes. Two groups have recently reported using such an approach to distinguish EV71 and CVA16 infection in primary clinical specimens [34, 134].

6.3 Serology

Serological diagnosis of an acute virus infection is classically established by demonstrating a fourfold rise in specific neutralizing antibody between the acute and convalescent samples [114]. However, in the case of EV71, very high levels of neutralizing antibodies are often detectable within the first few days of illness, and thus a fourfold rise cannot be demonstrated [40, 103]. Furthermore, although homologous antibody is produced when young children encounter their first enterovirus infection, heterologous cross-reacting IgG and IgM antibodies are produced by older children and adults following repeated infection with different enterovirus serotypes; this reduces their diagnostic usefulness [87]. Several rapid IgM ELISA tests for EV71 have recently been developed to try to overcome some of these limitations [153]; however, cross-reactivity remains an issue [168], and the duration of detectable EV71-specific IgM following an infection is also uncertain.

6.4 Diagnostic Imaging

Computer tomography scans are almost always normal in EV71 encephalitis, where the pathology is mostly in the brainstem. Conventional MRI may be normal in the early phase of EV71 encephalitis. Conversely, it may show characteristic high signal intensities on T2-weighted and fluid-attenuation inversion recovery (FLAIR) images in the dorsal pons and medulla, most of the midbrain, and the dentate nuclei of the cerebellum. Similar high-signal lesions may also be found in the anterior horn cells of cervical spinal cord (Fig. 12.3) [63, 131, 175]. Gadolinium-enhanced MRI examination improves the results [175]. Diffuse-weighted imaging (DWI), a sensitive tool for detecting of early changes in brain cellular function, seems to be better at detecting EV71 encephalitis than conventional MRI [81]. However, the value of MRI screening has yet to be demonstrated. In children with acute flaccid paralysis, MRI typically shows unilateral high-signal lesions in the anterior horn cells of spinal cord on T2-wieghted images and contrast-enhancing ventral root on T1-weighted images [28, 63, 132].

6.5 Predictors of Severe Disease

Several clinical features and laboratory abnormalities have been associated with neurological and fatal EV71 disease, but few have been prospectively validated as prognostic indicators [25, 60, 65]. Younger age is associated with increased risk of severe disease [21]. A prospective clinical study of nearly 1,500 children presenting to one hospital in three EV71 outbreaks in Sarawak over 7 years suggested that peak temperature of 38.5 °C or higher, duration of fever for 3 or more days, and a history of lethargy were useful clinical predictors for neurological involvement. The presence of at least two of those three factors was strongly associated with the subsequent development of neurological disease [108]. The study corroborated the findings from early retrospective studies. However, it did not confirm other findings from earlier studies, such as the association between the absence of mouth ulcers and development of complicated or fatal disease [38, 112]. Hyperglycemia and leukocytosis have also been associated with fatal EV71 disease in a retrospective evaluation [25], and although these findings were confirmed in the prospective study, because they were late changes occurring about the same time as the fulminant disease, they were not helpful clinically in identifying children at high risk of complications and death [108].

Not all children with CNS involvement in EV71 infection will progress to cardiorespiratory collapse. A recent study in Southern Vietnam revealed that, in general, about 6 % of children with CNS involvement would develop autonomic

system dysregulation. Children with frequent myoclonus observed by doctors and healthcare workers, with or without other focal neurological signs, were at higher risk of disease progression compared with those children with no history of myoclonus (22.2 % vs 4.4 %). A small study involving 46 patients showed that children developed abnormal heart rate variability, an index of autonomic nervous system disease, about 7 h before the clinical onset of cardiorespiratory instability [83]. The authors proposed that screening children with CNS involvement for abnormalities in heart rate variability may provide early warning of imminent cardiorespiratory failure and allow timely institution of appropriate interventions. Cardiac troponin I is a cardiac-specific biomarker for myocardial damage, used for early diagnosis of acute coronary syndrome in adults. Elevated cardiac troponin I has been observed in children with brainstem encephalitis and cardiopulmonary failure [65]; in some cases, it was elevated prior to the development of cardiopulmonary failure, suggesting that serial measurement of troponin may be helpful in identifying children at risk of left ventricular failure. However, neither the evaluation of heart rate variability nor the measurement of troponin I level has become routine clinical practice in the management of EV71 infection, probably because the former requires relatively sophisticated equipment not widely available and the latter is relatively expensive in developing countries. Overall simple clinical parameters such as length of illness, height of fever, and lethargy are probably more useful indicators of potentially severe disease.

6.6 Outcome

Follow-up for as long as 7 years after infection shows that children who present with aseptic meningitis generally have a favorable outcome, although a recent report documented the incidence of attention-deficit/hyperactivity disorder-related symptoms, as reported by parents and teachers, is higher (20 %) in children who had recovered from CNS infection compared with the matched controls (3 %) [49]. Approximately one-fifth of those with more severe neurological disease, including encephalitis, poliomyelitis-like paralysis, and encephalomyelitis, have sequelae, particularly focal limb weakness and atrophy [21, 110, 145]. Cerebellar dysfunction is observed in about 10 % of patients who had moderately severe brainstem encephalitis, including cranial neuropathies, myoclonus, tremor, and ataxia. However, only a quarter of those with more severe brainstem encephalitis associated with fulminant cardiorespiratory failure make a full neurological recovery. Common sequelae include focal limb weakness and atrophy, swallowing difficulties requiring nasogastric feeding, central hypoventilation, facial nerve palsies, seizures, and psychomotor retardation.

7 Pathogenesis

7.1 Viral Determinants of Virulence

The factors that determine whether EV71 infection will be asymptomatic or result in HFMD or severe neurological disease remain unknown. For poliovirus, the 5'UTR and VP1 genes are known to contain virulence determinants [72]. Several studies have therefore examined the nucleotide sequence of these genes, or the whole genome, comparing EV71 isolates from fatal and nonfatal cases, but for the most part the isolates have been nearly or entirely identical, and significant changes have not been found [135, 139]. The incidence of CNS disease and other severe complications of EV71 infection seems to have varied among the recent outbreaks in Asia, leading to the postulation that differences in the virulence of the various genotypes may have a role. However, comparisons between outbreaks have been hampered by the retrospective nature of many of the studies as well as differences in inclusion criteria, definitions of disease severity, and viral diagnostic approaches and capabilities. Perhaps the strongest data supporting the hypothesis that strain virulence determinants play an important role in the pathogenesis of severe neurological disease come from studies in Perth and Sarawak. During the Perth epidemic in 1999, two subgenogroups, B3 and C2, co-circulated, thus providing a unique opportunity to examine the role of virulence determinants in a single epidemic setting [96, 97]. In this outbreak, subgenogroup C2 viruses linked to the Taiwan epidemic of 1998 were almost exclusively isolated from children with severe neurological disease, and only a single isolate came from a case of uncomplicated HFMD. Conversely, subgenogroup B3 viruses, similar to those from the Sarawak 1997 epidemic, were isolated mainly from children with uncomplicated HFMD, aseptic meningitis, or post-infectious neurological disease, none of whom died [98]. A detailed prospective clinical study of EV71 disease in Sarawak, which included 277 children with EV71-associated HFMD, provided further clinical and epidemiological evidence for different biological behavior of EV71 subgenogroups, with regard to risk of CNS disease and transmissibility within families [112]. Two discrete EV71 epidemics, caused predominantly by subgenogroup B3 and B4, respectively, but with small numbers of cases caused by subgenogroup C1, occurred [112]. Children infected with subgenogroup B4 viruses were less likely to present with CNS infection than those infected with C1 or B5 viruses, and they were also less likely to be part of a family cluster. On the other hand, children infected with B5 were more likely to be part of a family cluster, and there was a trend toward a greater incidence of CNS disease in these patients. These results suggest that subgenogroups do indeed vary in their propensity to cause neurological disease.

7.2 Dual Infection

During the first of the Asian EV71 epidemics in Sarawak in 1997, which was due to subgenogroup B3, an adenovirus type 21 was also implicated in the fatal cases as well as in some cases with acute flaccid paralysis [13, 110]. The adenovirus was isolated from sterile sites such as CSF, brain, and heart in fatal cases and indeed was more frequently detected than EV71 itself; this led to the suggestion that the fatalities were due to dual infection, rather than EV71 alone [13]. However, subsequent detailed studies, including longitudinal studies from Sarawak, have not found evidence of adenovirus 21 infection in other HFMD or neurological cases, though dual infection of EV71 with other viruses, including dengue and Japanese B encephalitis, has been found [112]. Furthermore, adenovirus 21 has never been isolated in Sarawak since 1997.

7.3 Host Susceptibility

A range of host factors may affect pathogenesis, particularly partial cross-protective immunity from prior outbreaks. Lack of cross protection may partially explain why young age is a risk factor for severe disease [23, 24, 89]. The potential for host genetic variants to explain differential susceptibility, clinical severity, and outcome of EV71 has also been studied. One study in Taiwan reported that HLA-A33 is associated with increased susceptibility to EV71, although the role of the major histocompatibility complex in the virus infection is still unknown [19]. HLA-A33 is more frequently found in the populations of Asian ancestry than in those of European ancestry, and a causal association of this allele could help explain the higher frequency of EV71 epidemics in Asia. In the same study HLA-A2 was linked to the risk of cardiopulmonary failure often observed in fatal cases [19]. HLA-G, an important immunotolerant molecule, is involved in the suppression of T lymphocytes, NK cells, and antigen-presenting cells and in the induction of regulatory T cells and tolerant dendritic cells. Increased levels of cell surface bound HLA-G and plasma sHLA-G were found in EV71-infected children and in children with pulmonary edema [179]. Cytotoxic T lymphocyte antigen-4 (CTLA-4) is an important regulator of T cell cytotoxicity and is involved in the regulation of immune response. Studies of polymorphisms in the gene encoding CTLA-4 in children with meningoencephalitis have yielded conflicting results [19, 173]. Th17 cells are effector cells in human immune response, and its related cytokines, IL-17 and IL-23, are important mediators in pro-inflammatory response. Chinese children with the IL-17 F 7488C allele, which has been associated with a blunted pro-inflammatory response, were more likely to have milder EV71 infection, and patients who were homozygous for the T allele had significantly higher level of C-reactive protein, leukocytosis, and neutrophil counts when compared with patients with CC+CT genotypes [93]. Another Chinese study showed increased frequency of Th17 cells, as well as elevated serum IL-17 and IL-23, in peripheral blood of children infected with EV71 when compared to the healthy controls [32]. Interferon gamma, a Th1 antiviral cytokine, and IL-10, a potent anti-inflammatory cytokine that suppresses innate host defense, including interferon gamma production, have been implicated in the pathogenesis of severe EV71 infection. Susceptibility to EV71 encephalitis has been reported in children with the IFN-gamma+874 T/A genotype, previously associated with reduced IFN-gamma production, and in children with the IL-10-1082G/A genotype, previously associated with reduced IL-10 production [172]. All of these associations require replication.

7.4 Viral Entry and Spread

EV71 is transmitted predominantly via the feco-oral route, with respiratory spread also implicated [114]. As for other enteroviruses, initial viral replication is presumed to occur in the lymphoid tissues of the oropharyngeal cavity (tonsils) and small bowel (Peyer's patches), with further multiplication in the regional lymph nodes (deep cervical nodes, mesenteric nodes), giving rise to a mild viremia. The majority of infections are controlled at this point and remain asymptomatic. However, in vivo studies show that if enteroviruses disseminate further, they reach target organs, particularly the reticuloendothelial system (liver, spleen, bone marrow, and lymph nodes), heart, lung, pancreas, skin, mucous membranes, and central nervous system, coinciding with the onset of clinical features.

The mechanism by which enteroviruses enter the CNS is not completely understood. A number of epidemiological and experimental animal studies on polioviruses indicate that the virus can invade the CNS system by permeation through a disrupted blood-brain barrier or by retrograde axonal spread along cranial or peripheral nerves. For EV71, this latter route has been implicated both in mouse models and by examining the distribution of virus and inflammation in fatal human cases [31, 107, 163].

7.5 Pathological Findings

The topographical distribution of CNS inflammation has been stereotypic and is observed predominantly in the neuronal areas of spinal cord, the entire medulla oblongata except the pyramidal areas, the tegmentum and floor of fourth ventricles in the pons but not the anterior pons, and the whole midbrain sparing the cerebral peduncles. In addition, the hypothalamus, subthalamic and dentate nuclei, and to a lesser degree motor cortex of the cerebrum and meninges are involved (Fig. 12.4) [62, 63, 91, 92, 131, 133, 163].

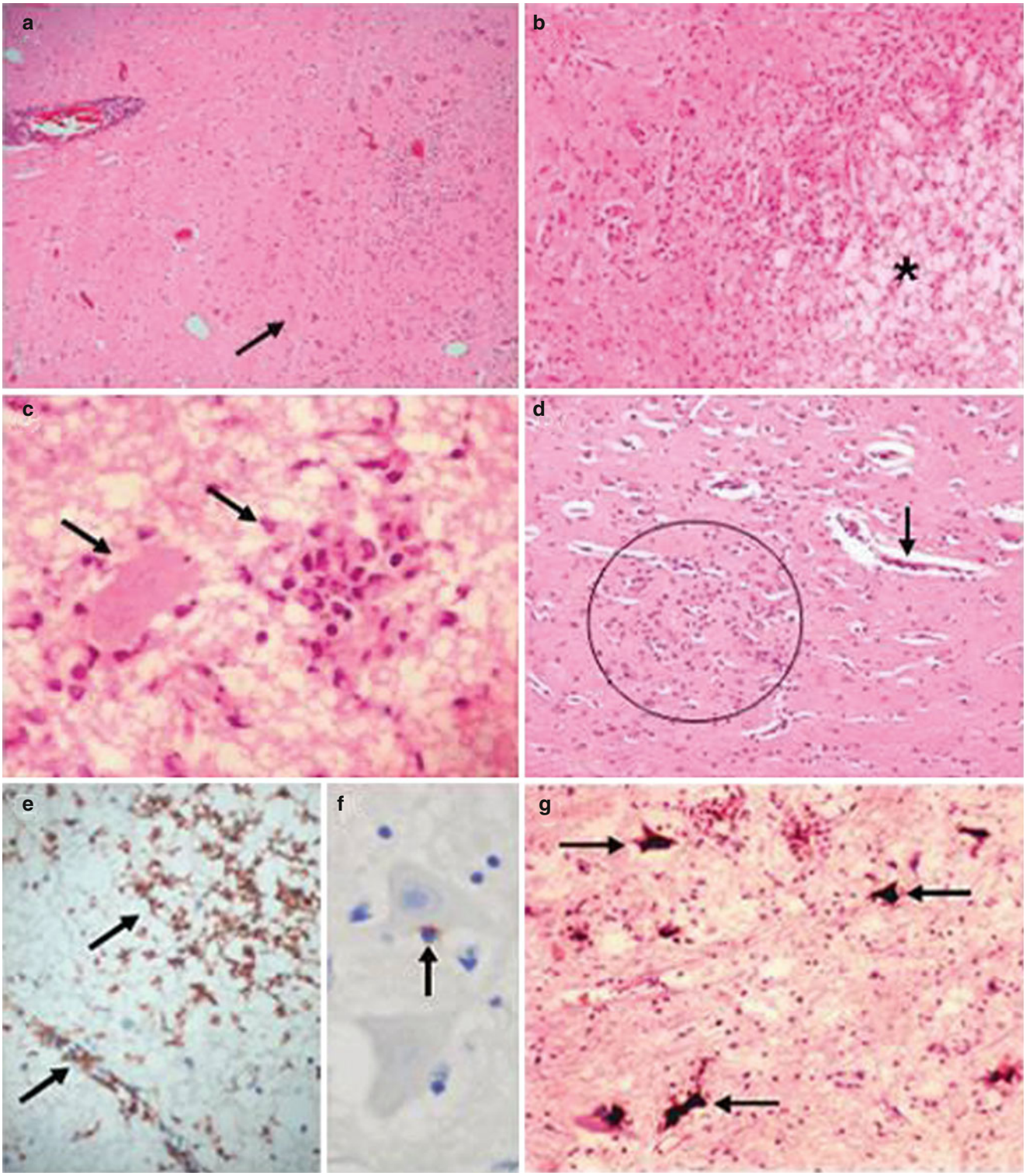

Fig. 12.4 Pathological findings in enterovirus 71 encephalitis (Modified from [163]). Parenchymal inflammation (*arrows*) and perivascular cuffing in the medulla (**a**); more severely inflamed areas (**b**), with edema (*) and neuronophagia (**c**, *arrows*). More subtle inflammation in the motor cortex with mild perivascular cuffing (*arrow*) and parenchymal inflammatory cells (*circle*) (**d**). Numerous CD68-positive macrophages/microglial (**e**), CD8-positive lymphocyte adjacent to a neuron (**f**). Viral RNA in the anterior horn cells of the spinal cord (**g**). (**a**–**d**: hematoxylin and eosin stains; **e**, **f**: immunohistochemistry/peroxidase/DAB; **g**: ISH/nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate stains. Original magnification: (**a**) 4×; (**b**, **d**) 10×; (**c**, **f**) 40×; (**g**) 20×

Inflammatory changes were absent in the cerebellar cortex, thalamus, basal ganglia, mammillary body, hippocampus, temporal lobe, peripheral nerve, and autonomic ganglia. The histopathological changes, characterized by perivascular cuffing of macrophages, lymphocytes, neutrophils and plasma cells, variable edema and necrosis, focal neuronophagia, and microglia nodules, are similar to those in encephalitis caused by other viruses [50]. Neither virus inclusion nor vasculitis has been observed, and viral antigens and RNA can only be seen in a small number of neuronal processes and phagocytic cells [163, 164].

7.6 Pathogenesis of Severe Pulmonary Edema and Heart Failure

While it is clear that fulminant pulmonary edema is closely associated with, and preceded by CNS involvement, there is no consensus on its cause, especially the relative contributions of neurogenic pulmonary edema, cardiac dysfunction, increased vascular permeability, and a cytokine storm (Fig. 12.5).

Neurogenic pulmonary edema is classically seen following a head injury, where the associated raised intracranial pressure is thought to be important. Although the pathogenesis is not completely understood, studies from animal models suggest that the hypothalamus, and vasomotor centers of the medulla, and nuclei in the cervical spinal cord are important; lesions to various nuclei in these regions can increase activity along the sympathetic trunk, resulting in profound systemic and pulmonary hypertension and consequent pulmonary edema [57]. Pulmonary edema was also seen in poliomyelitis, and because it was associated with damage to brainstem nuclei, it was thought to be neurogenic in origin [6]. Thus when severe pulmonary edema was first seen in EV71 encephalitis, and brainstem inflammatory changes were observed too, the development of edema was attributed to neurogenic origin. Autopsy examination and magnetic resonance imaging studies of children with EV71 brainstem encephalitis showed that there was extensive inflammation of gray matter of the spinal cord and the whole medulla oblongata, as described above [62, 63, 91, 92, 131, 163]. The observations of hyperglycemia and leukocytosis were also postulated to be due to increased sympathetic discharges [25].

However, detailed hemodynamic observations of children with EV71 and pulmonary edema have not always shown the profound systemic and pulmonary hypertension that would be expected [46, 86, 89, 165] This may be because the changes in vascular pressures in neurogenic pulmonary edema are only transient, as was shown for one child with EV71 [165]. Others have argued that cardiac impairment is a major contributor to the pulmonary edema. Although there is no histological or virological evidence of a viral myocarditis, increased cardiac-specific troponin I level suggests some degree of cardiac injury [22, 46, 65, 91, 92]. A detailed echocardiographic study of 11 children with EV71 brainstem encephalitis shows that their cardiac function was impaired, with significantly reduced left ventricular ejection fraction [46]. Two children whose cardiac output was supported with a left ventricular assist device survived, whereas all the others died [45].

Although there is no myocardial inflammation, histological examination of heart ventricular tissue from six fatal cases and one survivor, obtained though a biopsy, revealed significant coagulative myocytolysis, myofibrillar degeneration, and cardiomyocyte apoptosis which are characteristic of catecholamine cardiotoxicity [46, 47]. Thus it is argued by some that the massive release of catecholamine caused by brainstem encephalitis may have a direct effect on cardiac function, as well as causing pulmonary edema through raised pulmonary pressures.

The other potential contributor to pulmonary edema is increased vascular permeability, which is postulated to have occurred following the systemic inflammatory response. Early studies that examined a narrow range of cytokines and chemokines have shown that interleukin (IL)-6, IL-1B, IL-10, IL-13, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ are all significantly higher in EV71 patients with pulmonary edema than in those with uncomplicated encephalitis, and several of these, including IL-1, IL-6, IL-13, and IFN-γ, are known mediators of increased vascular permeability [84–86, 151]. In addition, increased plasma levels of several chemokines including interferon gamma-induced protein (IP-10), monocyte chemoattractant protein (MCP)-1, monokine induced by interferon gamma (MIG), and IL-8 have been found in children with pulmonary edema when compared to those with uncomplicated brainstem encephalitis [156]. A study of 30 cytokine and chemokine levels in EV71-infected Malaysian children confirmed that cardiorespiratory impairment was associated with a widespread elevation of both pro-inflammatory and anti-inflammatory mediators in the serum and CSF. In addition, the study also showed that serum IL-1Ra and G-CSF levels were significantly elevated in patients who died, with a serum G-CSF/IL-5 ratio of >100 being the most accurate prognostic marker [52]. Children with edema also had depleted lymphocyte population particularly in CD4, CD8, and natural killer cells [151, 173]. Thrombocytosis, neutrophilia, and hyperglycemia are all thought to be indicative of a systemic inflammatory response [84, 151]. Fewer studies have looked at cytokines in the CSF. Elevated CSF IL-1b was found in patients with encephalitis complicated by edema when compared to those with encephalitis alone [84]. It has previously been proposed that the CNS may be the source of the inflammatory cytokines detected in the serum of patients with EV71-associated cardiac dysfunction [156]. A study examining the relative abundance of inflammatory mediators in the serum and CSF samples from

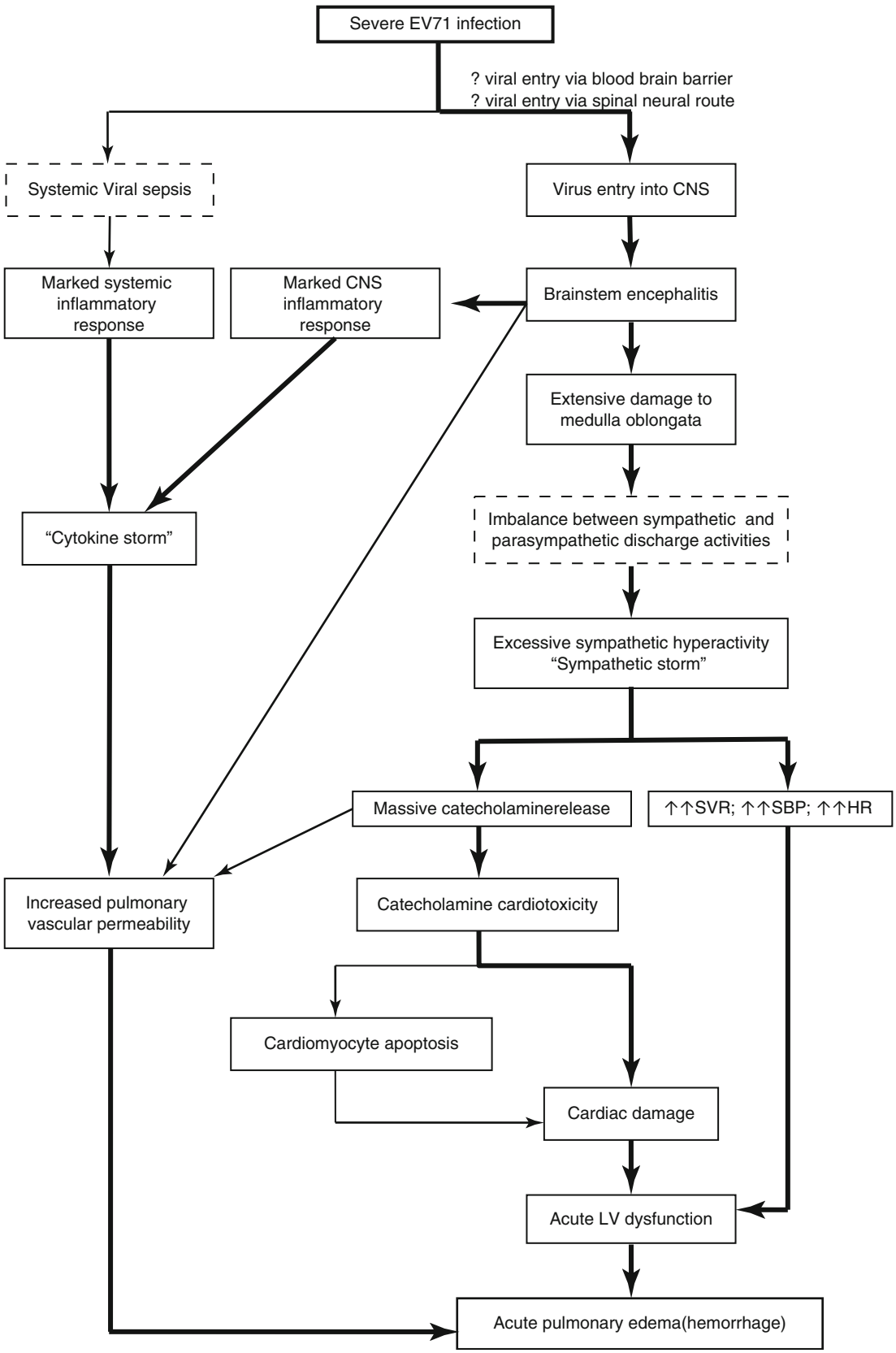

Fig. 12.5 The postulated pathogenesis of enterovirus 71-associated acute pulmonary edema Major postulated pathogenic pathways are shown with thick lines; lesser contributory pathways are shown with thinner lines. The solid boxes indicate strong supporting evidence from human clinical or pathological studies, while the dotted boxes indicate hypothetical but unproven steps or evidence from animal models only. *EV71* human enterovirus 71, *BBB* blood-brain barrier, *CNS* central nervous system, ↑↑ markedly high, *SVR* systemic vascular resistance, *SBP* systemic blood pressure, *HR* heart rate, *LV* left ventricular (Reproduced from Solomon et al. [140])

88 Malaysian children showed two distinctive immune response patterns occurring independently in these two compartments [52]. Because the development of pulmonary edema in patients with EV71 encephalitis appears to be strongly associated with a dysregulated systemic and CNS inflammatory response, this observation has at least partly formed the basis for treatment with intravenous immunoglobulin, which appears to be effective [20, 85, 86, 108, 112, 154].

In summary, although still ill-defined, brainstem inflammation appears to be an important neurogenic mechanism for pulmonary edema in EV71 encephalitis. However, similar pathological changes are also observed in other encephalitides without pulmonary edema as a prominent feature. Cardiac compromise and the effects of the systemic inflammatory response on the vascular endothelium may also make an important contribution. In vivo mouse and nonhuman primate models have replicated some of the features of severe EV71 disease, such as neuroinvasiveness with marked inflammatory changes; however, no model has yet reproduced the severe systemic features such as pulmonary edema [5, 31, 36, 107, 158, 163].

8 Control and Prevention

8.1 Treatment

8.1.1 Antiviral Agents

There are no established antiviral treatments for EV71. Pleconaril is an antiviral drug that inhibits entry into cells for a number of enteroviruses by blocking viral attachment and uncoating. It has been used in clinical trials of aseptic meningitis [122–124]; however, it is not active against EV71 [35]. Several other newer capsid-function inhibitors have been investigated, and some have demonstrated promising anti-EV71 activities in preclinical studies [35]. Two human transmembrane proteins, P-selectin glycoprotein ligand-1 (PSGL-1) and scavenger receptor class B member 2 (SCARB2), have recently been identified to be functional receptors of EV71 [106, 170]. Analysis of the much-awaited crystal structure of EV71 revealed important structural differences from other enteroviruses. The mature virion of EV71 has a shallower canyon (believed to be the receptor binding site) on the viral surface and a relatively exposed "pocket factor" (which stabilizes the virus) compared to other enteroviruses [118, 157]. These discoveries are major steps forward and will guide rational design of antivirals against EV71.

8.1.2 Intravenous Immunoglobulin

Intravenous immunoglobulin (IVIG) was used during the initial epidemics of EV71 in Sarawak and Taiwan in the late 1990s on the basis that its anti-EV71 neutralizing antibodies and nonspecific anti-inflammatory properties might be therapeutic [112, 152]. Retrospective comparisons of patients who received IVIG with those who did not suggested possible benefit from IVIG if given early [20, 108]; for example, in Sarawak over 3 seasons, 204 (95 %) of 215 children who survived despite severe CNS complications had timely IVIG treatment, typically once severe disease occurred, whereas only one (11 %) of nine fatal cases received this treatment (OR 148.36, 95 % CI 16.34–6609.04, $p<0.0001$) [108]. Proinflammatory cytokines measured before and after IVIG treatment were significantly lower in EV71 patients with autonomic dysfunction than patients with less severe disease [84–86, 151, 154]. IVIG has since become routine treatment for severe EV71 disease, and it has been recommended in the national treatment guidelines in Taiwan and Vietnam [20, 75, 85, 86, 108, 112, 150]. While remaining uncertainty over the efficacy of this treatment warrants randomized placebo-controlled trials, they would be logistically and ethically challenging to conduct in the face of the wide acceptance of IVIG as the current standard of care and the beliefs in their value strongly held by some. Carefully designed phase II trials would need surrogate end points for disease progression (e.g., failure of resolution of tachycardia).

8.1.3 Milrinone

Milrinone is a cyclic nucleotide phosphodiesterase (PDE) inhibitor currently used in the treatment of congestive heart failure. Inhibition of PDE subtype III by milrinone results in an increase in intracellular cyclic adenosine monophosphate (cAMP), which in turn leads to increased cardiac output and decreased peripheral vascular resistance. In a small nonrandomized retrospective comparison involving 24 children with EV71-induced pulmonary edema, those treated with milrinone had reduced tachycardia and lower mortality [150, 155]. Intriguingly, the peripheral white blood cell count, platelet count, and plasma IL-13 were also lower [155], suggesting an immunomodulatory as well as a cardiovascular effect of the drug. More recently a prospective, open-label, randomized controlled trial conducted by the same authors, involving 41 Vietnamese children 5 years of age and below, showed that milrinone significantly reduced the 1-week mortality from EV71-induced cardiovascular collapse without adverse effect. This encouraging finding has tentatively raised hope that earlier milrinone treatment might be useful in halting disease progression of cases with severe brainstem encephalitis [37].

8.1.4 Fluid Balance and Ionotrope Support

Hypovolemia and dehydration are the commonest causes of shock in children and are treated with rapid fluid resuscitation with good results. However, similar approaches used in the early EV71 epidemics in Asia precipitated massive pulmonary edema. Judicious use of intravenous fluid and early

institution of inotrope support are critical in children with severe EV71 infection. Where fluid management could be guided by central venous pressure monitoring, in Taiwan, management algorithms based on this approach appear to have improved outcome [20].

8.1.5 Novel Treatment Approaches

Although better recognition of early signs of CNS involvement and the disease progression has helped improve the clinical outcome, many children continue to succumb to severe EV71 infection because of late presentation or disease progression despite intervention. A left ventricular assist device with extracorporeal membrane oxygenation was associated with a higher survival rate in a small number of recent cases than in historical controls, but with significant neurological sequelae [69]. Hemofiltration has been employed to treat children with cardiovascular collapse in Vietnam [117].

8.2 Prospects for Prevention

8.2.1 Surveillance

The only measures available currently for disease control at the population level are public health approaches. Countries in Asia (Singapore, Taiwan, Japan, and Vietnam) have implemented heightened surveillance for EV71 [2, 3, 33, 101, 119]. HFMD has now become a notifiable disease in many places, including Malaysia, Singapore, Thailand, Taiwan, Vietnam, and China [3, 4]. However, HFMD may be caused by a number of enteroviruses including CVA8, 10, and 16, and concurrent virological surveillance is necessary. Virological surveillance also provides invaluable molecular epidemiological data about the circulating EV71 genotype and may thus help to track the spread of the virus across the region.

Because humans are the only known natural hosts of human enteroviruses, outbreak control measures are targeted primarily at interrupting person-to-person virus transmission via contact with throat and nose secretions, saliva, stool, and vesicular fluid but also at minimizing contact with contaminated surfaces, toys, or fomites. Because of the lack of a lipid envelope, EV71 has considerable stability in the environment. It can remain viable at room temperature for several days and has been recovered from surface and ground water and hot spas [30, 61]. Hence, health education focuses on personal hygiene and good sanitation including frequent hand washing, proper disposal of soiled diapers, and disinfection of soiled surfaces with sodium hypochlorite [73]. Like other enteroviruses, EV71 is resistant to alcohol. Consequently, use of the widely available alcohol-based (70 % ethanol or isopropanol) disinfectants alone for hand hygiene is ineffective in preventing EV71 transmission [26]. A recent study showed that EV71 can be destroyed by virucidal disinfectants such as Virkon [18].

Transmission of enteroviruses including EV71 is most efficient in overcrowded settings, and most countries in the region including Malaysia, Singapore, Taiwan, Hong Kong, and China have adopted "social distancing" measures during epidemics. These measures include closures of childcare facilities and schools and cancelation of public functions involving children [2, 3]. Although there has been little systematic research to examine the effectiveness of public health interventions, a few studies of school closure or other approaches from Singapore and Hong Kong appeared to show some benefit [2, 95]. It nevertheless remains uncertain whether social distancing measures are effective or what the optimal timing for instituting them may be—at the onset of an HFMD outbreak or at the time of laboratory confirmation of an EV71 etiology. Although this widely adopted empirical measure has considerable socioeconomic impact, the cost of the disease and its control measures have not yet been studied. If experience with other directly transmissible viruses applies to EV71, then such measures as school closure could decrease the peak incidence but prolong the outbreak, with no reduction in the overall number of cases (Fig. 12.6) [140].

Further epidemiological work to elucidate the transmission dynamics of the virus will guide the formulation of evidence-based interventions to control the spread of EV71. Critical pieces of information such as precise estimates of the incubation period, ratio of asymptomatic to symptomatic cases, and time and duration of infectiousness are needed. The reproduction number (R_0) for EV71 has been estimated to be higher than that of CVA16 (median 5.48, IQR 4.20–6.51 compared with 2.50 [1.96–3.67], respectively; $p = 0.002$) [94].

8.2.2 Vaccine Development

The success story of the control of poliomyelitis indicates that vaccines would be the best approach for future disease control, and the target population should be younger children, especially those less than 3 years of age, because they are at highest risk of severe disease. In fact, an inactivated EV71 whole virus vaccine was developed in Russia in 1976 after the Bulgaria epidemic. But it received no further evaluation because no further outbreaks of EV71 occurred [78]. More recent candidates for an EV71 vaccine include inactivated whole virus, live-attenuated, recombinant viral protein, virus-like particle, and DNA vaccines. These are at different stages of the development in China, Taiwan, and Singapore [78, 176]. Among these, inactivated whole virus vaccine candidates, the development of which modeled that of inactivated polio vaccine, are at the final stages of the clinical evaluation. A phase 3 randomized double-blinded, placebo-controlled trial of inactivated alum-adjuvant EV71 vaccine (Beijing Vigoo Biological, EV71 strain FY7VP5/AH/CHN/2008, genogroup C4) involving 10,245 Chinese children between the

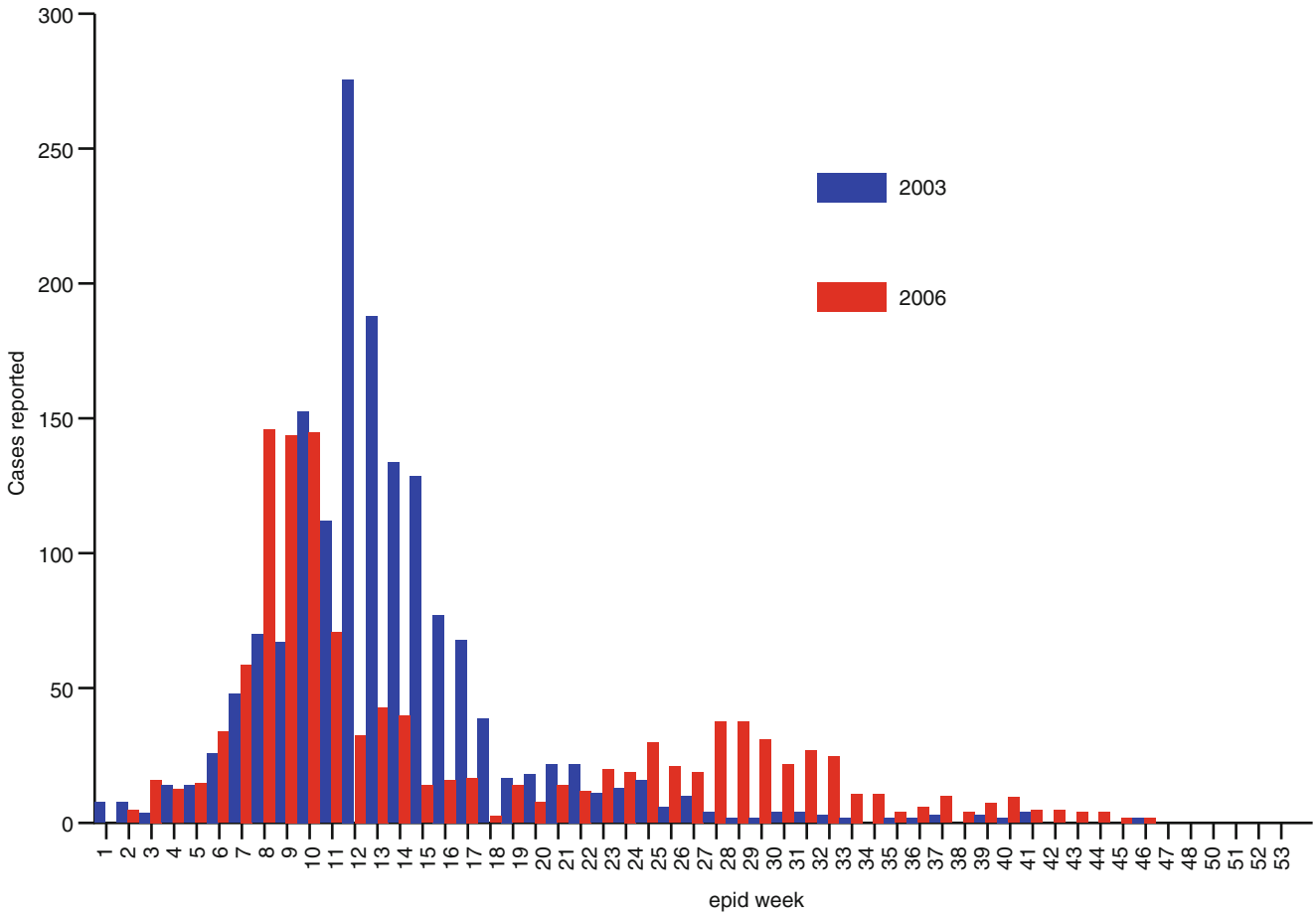

Fig. 12.6 The effect of public health interventions on hand-foot-and-mouth disease outbreaks, comparing sentinel surveillance data from the 2003 outbreak in Sarawak, Malaysia, when the public health response was limited, and the 2006 outbreak, when more rigorous social distancing measures were encouraged. (Reproduced from Solomon et al. [140])

ages of 6 and 35 months has shown encouraging vaccine efficacy, immunogenicity, and safety [180]. Another alum-adjuvant inactivated whole virus candidate (EV71 strain H07, genogroup C4) produced by Sinovac Biotech Co., LTD) was reported to be well tolerated and highly immunogenic in a phase 1 trial in infant populations in China [80]. While an eagerly awaited vaccine will likely be available for routine use in the near future, it remains uncertain whether the vaccine developed from a specific genogroup would provide adequate cross protection against all genogroups. This concern is critical because genogroup B and C virus so far appear to have different geographical distributions in Asia, and the data to date on cross protection between genogroups are conflicting. Another important issue pertaining to manufacturing processes of inactivated whole virus vaccine is that no international reference standards for potency assays and quantification of EV71 vaccine antigens exist [78]. Establishment of such standards is urgent in Asia, where the vaccine will likely be used soonest and most extensively.

9 Concluding Remarks and Unsolved Problems

The emergence of EV71 in the Asia-Pacific region over the last 15 years has had a major public health impact. Molecular epidemiological studies suggest that some subgenogroups appear to have massive potential for explosive epidemics, while others circulate in a more indolent pattern. However, the biological determinants of these differences are poorly understood. The epidemiological differences observed between EV71 in the Asia-Pacific region and the strains found in Europe and USA also represent an unsolved puzzle. There are no reliable and easy-to-use clinical tools to predict who will develop neurological complications and which patients with CNS involvement are at risk of disease progression. The virological and host determinants of the wide-ranging clinical phenotypes in those infected remain unclear. There are relatively good animal models of neurological disease caused by EV71, but there is an urgent need for an animal model of cardiorespiratory dysfunction to advance

understanding of its pathogenesis. Despite lack of solid evidence for its efficacy, the wide use of IVIG for severe EV71 infection in many Asian countries will make efficacy trials difficult. There is still no specific antiviral drug available for EV71 infection although the determination of its crystal structure and identification of several EV71 receptors should accelerate drug discovery. An inactivated whole virus vaccine is nearing clinical availability, but important steps must be taken to ensure that it ultimately reaches the populations in greatest need. Public health intervention and control measures of EV71 epidemics so far have been empirical and generic, not stringently evidence based. Because they have significant socioeconomic impact, further research is needed on the transmission dynamics of the virus and which of these public health intervention strategies most effectively limit the havoc wreaked by future EV71 outbreaks.

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Suggested Reading

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Enteroviruses: Polio

13

Olen M. Kew

1 Introduction

Six decades ago, every child faced the threats of lifelong paralysis or death from poliomyelitis. Poliomyelitis, an infectious disease dating back to antiquity (Fig. 13.1), suddenly appeared in epidemic form in the late nineteenth century in northern Europe and the United States and emerged as one of the great epidemic diseases of the twentieth century [1–3]. The threat of poliomyelitis quickly receded in developed countries following the introduction of the inactivated poliovirus vaccine (IPV) in 1955 [4, 5] and the oral poliovirus vaccine (OPV) in 1961 [6, 7] and had all but disappeared in high-income countries by the early 1970s [8, 9]. In sharp contrast, poliomyelitis remained largely uncontrolled in the developing countries of Latin America, Asia, and Africa and continued to threaten the majority of the world's children with crippling disease [8, 9].

Today, poliomyelitis is on the verge of eradication, and its etiologic agents, the three poliovirus serotypes, are on the brink of extinction from the natural environment (Figs. 13.2 and 13.3) [12]. Circulation of indigenous wild type 2 poliovirus ceased in 1999 [13], and wild type 3 poliovirus may be nearing eradication [12]. Wild type 1 poliovirus circulation is localized to a small and decreasing number of districts in parts of three countries (for updates see http://www.polioeradication.org/) [12].

This brightening picture is the direct result of the initiatives launched in 1985 by the Pan American Health Organization (PAHO; the Regional WHO Office for the Americas) to eradicate poliomyelitis in the Americas by 1990 [14], and subsequently by the World Health Organization (WHO) to eradicate poliomyelitis worldwide by the year 2000 [15]. The Global Polio Eradication Initiative (GPEI), established by a landmark 1988 resolution of the World Health Assembly (the governing body of the WHO) [15], has grown to become the largest public health program in history [16], engaging key segments of both the public and private sectors [17]. The launch of the GPEI was made in light of dramatic progress by PAHO toward achieving its regional eradication goal, attained in 1991 [18]. Like the PAHO initiative, the GPEI achieved early rapid progress, reducing poliomyelitis incidence worldwide by >99 %, from an estimated 350,000 cases in 125 countries in 1988 to a low of 493 cases reported in 10 countries in 2001, raising the long-held hope that a polio-free world would soon be realized [19]. Optimism was fueled by the eradication of wild poliovirus type 2 and reinforced by the cessation of wild poliovirus transmission in many highly challenging settings. However, progress stalled between the years 2000 and 2010 as the global incidence poliomyelitis stabilized at ~500–2,000 cases per year (Fig. 13.2a) [10, 19, 20]. With intensified efforts, the GPEI steadily reduced the number of endemic reservoirs, such that by the end of 2012 the global poliomyelitis case count again fell to a new all-time low of 223, and only three countries (Nigeria, Pakistan, and Afghanistan) had never stopped wild poliovirus transmission (Figs. 13.2 and 13.3) [12, 21]. Despite setbacks, the GPEI achieved many landmark successes: coordinating the vaccination of 2.5 billion children, many of them among the most vulnerable living in the most under-resourced communities in the world, and saving more than ten million people (mostly children <2 years of age) from lifelong paralysis and sparing the lives of more than 250,000 others [22]. The WHO GPEI is now developing a detailed endgame strategic plan to secure forever the many gains achieved by polio eradication (http://www.polioeradication.org/portals/0/document/resources/strategywork/endgamestratplan_20130414_eng.pdf) [22].

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Fig. 13.1 Thirty-three centuries of poliomyelitis. (**a**) Stele from the Eighteenth Dynasty of Egypt (c.1550 to c.1292 BCE) portraying a young man with an atrophied right leg and flaccid foot drop characteristic of the long-term sequelae of paralytic poliomyelitis. The stele is in the Ny Carlsberg Glyptotek, Copenhagen, Denmark; the photograph was downloaded from Wikimedia Commons (http://commons.wikimedia.org/wiki/File:Polio_Egyptian_Stele.jpg). (**b**) Poliomyelitis in Delhi, India, 2002. The last case of poliomyelitis associated with wild poliovirus in India had onset in January 2011. The photograph was downloaded from the WHO Media Centre (http://www.who.int/mediacentre/multimedia/2002/ind_polio211460.jpg)

Polioviruses are members of the *Enterovirus* genus of the family *Picornaviridae* (*pico*, L., small; *rna*, RNA genome) (Chap. 11) [23]. The *Enterovirus* genus, comprising >100 serotypes, is divided into 12 species (enterovirus species A to J and rhinovirus species A to C); poliovirus, along with >20 other serotypes are members of human enterovirus species-C (for updates see: http://www.ictvonline.org/) [24, 25]. Enteroviruses inhabit the intestinal tracts and/or the nasopharyngeal tissues of humans and other mammals. Polioviruses, for which humans are the only natural reservoir host, occasionally invade the central nervous system (CNS) and cause destruction of motor neurons in the spinal cord, resulting in acute flaccid paralysis (AFP). However, poliovirus invasion of the CNS occurs in less than 1 % of infections and represents a dead end for transmission, which occurs by the fecal–oral or respiratory routes [23, 26]. The abrupt appearance of large poliomyelitis outbreaks generated intense interest in the disease and prompted intensive studies of poliovirus epidemiology, pathology, immunology, and virology, leading to the development and worldwide deployment of effective poliovirus vaccines and many groundbreaking contributions to public health, medical science, and basic research [3, 19, 23, 27].

2 Historical Background

The rich history of research on poliomyelitis and polioviruses has been chronicled in numerous excellent books, chapters, and reviews and in an extensive scientific literature

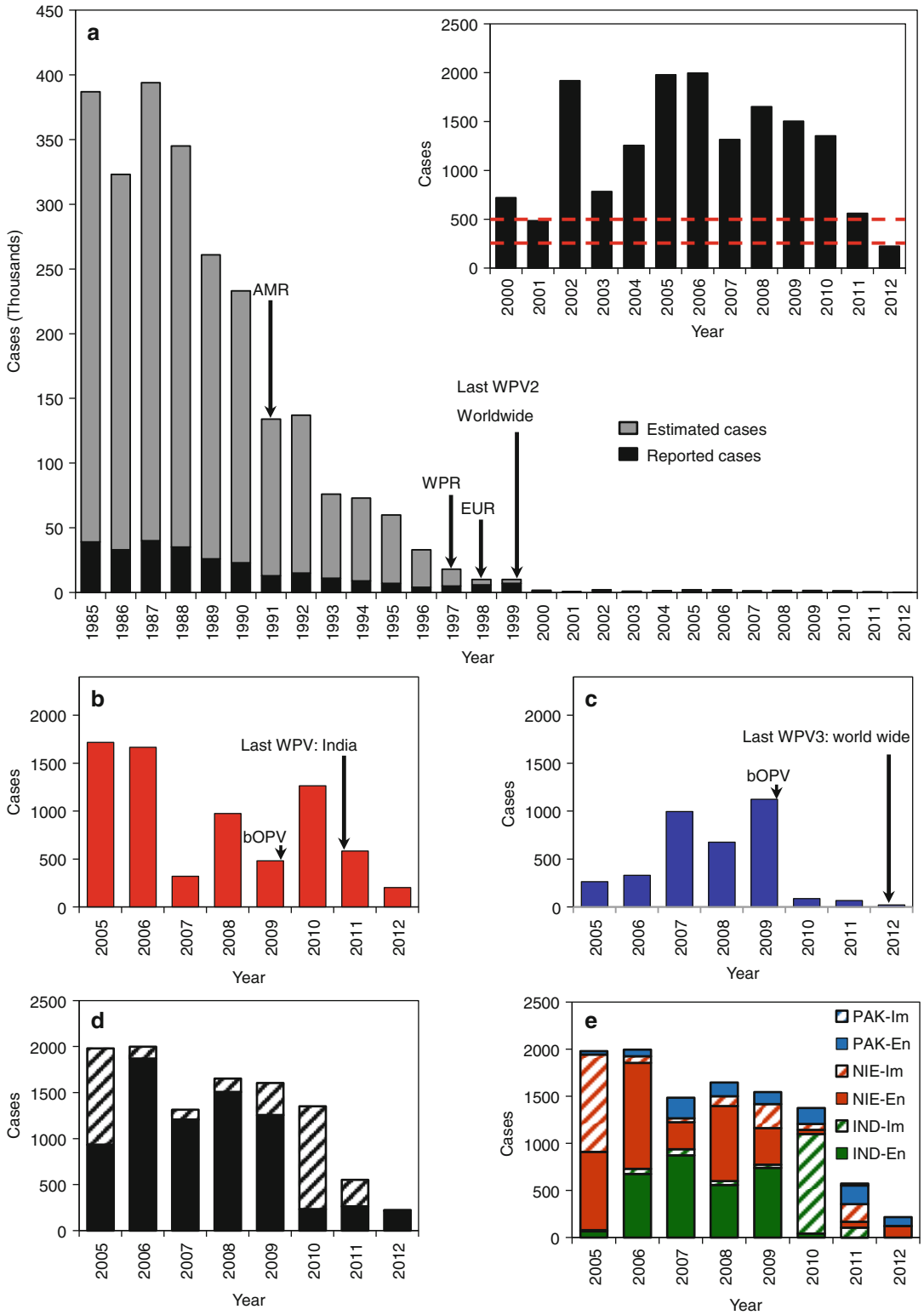

Fig. 13.2 (**a**) Incidence of paralytic poliomyelitis cases associated with wild poliovirus (*WPV*) infections worldwide, 1985–2012 (source http://www.polioeradication.org/). Estimated cases are shown as gray bars; reported, clinically confirmed, and virologically confirmed cases are shown as *black bars*. Starting in 2001, all WPV case counts were based on virologic confirmation by the GPLN. *Arrows* below three-letter codes for WHO regions (*AMR* Americas, *EUR* Europe, *WPR* Western Pacific) indicate year of last detection of indigenous WPV. *Red dashed lines* in inset indicate estimated number (250–500) of cases of VAPP per year worldwide. (**b**) Wild poliovirus type 1 (*WPV1*) poliomyelitis cases, 2007–2012. Introduction of bivalent OPV (bOPV; types 1 + 3) in late 2009 is indicated by the arrow. (**c**) Wild poliovirus type 3 (*WPV3*) poliomyelitis cases, 2007–2012. (**d**) Poliomyelitis cases from endemic (*solid bars*) and imported (*hatched bars*) WPV, 2007–2012. (**e**) Poliomyelitis cases associated with endemic (En) and imported (Im) WPV, 2007–2012 (*PAK* Pakistan [and Afghanistan], *NIE* Nigeria, *IND* India). (**e**) Poliomyelitis cases associated with endemic and imported WPV3, 2007–2011 (Modified from reference Kew [10])

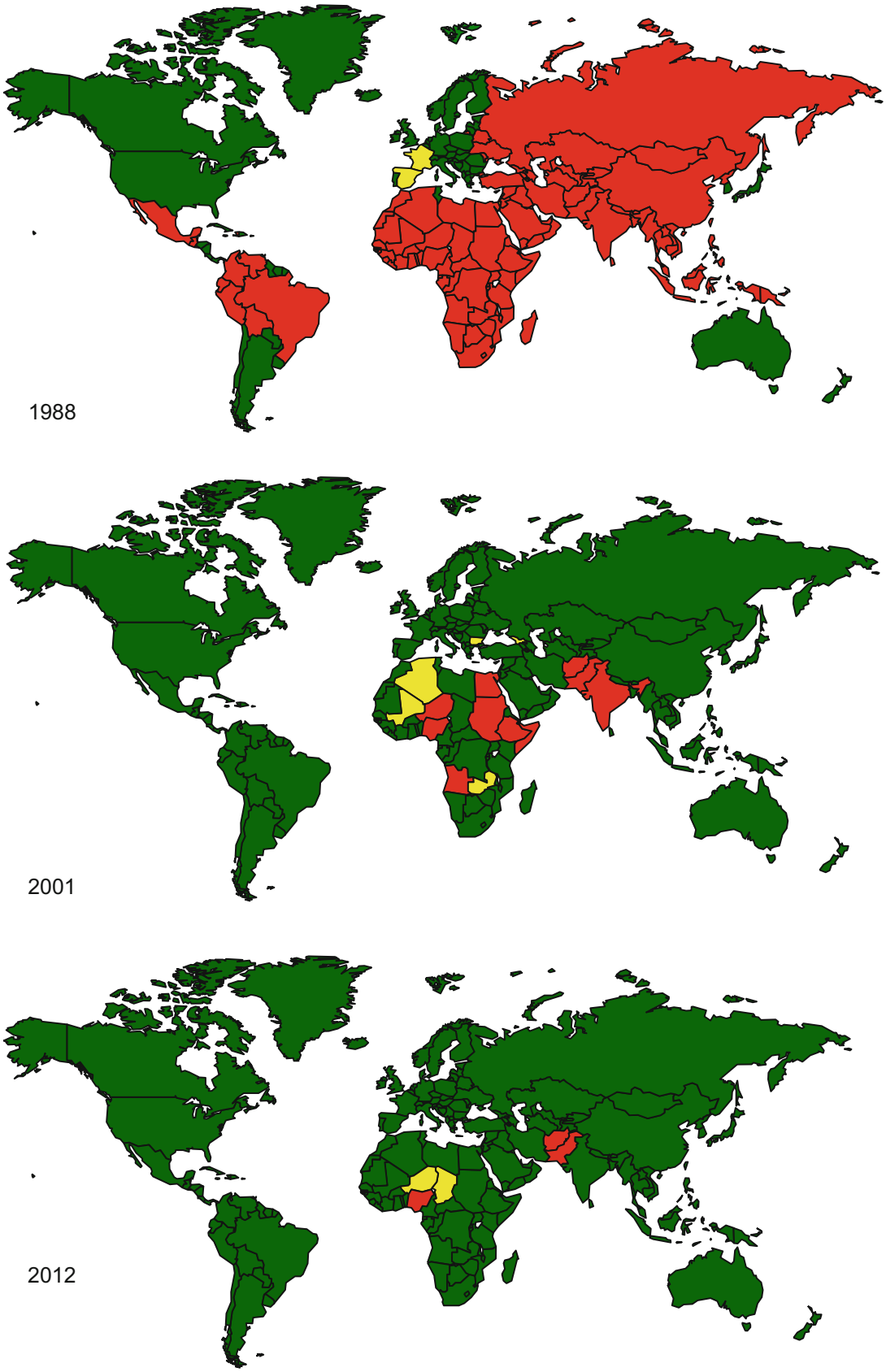

Fig. 13.3 Progress toward global polio eradication, 1988 to 2012. *Red* countries with indigenous wild polioviruses (WPVs), *yellow* countries with one or more case associated with imported WPV, *green* polio-free countries (Modified from reference Kew and Pallansch [11])

dating back over a century [1–3, 7, 23, 28–35]. The disease probably emerged at the dawn of civilization, when population centers grew in size and density sufficient to support continuous endemic circulation. The earliest evidence for endemic poliomyelitis comes from Egypt, recorded on a small funerary stele from the Eighteenth Dynasty (c.1550 to c.1292 BCE), depicting a crippled young man, standing with the aid of a staff, with an atrophied right leg and flaccid foot drop characteristic of the long-term sequelae of paralytic poliomyelitis (Fig. 13.1) [1]. Three millennia later, in 1789, Underwood in England wrote the first clear clinical description of poliomyelitis as a "debility of the lower extremities" [36]. In 1840, the German orthopedist, von Heine, described "Spinale Kinderlähmung" (infantile spinal paralysis) and postulated that the disease could be contagious [37]. In Sweden, Medin conducted the first investigations of the epidemiology of poliomyelitis during the outbreak in Stockholm in 1887 [38]. Sporadic small outbreaks of paralytic disease had been described in the United States since 1841 [33, 39], but the first large outbreak (132 cases) occurred in Rutland, Vermont, in 1894 [40]. In northern Europe, major epidemics (>500 reported cases) erupted in Norway and Sweden (1905), Austria (1908–1909), Germany (1909), and England and Wales (1911) [34, 39]. During this period, Wickman in Sweden firmly established that poliomyelitis was transmitted by person-to-person contact, that the disease spread along the major lines of transportation, and that it gave differing clinical presentations. Wickman hypothesized that all infections, both severe and mild, contributed to spread, and that the incubation time was 3–4 days [41]. In Vienna in 1908, Landsteiner and Popper demonstrated that monkeys became paralyzed after intraperitoneal injection of a filtered homogenate from the spinal cord of a 9-year-old boy (who died within 3 days of onset of paralysis), and that they developed neural lesions similar to those observed in paralyzed humans [42]. Landsteiner and Popper were unable to passage the virus, but several groups, including Flexner and Lewis in 1909, achieved serial passage of poliovirus by nasal inoculation of monkeys [43]. With continued passage in monkeys, they selected a strictly neurotropic type 2 variant, MV, leading them to postulate that poliovirus grew only in neural tissues. However, in 1912, Kling, Wernstedt, and Pettersson isolated poliovirus not only from neural tissues but also from the oropharynx and small intestine, as well as from intestinal contents and throat swabs [44], but the views of Flexner and colleagues prevailed, and the critical findings from human pathology were overlooked for nearly three decades [1, 2].

After 1906, large epidemics appeared in the northeastern and north central states of the United States, culminating in the epidemic of 1916 centered around New York City, far larger at 23,000 reported cases than any previous outbreak [34, 45]. Nationwide poliomyelitis surveillance had begun in the United States in 1910, when all states were requested to forward monthly poliomyelitis case counts to the Surgeon General's Office [46]. The Drinker respirator, commonly known as the "iron lung," was introduced in 1929 as a device to save the lives of patients with respiratory paralysis (many of whom would subsequently recover unassisted respiratory function) [47]. In 1931, reinfection experiments in monkeys by Burnet and Macnamara provided the first evidence of more than one poliovirus serotype [48]. Seven years later, President Franklin D. Roosevelt, who had been paralyzed in both legs by poliomyelitis in 1921, cofounded with Basil O'Connor the National Foundation for Infantile Paralysis and the March of Dimes campaigns, providing a critical source of support for poliovirus vaccine research [1, 3]. Adaptation of the type 2 Lansing strain to cotton rats and mice by Armstrong in 1939 opened the way for much broader and more quantitative virologic and serologic studies on a scale previously unattainable with titrations in monkeys [49]. By the early 1940s, Trask and Paul [50, 51] and Sabin and Ward [52] recognized that poliovirus replicated in the tissues of the intestinal tract as well as the CNS, confirming the earlier observations of Kling. In 1949, Enders, Weller, and Robbins cultivated the type 2 Lansing strain of poliovirus in nonneural cells from human embryonic tissue—including skin, muscle, and intestine—yielding large quantities of virus, thereby accelerating the pace of poliovirus research and opening the way for expanded vaccine development and large-scale vaccine production [53]. That same year, Bodian and colleagues established that there were only three poliovirus serotypes [54, 55]. In 1953, following the peak year for poliomyelitis cases reported in the United States (57,628) [56], Hammon et al. demonstrated that administration of immune gamma globulin was protective against paralytic disease [57], and the following year Horstmann et al. showed that viremia preceded paralysis in humans [58]. With these strong experimental underpinnings, the stage was set in 1954 for Francis to conduct a field trial enrolling 1,800,000 children in the United States [59], demonstrating the safety and efficacy of the IPV developed by Salk and colleagues [4]. The new IPV was promptly licensed and distributed following announcement of the field trial findings in April 1955 [1, 3, 35]. In 1959, large field trials of the OPV of Albert Sabin were conducted in the Soviet Union, Poland, and Czechoslovakia [60], leading to the licensure and distribution of monovalent OPV types 1 and 2 in 1961 and type 3 in 1962 [6].

3 Methodology Involved in Epidemiologic Analysis

3.1 Sources of Data

Poliomyelitis has been a notifiable disease in the United States since 1910 [46], when case reports from state and territorial boards of health and health departments were summarized

monthly (1910–1926) and then weekly (1927–1951) in *Public Health Reports* [61]. Starting in 1952 [62], reports were regularly published in the CDC (Communicable Disease Center, later Centers for Disease Control and Prevention) *Morbidity and Mortality Weekly Report* (*MMWR*) [63]. Special annual poliomyelitis surveillance summaries were also published by CDC through 1974 [64]. In addition to the United States, many European countries and Canada established systems early in the last century for reporting cases of poliomyelitis, allowing epidemiologists to monitor rising disease incidence up to mid-century [65, 66] and the sharp decline after the introduction of poliovirus vaccines [8, 9]. In contrast, data on poliomyelitis incidence in developing countries was very incomplete, with only a small fraction of cases reported and with many populous countries not reporting any cases at all [8, 9]. In 1969, the World Health Assembly adopted a resolution that placed poliomyelitis "under international surveillance" [67]. However, systematic and sensitive surveillance for poliomyelitis in developing countries only followed the launch of polio eradication efforts in the Americas [14, 18] in 1985 and the GPEI in 1988 [15] and the establishment of field surveillance for cases of acute flaccid paralysis (AFP) [68] closely integrated with virologic testing of clinical specimens [69]. The quality of the integrated surveillance data improved gradually, usually in step with improvements in OPV coverage, and the findings were published regularly in reports by PAHO [70], WHO [71], and CDC [72]. Current weekly and monthly reports are posted on the WHO website (http://www.polioeradication.org/Dataandmonitoring/Poliothisweek/.aspx), and lists of wild polioviruses by country and year are posted on http://www.polioeradication.org/Dataandmonitoring/Poliothisweek/Wildpolioviruslist.aspx.

Over the past century, the large majority of case counts were based on clinical diagnoses, and it was soon recognized that the most accurate counts were obtained in outbreak settings [73]. Cases not associated with large outbreaks were more likely to be underreported. In the PAHO and WHO eradication initiatives, AFP cases were systematically reported and investigated, and as national and regional laboratory networks developed, all specimens from AFP cases were tested for the presence of poliovirus [69, 74, 75]. Countries and regions shifted from a clinical case definition to a virologic case definition once field surveillance for cases of AFP was tightly integrated with laboratory investigations for poliovirus. By 2001 global poliomyelitis case counts were based on virologic findings.

Data on case/fatality (CFR) ratios are not routinely available as ratios vary with the age distribution of population susceptibility and by setting [9, 23]. CFRs increase with age and are generally on the order of 2–5 % in children <5 years of age and 10–30 % in adults [23, 76, 77]. The epidemics early in the twentieth century were associated with high CFRs (27 % in the 1916 New York epidemic) [45], and more recent outbreaks from importation of wild poliovirus into previously polio-free countries have also been characterized by high CFRs in older age groups: Albania, 1996 (18 % for ages 19–24 years) [78]; Cape Verde Islands, 2000 (57 % for ages >15 years) [79]; Namibia, 2006 (31 %; most paralytic cases were among adults) [80]; the Republic of Congo, 2010–2011 (43 %; most paralytic cases were among adults) [81]; and Xinjiang, China, 2011 (10 %) [82]. During the outbreak year of 2006 in India, highly sensitive surveillance documented a CFR of 7.1 % among children <2 years [77].

3.2 Serologic Surveys

Serologic studies of infectious diseases were initially applied to the diagnosis of individual cases [83]. The first applications of serology to epidemiology were the studies by Aycock and Kramer in 1930, who used the newly developed neutralization test to show that antibodies to poliovirus appeared at younger ages in urban compared with rural populations [84]. Despite the methodological limitations (neutralization tests were performed in monkeys and predated recognition of more than one poliovirus serotype), these early studies heralded a powerful new tool to address fundamental questions about the epidemiology of poliomyelitis. In the pre-vaccine era, serologic surveys played an indispensable role in defining the prevalence of poliovirus infection, the intensity of transmission of each poliovirus serotype, the age profiles of exposure in different settings, the years and the associated serotypes of past outbreaks in isolated populations, the duration of type-specific immunity, the identification of susceptible populations, and key aspects of the pathogenesis of paralytic disease—including estimates of age-specific case/infection ratios [23, 28, 85, 86]. In the post-vaccine era, seroepidemiology has been used to detect immunity gaps in underserved populations [87, 88], to estimate the extent of wild poliovirus circulation in populations [89], to determine the efficacy of different OPV formulations in inducing neutralizing antibodies [90], and to provide evidence for eradication of indigenous wild polioviruses [91]. In the GPEI (and in the earlier PAHO initiative), serology was found not to be useful in the diagnosis of individual cases, because the response to detection of an AFP case was prompt administration of trivalent OPV (tOPV) to the patient and to the community ("mop-ups"; Sect. 10.4), such that many initially seronegative children had seroconverted to all three poliovirus serotypes by the time of the second blood sample [18]. However, seroprevalence studies continue to be important in measuring the immunogenicities of different OPV formulations [92], in detecting otherwise inapparent spread of OPV-derived viruses in unimmunized populations [93, 94], and in providing objective data on population immunity [95, 96],

including in polio-free countries where OPV coverage rates have fallen and the rising risks of outbreaks might not otherwise be recognized [22]. In recent outbreaks, primarily associated with poliovirus type 1 (Sect. 10.6), determination of the prevalence of neutralizing antibodies to poliovirus type 2 is a surrogate for vaccine-induced population immunity when the initial immunization response is deployment of type 1 monovalent OPV (mOPV1).

3.3 Lameness Surveys

Severe underreporting of poliomyelitis cases in many developing countries led to the misperception that the disease was not a source of serious morbidity in the tropics [8, 66]. Lameness surveys conducted in the 1970s and 1980s in Africa, Asia, and the Middle East confirmed the high prevalence of paralytic disease in developing countries and prompted many countries to begin polio vaccination programs [97, 98]. For example, in India in 1981–1982, estimates of the incidence of poliomyelitis from lameness surveys were as high as 200,000 cases per year, more than tenfold higher than officially reported case counts, with 83 % of cases occurring before 3 years of age [99]. Methods to improve the comparability of lameness surveys in different settings, including the use of standardized case definitions, has been reviewed [97].

3.4 Acute Flaccid Paralysis (AFP) Surveillance

AFP is the most serious clinical manifestation of wild poliovirus infection (Sect. 8.1) [100]. Poliomyelitis outbreaks are readily recognized, but low-level circulation in interepidemic periods may be missed in the absence of a sensitive surveillance system. This is especially true for endemic circulation of poliovirus types 2 and 3, which have much lower paralytic case/infection ratios than type 1 (estimated case/infection ratios [assuming an overall case/infection ratio of 1/150]: type 1, ~1/190; type 2, ~1/1,900; type 3, ~1/1,150) [34, 101]. Recent importation of wild poliovirus or emergence of circulating vaccine-derived polioviruses (cVDPVs; Sect. 10.8) may also be missed by the AFP surveillance system unless high sensitivity is maintained. Starting in 1985, PAHO built an AFP surveillance system to support the regional Polio Eradication Initiative [18]. Performance indicators for reporting of AFP cases were established assuming a background rate of nonpolio AFP of at least 1 case per 100,000 population <15 years. In addition, surveillance sites were required to report weekly, including "zero reporting" when no AFP cases were identified during the previous week [75]. AFP surveillance was closely integrated with virologic surveillance whereby stool samples from at least 80 % of patients with AFP were tested for the presence of poliovirus (Sect. 3.6) [69]. The successful PAHO strategy was adopted by the GPEI and implemented in all polio-endemic countries [68, 74, 102]. The benchmark AFP rate was raised to at least 2 cases per 100,000 population <15 years in endemic areas, and the global rate has been >4 since 2007 (http://apps.who.int/immunization_monitoring/en/diseases/poliomyelitis/afpextract.cfm). In the last stages of polio eradication in India, AFP surveillance sensitivity reached the extraordinarily high levels of >25 AFP cases per 100,000 population <15 years in the remaining polio-endemic states of Uttar Pradesh and Bihar (http://www.npspindia.org/bulletin.pdf) [91]. Only a small fraction of the AFP case-patients had wild poliovirus infections, and the integrated AFP and poliovirus surveillance system was approximating a community stool sampling survey of a population of ~300 million. It was critical to integrate AFP surveillance with laboratory-based poliovirus surveillance because AFP has multiple etiologies (including Guillain–Barré syndrome, transverse myelitis, infections by other neurotropic viruses, and traumatic neuritis; Sect. 8.1) [7], and the large majority of wild poliovirus infections are inapparent.

3.5 Environmental Surveillance

Sewage sampling was used as early as the 1940s to monitor the seasonal variation of poliovirus circulation in urban communities [51, 103]. Because most poliovirus infections are inapparent (Sect. 3.4 and 8.1), sewage sampling can greatly increase the overall sensitivity of poliovirus surveillance. For example, during the pre-vaccine era in the United States, poliovirus could be detected in sewage shortly before and after the seasonal appearance of paralytic cases, and the combined clinical data and environmental poliovirus isolation rates permitted estimation of the ratio between inapparent infections and paralytic cases [103]. Sewage sampling is widely implemented in Europe (conducted by 20 countries, including Israel [104–108]) and Japan [109] as a component of enterovirus (and poliovirus) surveillance. During the 1984 poliovirus outbreak in Finland, environmental surveillance demonstrated widespread circulation of the wild type 3 outbreak virus and provided a basis for the estimate of the occurrence of at least 100,000 inapparent infections despite the appearance of only nine paralytic cases [104, 110]. Wild poliovirus type 3 was found to be present in the environment 3 weeks before the appearance of the first paralytic case during the 1992–1993 outbreak in the Netherlands, and circulation of the outbreak virus was found to be localized to communities that refused immunization [106]. Environmental surveillance coupled with sequencing of wild poliovirus isolates was introduced on a limited basis in the PAHO program [111, 112]. Sampling of wastewater in a high-risk community

in Cartagena, Colombia, revealed close sequence relationships between sewage isolates, stool survey isolates, and paralytic case isolates obtained from the community over the same period, but also detected circulation of lineages not found by AFP surveillance [111]. Sewage sampling in Israel and adjoining Palestinian territories detected outbreaks of wild poliovirus infections in Gaza, Ashdod, and the West Bank in 1990 (type 3), 1991 (type 1), 1994–1995 (type 1), and 1996 (type 1) [107]. The outbreaks were described as "silent" because no poliovirus-associated paralytic cases had been detected by the AFP surveillance systems. All of the wild poliovirus sewage isolates were found to be related to viruses circulating in Egypt. The continued detection in Gaza of wild polioviruses of Egyptian origin at times when none were reported by the AFP surveillance system in the source reservoir communities prompted the implementation of environmental surveillance in Egypt in September 2000, which by 2004 sampled 33 sites in 18 governorates [113–115]. This approach, combined with strengthened AFP surveillance, improved the overall sensitivity of the poliovirus surveillance system, and wild polioviruses disappeared from the environment soon after their disappearance in specimens collected from patients with AFP [113, 115]. Implementation of sewage sampling in the open canals in the large slum communities of Mumbai, India [116], coupled with sequencing (section "Nucleotide sequencing of poliovirus isolates"), confirmed the disappearance of the local wild polioviruses and the repeated importation of wild polioviruses from known reservoirs in the northern Indian states of Uttar Pradesh and Bihar [91]. Because no suitable sampling sites were available in the highest-risk rural reservoir communities, additional sites were established in Delhi and Patna, Bihar, which receive migrants from the endemic rural areas [91]. As in Egypt, the findings from the environmental and AFP surveillance systems were in agreement: the last environmental wild poliovirus isolate (a type 1) was found in Mumbai sewage in November 2010, and the last wild poliovirus isolate (a type 1) from an AFP case-patient was in West Bengal in January 2011 (http://www.npspindia.org/bulletin.pdf) [91]. The GPEI and the Global Polio Laboratory Network (Sect. 3.6.1) have extended environmental surveillance to six cities in Pakistan and three cities in Nigeria and are planning further expansions in countries at high risk of reestablishment of poliovirus circulation either by importation or by the emergence of cVDPVs (see below and Sect. 10.8) [22].

VDPVs closely resembling those excreted by individuals with primary immunodeficiencies (Sect. 10.8.2) have been detected in sewage in Israel [117], Estonia [118], Slovakia [119], and Finland [120]. Despite efforts in each country to identify the source of the excreted virus, no poliomyelitis cases or chronically infected individuals have so far been identified.

Environmental surveillance has also helped inform the endgame strategy for the GPEI (Sect. 11.2). A key question is the persistence of vaccine-related viruses in the population and environment following cessation of OPV use. In Cuba, where OPV is delivered only in mass campaigns in the form of two rounds of National Immunization Days (NIDs), vaccine-related viruses were detected in stool surveys for up to 8 weeks and in the environment for up to 15 weeks after the second NID round [121]. New Zealand shifted from OPV to IPV in February 2002, and vaccine-related viruses were regularly detected in sewage samples until May 2002. Sporadic vaccine-related isolates detected subsequently showed very limited sequence divergence from the parental OPV strains, indicating that they were recent imports from OPV-using countries rather than persistence of vaccine-related viruses in the community [122].

An important difference between environmental sampling and AFP surveillance is that environmental sampling is most sensitive in locations with developed sewage systems or open sewage canals in large slums and therefore is usually established in more urbanized settings. Sewage sampling sites in urban centers are selected to include communities of migrant populations from rural areas. Environmental surveillance is necessarily localized, targeted, and intermittent, in contrast to a well-functioning AFP surveillance system that monitors the entire population on a continuous basis. Consequently, poliovirus isolates obtained by the two surveillance approaches may yield different kinds of public health information. For example, a wild poliovirus isolate from an AFP case is directly linked to a specific patient from a specific locale and usually signals many other inapparent infections in the community. In contrast, the high sensitivity of environmental sampling can result in multiple poliovirus isolations from a single infected person but does not yield further information about the specific source of infection. However, some information about the extent of poliovirus circulation can be obtained from the extent of genetic diversity of polioviruses obtained at a sampling site [111, 115].

3.6 Laboratory Methods

3.6.1 The Global Polio Laboratory Network (GPLN)

The GPLN was established by the WHO to support the GPEI [123]. Currently the GPLN consists of 145 laboratories, initially organized in three tiers: (1) National and Subnational Laboratories ($n=122$), (2) Regional Reference Laboratories ($n=16$), and (3) Global Specialized Reference Laboratories ($n=7$) (Fig. 13.4). As the GPLN developed, activities once assigned to Regional Reference Laboratories are frequently performed by many National and Subnational Laboratories, and Regional Reference Laboratories currently perform

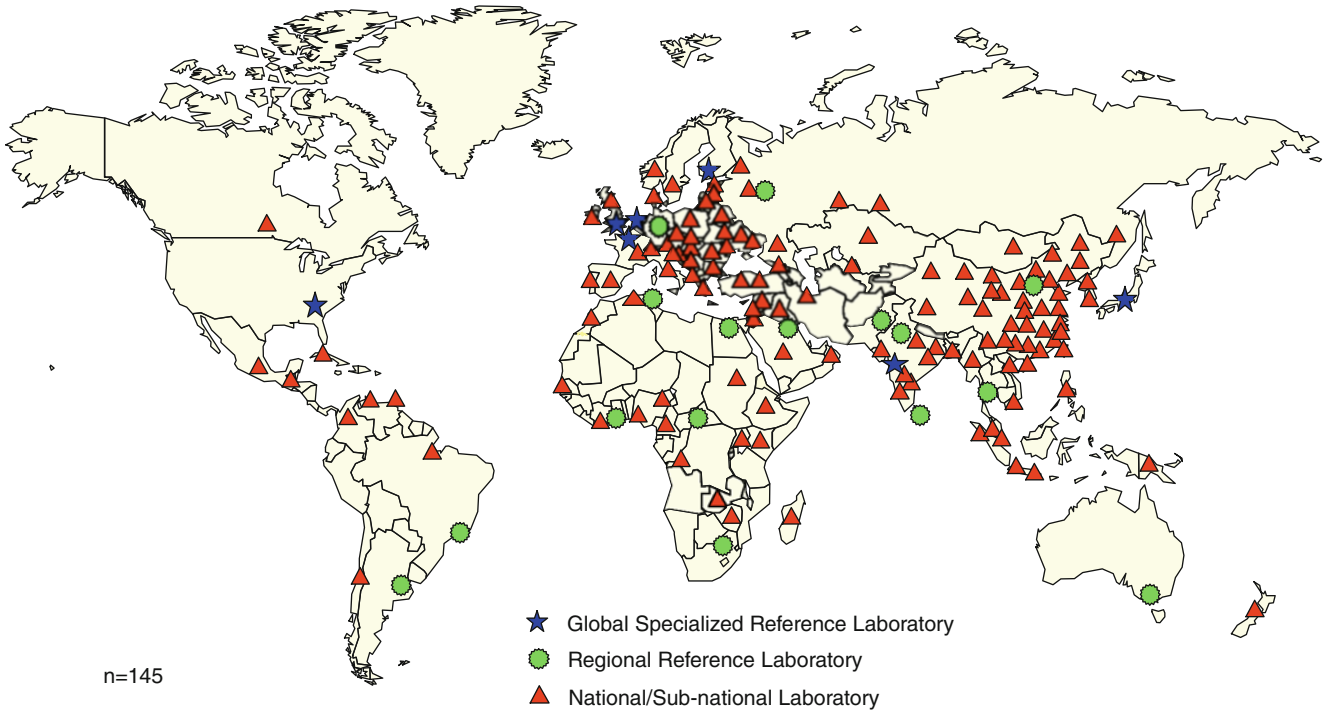

Fig. 13.4 Distribution of laboratories of the Global Polio Laboratory Network (GPLN). *Triangles* National and Subnational Laboratories, *circles* Regional Reference Laboratories, *stars* Global Specialized Reference Laboratories

many functions (such as genomic sequencing) originally assigned to Global Specialized Reference Laboratories. These trends have strengthened the GPLN, permitted continuous technical innovation, and moved many of the diagnostic activities closer to the endemic areas of highest priority. The GPLN was patterned after the PAHO Regional Laboratory Network established in parallel with development of AFP surveillance [69]. Close integration of AFP and poliovirus surveillance was facilitated by the use of standardized case ("EPId") numbers accessible to surveillance officers, virologists, and program managers [100, 124].

Methods for poliovirus isolation, identification, and serology have been described in detail previously [23, 125, 126]. Laboratory manuals were developed by the GPLN to standardize methods for detecting and characterizing polioviruses in clinical specimens and environmental samples [124], and a GPLN Quarterly Update was published by the WHO to keep GPLN virologists and others abreast of new developments and innovations [127]. Because the overriding emphasis of the GPLN is to monitor poliovirus circulation, less attention has been given to the routine typing and characterization of nonpolio enterovirus isolates, and readers are referred to Chap. 11 for details on those methods. The WHO Polio Laboratory Manual [124] is regularly updated as new methods are developed and tested for suitability for use by GPLN laboratories. New methods, designed to increase sensitivity, specificity, and work efficiency, are developed in concert with the rising technical capabilities of the GPLN and in anticipation of (or in response to) increasingly focused surveillance questions posed by the GPEI [22]. All GPLN laboratories participate in a formal accreditation process which includes review of performance in standardized proficiency tests as well as routine diagnostic work as confirmed by GPLN reference laboratories [128]. Well-characterized cells, reference OPV virus stocks, serologic reagents, and molecular reagents are distributed by GPLN reference laboratories to ensure a high degree of standardization, and internal quality control procedures are regularly implemented by all GPLN laboratories to ensure high routine performance [128]. GPLN laboratories participate in annual regional and global meetings to review performance, discuss effective implementation of new methods, develop approaches to improve coordination, and plan research and other future activities (http://www.polioeradication.org/Dataandmonitoring/Surveillance/GlobalPolioLaboratoryNetwork.aspx) [128]. The GPLN is interdependent, applying common approaches to problem solving, parallel testing as needed, training, and other kinds of technical support. The GPLN is guided by expert WHO virologists who serve as global and regional laboratory coordinators. The GPLN, with its close integration with program, has served as the model for newer regional and global networks supporting laboratory-based surveillance for other viral and bacterial vaccine-preventable diseases [129–131]. Many of the methods and reagents used by the GPLN have also been shared with state laboratories in the United States [132].

3.6.2 Virus Isolation and Identification

Categories of Poliovirus Isolates

The primary purpose of infectious disease surveillance is to identify agents that present potential public health risks. Poliovirus isolates of each serotype are grouped into three categories, correlating with the risk of transmission and spread, and based on the extent of divergence of the VP1 nucleotide region compared to the corresponding OPV strain: (1) wild polioviruses (no genetic evidence of derivation from any vaccine strain and demonstrated capability of continuous person-to-person transmission); (2) vaccine-derived polioviruses (VDPVs) (vaccine-related polioviruses that are >1 % divergent [types 1 and 3] or >0.6 % divergent [type 2] from the corresponding OPV strain and potentially capable of causing paralytic disease and establishing person-to-person transmission) (Sect. 10.8); and (3) "OPV-like" polioviruses (vaccine-related polioviruses that are ≤1 % divergent [types 1 and 3] or ≤0.6 % divergent [type 2] from the corresponding OPV strain) that are ubiquitous wherever OPV is used [133]. VDPVs are further categorized as (1) circulating VDPVs (cVDPVs), when there is evidence of person-to-person transmission in the community; (2) immunodeficiency-associated VDPVs (iVDPVs), which are isolated from persons with primary immunodeficiencies who have prolonged VDPV infections; and (3) ambiguous VDPVs (aVDPVs), which are either clinical isolates from persons with no known immunodeficiency or sewage isolates whose ultimate source is unknown [133].

Clinical Specimens

The specimens of choice for AFP and poliovirus surveillance are stool samples collected as soon after onset of paralysis as possible. The GPEI has defined "adequate" stool specimens as "two stool specimens of sufficient quantity (~8 g) for laboratory analysis, collected at least 24 h apart, within 14 days after the onset of paralysis, and arriving in the laboratory in good condition and with proper documentation" (http://www.polioeradication.org/Dataandmonitoring/Surveillance.aspx). Two samples are collected because poliovirus shedding is often intermittent [134]. The GPEI has established clear performance guidelines and training procedures for proper specimen transport to the laboratory via a "reverse cold chain" similar to the forward cold chain used for deployment of OPV [100, 124]. Such importance has been assigned to specimen collection from AFP cases that runners with specimens in insulated backpacks were allowed safe passage through combat lines in war zones where use of motorized transport was very hazardous.

Poliovirus may be isolated at lower frequencies from rectal swabs [135] and throat swabs (if taken within the first few days of infection) and only rarely from cerebrospinal fluid (CSF); however, none of these specimens are recommended by the GPEI and the GPLN [100, 124].

Virus Isolation in Cell Culture

The most critical and basic procedure is virus isolation in cell culture. Polioviruses can be grown in a wide range of human cells (RD, HeLa, HEp-2, WI-38, MRC-5, HEK293) and simian cells (from rhesus macaques and African green monkeys; primary monkey kidney cells, Vero, LLC-MK2, BGM) (http://www.atcc.org/) [23], but two cell lines are routinely used in combination by the GPLN for virus isolation [124]: (1) RD cells (a continuous line from human rhabdomyosarcoma [136]) which are highly sensitive to poliovirus infection and yield virus at high titers [23] and (2) L20B cells (a derivative of the mouse L cell line engineered to express the human poliovirus receptor, CD155) which are highly selective for growth of poliovirus [124, 137]. Viruses that grow in L20B are usually polioviruses (although some Coxsackie A viruses can grow in L20B cells [124, 138]) and are further characterized by molecular identification methods.

Molecular Characterization and Intratypic Differentiation of Isolates

The original methods for identification of polioviruses and other enteroviruses were based on antigenic properties. Virus isolates were typed by testing for growth in the presence of pools of antisera containing different combinations of high-titer neutralizing antibodies [23]. Typing of individual poliovirus (or enterovirus) isolates was then confirmed by use of type-specific antisera. Heterotypic poliovirus mixtures were resolved by growth in the presence of different pairs of type-specific neutralizing antisera.

Intratypic differentiation (ITD; distinguishing wild polioviruses from vaccine-related isolates) was originally based on antigenic or phenotypic properties [139]. Before the development of molecular methods, the most reliable of these were the antigenic methods, and isolates were described as "vaccine-like," "non-vaccine-like," or "intermediate." Most assignments based on antigenic methods were confirmed by the more precise molecular methods. It is remarkable that the antigenic methods worked so well in view of the fact that the Sabin OPV strains undergo frequent antigenic evolution toward "non-vaccine-like" antigenicity during replication in the human intestine [139–141], and that the wild polioviruses themselves are antigenically diverse [139, 142]. Although the Sabin type 1 OPV strain (Sabin 1) has multiple non-consensus antigenic changes in its neutralizing antigenic sites [139, 143, 144], Sabin 2 and Sabin 3 are usually less antigenically divergent from the corresponding wild polioviruses [139]. ITD based on antigenic properties was improved by use of highly specific cross-absorbed monotypic sera which contained antibodies that reacted specifically with "vaccine-like" or "non-vaccine-like" antigens [143]. ITD using cross-absorbed antisera was adapted to an ELISA format [145] and widely used by the GPLN, especially in recent years for the characterization of VDPVs

Table 13.1 Poliovirus identification by real-time RT-PCR

RT-PCR primer–probe set	Poliovirus/enterovirus isolate category[a]								
	NPEV	WPV1[b]	WPV3[b]	S1	S2	S3	WPV1/WPV3	VDPV1	VDPV2
panEV	+	+	+	+	+	+	+	+	+
panPV	–	+	+	+	+	+	+	+	+
Sero1	–	+	–	+	–	–	+	+	–
Sero2	–	–	–	–	+	–	–	–	+
Sero3	–	–	+	–	–	+	+	–	–
Sab1	–	–	–	+	–	–	–	+	–
Sab2	–	–	–	–	+	–	–	–	–
Sab3	–	–	–	–	–	+	–	–	–
VDPV1	–	–	–	–	–	–	–	+	–
VDPV2	–	–	–	–	–	–	–	–	+
VDPV3	–	–	–	–	–	–	–	–	–

Based on data from references [151, 153, 157–159]. Reagents for identification of VDPV3 isolates have also been prepared. All suspected wild poliovirus and VDPV isolates are routinely further characterized by VP1 sequencing. Special sequencing primer sets have been developed to resolve both heterotypic and homotypic mixtures. The combinations shown, along with others (especially different combinations of Sabin vaccine-related isolates), have been observed in clinical isolates

[a]*Abbreviations*: *NPEV* nonpolio enterovirus, *WPV1* wild poliovirus type 1, *WPV3* wild poliovirus type 3, *S1* Sabin type 1 vaccine-related, *S2* Sabin type 2 vaccine-related, *S3* Sabin type 3 vaccine-related, *VDPV1* vaccine-derived poliovirus type 1, *VDPV2* vaccine-derived poliovirus type 2

[b]As previously described for other wild poliovirus genotypes [152], wild genotype-specific real-time RT-PCR primers and probe sets have been prepared for the Nigeria wild type 1, Nigeria wild type 3, Pakistan–Afghanistan wild type 1, and Pakistan–Afghanistan wild type 3 genotypes (D. Kilpatrick, manuscript in preparation)

[133]. Other ITD methods based on antigenic properties used panels of neutralizing monoclonal antibodies [140, 142, 145, 146]. However, none of the antigenic ITD methods could overcome the basic biological limitations arising from the antigenic evolution of the OPV strains [140, 141, 145], and antigenic methods have been replaced by methods based on the nucleotide sequence properties of poliovirus isolates.

The earliest molecular method for routine ITD was oligonucleotide fingerprinting [147, 148]. This approach had the high reliability required for poliovirus surveillance in support of eradication, but it was also laborious, expensive, and difficult to scale up and required the use of radioisotopes. Therefore, oligonucleotide fingerprinting was not appropriate for developing country laboratories. Consequently, oligonucleotide fingerprinting was replaced by nucleic acid probe hybridization [149, 150], and the transfer of this ITD method to the PAHO Polio Laboratory Network commenced in the late 1980s. The reverse transcriptase-polymerase chain reaction (RT-PCR) offered specificities and sensitivities unattainable with probe hybridization, and it became the method of choice within the GPLN [151, 152], although full deployment awaited adaptation to a real-time format, which greatly reduced the risks of contamination by PCR products [153]. RT-PCR coupled to restriction fragment length polymorphism analysis was also widely used, because this routine test provided insights into the origins of wild poliovirus isolates [154]. An elegant approach based on microarrays was also developed [155, 156], but it was less readily transferrable to developing country laboratories at the front lines of global poliovirus surveillance.

Currently the GPLN uses real-time RT-PCR for ITD [124]. A series of primer pairs and specific fluorescent probes have been developed that identify isolates hierarchically: (1) as enteroviruses (panEV), (2) as polioviruses (panPV), (3) by poliovirus serotype (Sero1, Sero2, Sero3), and (4) whether vaccine-related (Sab1, Sab2, Sab3) (Table 13.1) [153, 157, 158]. The sets of real-time RT-PCR reagents are deployed as kits for routine use by the GPLN [160] and can be supplemented with additional real-time RT-PCR reagents that further identify wild polioviruses by genotype (Sects. 3.6.3 and 10.7) and can facilitate screening for genetically divergent VDPVs (VDPV1, VDPV2, VDPV3) [133, 161]. The rapid evolution and high genetic diversity within and across poliovirus serotypes presented special challenges to development of the panPV and serotype-specific primer and probe sets (Table 13.1), as it was necessary to use degenerate and inosine-containing oligonucleotides to base pair with the appropriate specificities at positions of codon degeneracy [157, 158]. Although the nondegenerate Sabin vaccine strain-specific RT-PCR reagents can be used in a multiplex format, the complexity of the degenerate reagents limits the number of reactions that can be combined in multiplex.

Nucleotide Sequencing of Poliovirus Isolates

ITD screens for wild polioviruses and VDPVs and screens out OPV-like polioviruses that are unlikely to be of current epidemiologic importance. Since 2001, all wild poliovirus and VDPV isolates are sequenced by GPLN laboratories following standardized procedures and using standardized sequencing primer sets. The ~900-nucleotide interval

(representing ~12 % of the total genome) encoding the major capsid protein, VP1, is routinely sequenced. VP1 sequences are used for routine comparisons because they encode several serotype-specific antigenic sites [162] and evolve primarily by successive fixation of nucleotide substitutions rather than by recombination [163, 164]. Wider genomic intervals, up to the complete genome, may be sequenced to obtain higher epidemiologic resolution or to address specific virologic questions [165–168]. Serotype- and genotype-specific sequencing primers have been developed to specifically amplify components of heterotypic and homotypic poliovirus mixtures, bypassing selective cultivation in the presence of neutralizing antibody or incubation at supraoptimal temperatures [169]. Sequence relationships among poliovirus isolates are summarized in phylogenetic trees and genotypic maps that are distributed monthly by GPLN laboratories to Ministries of Health, WHO country and regional offices, WHO-Geneva, and other GPLN laboratories.

3.6.3 Molecular Epidemiology of Polioviruses

The application of genomic sequencing of poliovirus isolates has added a new dimension and resolving power to the understanding of the epidemiology of poliomyelitis [170]. Because poliovirus genomes evolve rapidly (typically just over 1 % nucleotide substitutions per site per year at all sites, equivalent to one to two nucleotide substitutions per week) [164, 165, 168, 171–174], links between poliomyelitis cases can be determined with precision, and the sources and timing of importations from the remaining poliovirus reservoirs can be established [11, 163, 164, 168, 175–178]. Sequence analyses offer an additional tool to monitor the progress of the GPEI and has shown that poliovirus genotypes (viruses within a genotype differ by <15 % in their nucleotide sequences) and genetic clusters within genotypes (viruses within a cluster differ by <5 % in their nucleotide sequences) disappear sequentially through intensive immunization efforts (Sect. 10.7) [11]. Experience in the Americas has found that in settings of sensitive surveillance, a genotype that is not detected for more than a year has probably become extinct [11, 170]. Molecular epidemiology has established the existence of numerous poliovirus genotypes endemic to different regions of the world (Sect. 10.5.3) [11, 170], demonstrated that poliovirus type 2 is usually the first serotype to be eliminated [13], that poliovirus type 3 appears to circulate more locally than type 1 [10], and that poliovirus type 1 appears to be most commonly associated with importations from neighboring countries and with intercontinental or global spread of the virus (Sect. 10.6) [10, 11, 163, 175, 176, 179–181]. In some settings, different genotypes of poliovirus type 1 have been found to have co-circulated in a geographically limited area [163, 179, 182, 183].

Molecular epidemiologic methods are routinely used to help identify reservoir communities with low population immunity and where demographic and environmental conditions favor poliovirus circulation. During the peak months of poliovirus circulation, virus spreads from the reservoir communities to adjacent non-reservoir indicator communities (where the density of nonimmune susceptible children can support some poliovirus circulation during the peak transmission season). This has led to a refinement in the concept of virus importation, which in previous usage referred to virus transmission across national boundaries. Although many importations over long distances have been documented [10, 180], reservoir communities and their associated indicator communities frequently overlap international borders [10, 184], underscoring the importance of regional synchronization of NIDs and Subnational Immunization Days (SNIDs). Equally important are the patterns of importation from reservoir communities to indicator communities within a country [168, 184, 185]. High vaccine coverage in the reservoir communities, especially in mass campaigns conducted during the low transmission season, prevents the subsequent spread to indicator communities.

Sequence analysis led to the recognition of highly divergent iVDPVs [165, 186] and cVDPVs [166, 187, 188] (Sect. 10.8), and it has been used to resolve at high-resolution chains of cVDPV transmission [132, 188–190] and separate iVDPV lineages in individual immunodeficient patients with prolonged infections [165, 173, 186, 191, 192].

Molecular epidemiologic methods have also opened a new avenue for detecting gaps in polio surveillance. In areas with good surveillance, poliovirus isolates representing frequent sampling of a single chain of transmission are typically closely related (usually >99.5 % VP1 sequence identity among the closest relatives). These closely related viruses are routinely visualized as sequences connected by short branches on phylogenetic trees [193]. Long-branch connections between isolate sequences indicate missing information. If the virus is imported, the missing information may be recovered from the sequence relationships to viruses from the source reservoir [11]. However, in many other circumstances, no closely related viruses can be found, and the recent virologic history of the isolate lineage is indeterminate. For example, gaps in AFP surveillance in southern Egypt were inferred from the sequence data, because indigenous type 3 isolates in 1999 appeared as “orphan lineages” at the tips of long branches on phylogenetic trees, and the closest relatives were isolated nearly 3 years earlier [194], observations that highlighted the importance of environmental surveillance to improve sensitivity. Orphan lineages have been repeatedly found in areas with insensitive surveillance. The GPLN regularly monitors for the appearance of poliovirus orphan lineages as a means to assess surveillance sensitivity.

A serious challenge to the integrity of poliovirus surveillance data is the occurrence of poliovirus contamination of

cultures. High workloads in many GPLN laboratories potentially increase the risk of contamination. Fortunately, sequence analysis can distinguish contaminants from true clinical isolates. Contaminants are easily recognized when they are standard wild reference strains, such as Mahoney, MEF-1, or Saukett (OPV-like contaminants are usually of little current programmatic importance), but are more difficult to recognize when they are the wild polioviruses indigenous to a country or community. However, when wild polioviruses isolated at different times and locations have identical VP1 sequences, contamination is suspected, because such sequence identities are inconsistent with the rapid rate of evolution of the poliovirus genomes. Contamination can be definitively confirmed (or ruled out) by complete genomic sequencing. At the advanced stages of polio eradication, laboratory contamination could have severe programmatic consequences if unrecognized, prompting the diversion of resources into unnecessary immunization campaigns mobilizing large populations and costing many millions of dollars.

3.6.4 Tests for Antibody

Because precise and detailed epidemiologic information is routinely obtained from characterization of poliovirus isolates, virologic methods are the mainstay for global poliovirus surveillance [195]. However, antibody tests, especially those measuring population immunity or vaccine efficacy, have assumed greater prominence in recent years [92, 95, 196–200]. The "gold standard" is the neutralization test, as the presence of neutralizing antibody is regarded as the key indicator of protective immunity to poliovirus [7]. Current automation methods permit tests for neutralizing antibody to be performed at scales previously unattainable.

4 Biological Characteristics of Poliovirus

4.1 General Properties

Polioviruses, as members of species-C of the *Enterovirus* genus, share most properties with other members of that species and genus (Chap. 11) [201, 202]. Polioviruses are small (~30 nm in diameter [203]), non-enveloped viruses with capsids of icosahedral symmetry enclosing a single-stranded, positive-sense RNA genome. The genome is ~7,500 nucleotides long, has a small (22-amino acid) basic protein, VPg, covalently linked to the 5′-end, and is polyadenylated at the 3′-end (Fig. 13.5). The single open reading frame (ORF) is flanked by a long (~740 nucleotides) 5′-untranslated region (5′-UTR) and a short (~70 nucleotides) 3′-UTR. Complete genomic sequences have been determined for numerous representatives of each of the three serotypes, including those of the three Sabin OPV strains [204]. Only the sequences encoding the capsid proteins are unique to polioviruses, as the flanking sequences are frequently exchanged by recombination with the closely related species-C enteroviruses during circulation (Sect. 4.6.2) [24, 166, 188, 205, 206]. The poliovirion consists of 60 copies each of 4 capsid proteins (VP1–4) that form a highly structured capsid shell [203]. The three major proteins (VP1, VP2, VP3) share a similar basic architecture and were probably derived from a common ancestral protein [203]. The smallest protein, VP4, internalized in the native virion, is formed by the cleavage of the precursor VP0 (VP4 + VP2) during final maturation of the virion. The external surface of the poliovirion is decorated by peptide loops extending from VP1, VP2, and VP3, which form the neutralizing antigenic sites (Fig. 13.5) [162, 207]. Polioviruses attach to and enter cells via the specific poliovirus receptor (PVR) on the cytoplasmic membrane; the PVR was later identified as CD155, a glycoprotein of the immunoglobulin superfamily [208–210]. The key distinguishing properties of poliovirus capsids are their antigenic surfaces and their abilities to specifically bind to CD155, as the sequences and structures of the internal capsid domains are largely conserved among species-C enteroviruses.

4.2 Physical Properties

Poliovirus capsids contain no essential lipids, and infectivity is insensitive to inactivation by detergents and lipid solvents such as ether, chloroform, or alcohol [23]. The viruses are stable at pH 3–5 for 1–3 h and can therefore pass through the stomach without inactivation. Exposure to 0.3 % formaldehyde, pH <1, pH >9, or free residual chlorine at 0.3–0.5 ppm causes rapid inactivation. Infectivity is stable indefinitely at –20 °C or lower, and stable for weeks at 4 °C, but is rapidly inactivated at temperatures above 50 °C [211]. Molar concentrations of $MgCl_2$ significantly increase the thermal stability of poliovirions [212], both at elevated and ambient temperatures, and $MgCl_2$ is added to many OPV preparations to preserve potency [7, 213]. High intensity ultraviolet light or desiccation inactivate infectivity by causing an irreversible conformational transition from D-antigenicity to C-antigenicity (Sect. 4.3) [211, 214]. The three-dimensional crystal structures of representatives of all three serotypes have been determined [203, 215, 216]. Poliovirions have a buoyant density of 1.34 g/ml and a sedimentation coefficient of 160S, properties that can be exploited to obtain highly purified virus preparations [217].

4.3 Antigenic Properties

There are three poliovirus serotypes [54, 55]. Three (or four) neutralizing antigenic sites have been identified by patterns

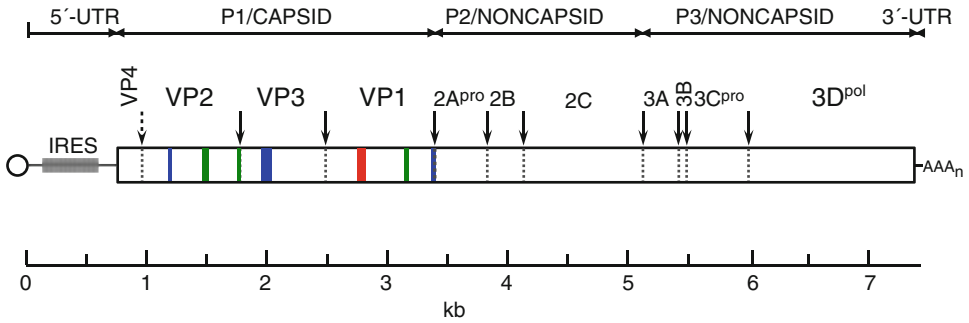

Fig. 13.5 Schematic of the poliovirus genome. The single open reading frame (ORF) is indicated by a rectangle, flanked by the 5′- and 3′-untranslated regions (*UTRs*); the small protein VPg (encoded by the 3B sequence interval) is covalently attached to the 5′-UTR and is represented by a circle at the 5′ end. The internal ribosome entry site (*IRES*; nucleotide positions 130–600) in the 5′-UTR is shown as a shaded rectangle. A single polyprotein is translated from the ORF, which is cotranslationally processed by virus-encoded proteases $2A^{pro}$ (catalyzes cleavage between VP1 and $2A^{pro}$; the cleavage site is indicated by a *dashed arrow*) and $3C^{pro}$ (catalyzes all other cleavages except the VP4/VP2 maturation cleavage; the cleavage sites are indicated by the *solid arrows*). Mature cleavage products are bounded by *dashed lines*. Protein $3D^{pol}$ is an RNA-dependent RNA polymerase (RdRP). *Colored bars* symbolize virion surface loops forming neutralizing antigenic sites 1 (*red*), 2 (*green*), and 3 (*blue*) (Redrawn from reference Kew et al. [94])

of reactivity with neutralizing murine monoclonal antibodies (Fig. 13.5) [162], and the assignments have been confirmed by high-resolution x-ray crystallography [216, 218, 219]. Neutralizing antigenic site 1 is continuous and formed by a loop in VP1; sites 2 and 3 are discontinuous and formed from loops contributed by different capsid proteins. The major type-specific differences in the capsid polypeptides primarily reside on the most surface-accessible peptide loops, which represent less than 4 % of the total capsid protein [204]. Although the neutralizing antigenic sites vary within each serotype [139, 142, 164, 165, 174, 220–223], the range of variability is constrained, possibly because of steric requirements for interaction with CD155 [224, 225], such that all polioviruses within a serotype can be neutralized by type-specific antisera, and poliovirus vaccines (both IPV and OPV) can induce protective immunity to all known antigenic variants. Poliovirus antigenic evolution differs importantly from that of influenza virus in that there is no cumulative antigenic divergence from ancestral viruses during person-to-person transmission, and genetically unrelated viruses may have similar antigenic properties and shared epitopes.

Limited cross-neutralization has been observed for all three poliovirus serotypes [226], and a shared epitope between types 1 and 2 has been identified by mapping escape mutants to cross-reactive neutralizing monoclonal antibody [227]. Recently, chimeric chimpanzee–human monoclonal antibodies have been produced showing patterns of strong cross-neutralization [228]. Epitope mapping with these primate monoclonal antibodies have identified shared determinants not previously recognized by studies using murine monoclonal antibodies [228], suggesting that the poliovirus antigenic surface may be more complex than previously thought.

Within each serotype there are two basic antigenic conformations: D-antigen ("dense"; sometimes also called N or "native" antigen) and C-antigen ("coreless"; corresponding to H or "heated" antigen) [211, 229]. The D-antigen corresponds to that of the intact native virion, and IPV potency is measured in D-antigen units [5]. The C-antigen contains no RNA and is not cross-reactive with the D-antigen [211]. Transitions between the D and C conformations are rapid in empty capsids, but D-antigen is stabilized by RNA packaging [214].

Poliovirus antigenic properties have been reviewed by Minor [162].

4.4 Host Range In Vivo and In Vitro

Humans are the only reservoir host for poliovirus [230]. Chimpanzees, gorillas, and orangutans have been infected while in captivity [231, 232], and chimpanzees can be experimentally infected by the oral route [233]. Poliomyelitis cases appeared in a natural chimpanzee colony following an outbreak in an upstream African village [234]. Old World monkeys are susceptible to experimental poliovirus infection upon intraspinal or intracerebral injection, and macaques (cynomolgus, rhesus, and bonnet) can be infected by the oral route, but high virus titers are required for infection [235–238]. New World monkeys are not susceptible to poliovirus infection by any route of administration because of substitutions in the variable domain of their CD155 orthologs [239, 240]. Paralytic attack rates in humans differ by poliovirus serotype in the order type 1>type3>type 2 (Sect. 3.4) [8, 9, 34, 241]. Susceptibility to oral infection is in the order of humans>chimpanzees>macaques, whereas neural susceptibility is in the order macaques>chimpanzees>humans [242–244].

Poliovirus variants of all three serotypes have been selected for growth in mice [245–247] and a type 2 variant has also been selected for growth in chick embryos [248]. Polioviruses normally cannot directly infect cultured mouse or chick cells, but can replicate efficiently when viral RNA is introduced by transfection, an observation that led to the concept of a specific viral receptor [249]. The CD155 PVR is a transmembrane glycoprotein with three extracellular immunoglobulin-like domains, encoded by a gene mapped to human chromosome 19 [250]. The normal function of CD155 is as a receptor for establishment of intercellular junctions between epithelial cells, a function that is "misused" by poliovirus to gain entry into human cells [251].

4.5 Poliovirus Replication Cycle

An overview of the poliovirus replication cycle is shown in Fig. 13.6. Virus attaches to cells through specific interactions between the amino-terminal variable domain 1 of CD155 and a "canyon" that surrounds the fivefold axis of the virion [225, 253–257]. After endocytosis, viral RNA is uncoated and released into the cytoplasm [257], VPg is cleaved from 5′-end of the RNA, and the RNA is translated. Translation is under the control of the internal ribosome entry site (*IRES*; Fig. 13.5), an element (nucleotides ~130–600) within the 5′-UTR that has a highly conserved stem-loop structure [258, 259]. The translation product is a single polypeptide, the

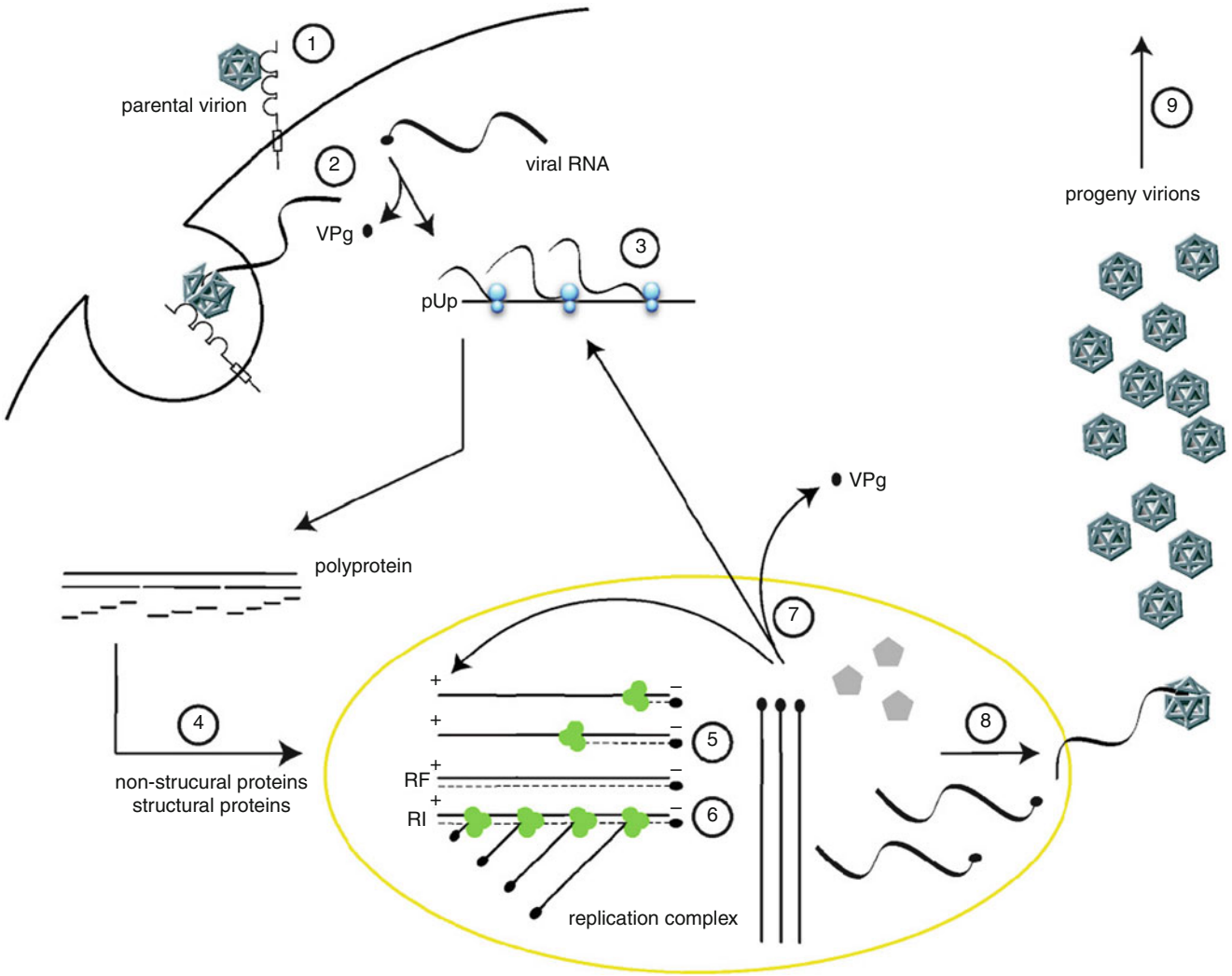

Fig. 13.6 Overview of the poliovirus replication cycle: *1* attachment of polio virion to poliovirus receptor (PVR; CD155) on cytoplasmic membrane, *2* endocytosis and uncoating of RNA, release into cytoplasm, and cleavage of VPg from 5′-end of RNA, *3* translation of viral proteins from viral RNA serving as mRNA, *4* proteolytic processing viral proteins, *5* replication of negative (–) strands of viral RNA by poliovirus RNA-dependent RNA polymerase (RdRP), *6* replication of positive (+) strands of viral RNA by RdRP in replication intermediates (RI), *7* cleavage of VPg from some + RNA strands for programming as mRNA, *8* encapsidation of other + RNA strands into virions, and *9* release from cytoplasm of infected cell. In cell culture, the entire infectious cycle is complete within ~6 h with release of up to 10,000 infectious virions per cell (Reproduced from reference De Jesus [252] with permission from BioMed Central)

polyprotein, which is cleaved by virus-encoded proteinases, $2A^{pro}$ and (primarily) $3C^{pro}$, into mature viral proteins [260]. Host protein synthesis is rapidly inhibited by the cleavage by $2A^{pro}$ of the translation initiation factor eIF4G, required for initiation of translation of capped host messenger RNA but not for the internal initiation of translation from the poliovirus IRES [259, 261]. One cleavage product is $3D^{pol}$, an RNA-dependent RNA polymerase (RdRP), that catalyzes the synthesis of negative-polarity (–) RNA strands from the genomic and mRNA-polarity (+) strands forming a duplex called the replicative form (RF) [262, 263]. Multiple copies of positive RNA strands are produced from negative-strand templates in replicative intermediates (RI) arrayed in intracellular membrane complexes [262, 263]. VPg is cleaved from some newly synthesized positive RNA strands for programming as mRNA and further translation [260]. Other positive strands are encapsidated during the maturation step in which the VP0 precursor to VP4 and VP2 is cleaved followed by release of infectious virions from the infected cell. The entire replication cycle takes place within the cytoplasm, and poliovirus can replicate in anucleate cells. Infected cells show cytopathic effects within 6 h and can release up to 10,000 infectious virus particles upon cell lysis and death. This rapid rate of cellular destruction accounts for the rapid progression of paralysis when poliovirus infects motor neurons [264].

Poliovirus (and picornavirus) replication has recently been reviewed in depth [27, 202, 259].

4.6 Poliovirus Genetics

4.6.1 Rapid Evolution of Poliovirus Genomes

Poliovirus is one of the most rapidly evolving viruses known [147, 164, 171, 172, 265]. Most of the nucleotide substitutions generate synonymous codons [164], and the basic biological properties of wild polioviruses remain unchanged, although the Sabin OPV strains can undergo important phenotypic changes (Sects. 9.1.4 and 10.8). Estimates of the rates of total nucleotide substitution into poliovirus capsid regions average ~10^{-2} substitutions per site per year [164–166, 171–173, 188, 191]. The rates appear to be similar across the three poliovirus serotypes and for both circulating polioviruses and polioviruses associated with chronic infections, and constitute a robust poliovirus molecular clock. Underlying the rapid pace of poliovirus genomic evolution are the high rates of base misincorporation (in the range of 10^{-5} to 10^{-3} per base per replication) by the poliovirus RdRP [266–271]. These high mutation rates are attributable to the absence of $3' \rightarrow 5'$ exonuclease proofreading mechanisms for the viral RNA polymerases [267], although other mechanisms may also be involved [272]. This exceptionally rapid rate of genomic evolution has facilitated high-resolution molecular epidemiologic studies (Sects. 3.6.3 and 10.7), even as deeper evolutionary relationships among poliovirus genotypes are obscured by saturation of variable nucleotide sites [164].

Poliovirus populations in cell culture and in humans [173] are a spectrum of mutational variants termed "quasispecies" [273, 274]. On average, each genome in a virus population contains one nucleotide substitution difference from the consensus "master sequence" of the quasispecies population. Two important consequences are that the virus populations in the live, attenuated OPV contain preexisting variants of higher potential neurovirulence [275], and that antigenic variants can be rapidly selected in cell culture [218, 276] and in humans [141, 220–223].

4.6.2 Recombination

Recombination occurs continuously during poliovirus infection of cultured cells [271, 277, 278] and individuals [173, 279]. Wild polioviruses undergo frequent recombination with the closely related human species-C enteroviruses during circulation [24, 167, 205, 206]. Indeed, the 5′-UTR and P2/P3 noncapsid sequences (Fig. 13.5) of wild polioviruses are drawn from a potentially large and constantly exchanging genetic pool that includes the locally circulating human species-C enteroviruses [167, 206, 280]. Crossovers usually map outside the capsid region when the exchange partners are different poliovirus or enterovirus serotypes, but crossovers may occur within the capsid region when the partners are of the same poliovirus serotype [173, 271, 277]. The biological role of recombination in poliovirus is unclear. Recombination may facilitate maintenance of replicative fitness by countering the accumulation of deleterious mutations [281]. However, natural selection apparently maintains wild poliovirus near its fitness optimum, and multiple recombinational variants can co-circulate locally [168]. Children fed tOPV regularly excrete vaccine/vaccine recombinants [279] and most circulating VDPVs (Sect. 10.8) are vaccine/non-vaccine recombinants [133, 166–168].

The genetics of poliovirus and other RNA viruses has been comprehensively reviewed [268, 273, 274, 277, 282, 283].

5 Descriptive Epidemiology

The epidemiology of poliomyelitis remained obscure until inapparent infections and mild cases were recognized [40, 41]. Unlike smallpox, where every infection of a susceptible person is associated with overt and characteristic clinical signs [284], the first poliomyelitis outbreaks erupted with no evident source [1, 34]. Five phases in the natural history of poliomyelitis can be recognized: (1) the endemic phase, (2) the epidemic phase, (3) the vaccine era, (4) the eradication era, and (5) the post-eradication era [22, 23]. Most developed countries eradicated their indigenous wild polioviruses four

to five decades ago [8, 9, 33, 34]. Similar progress has now been achieved by all but three developing countries (Fig. 13.3), parts of which remain in the endemic and epidemic phases because the vaccine era has not yet been fully implemented [12].

5.1 Endemic, Epidemic, Vaccine Era, and Eradication Phases

During the endemic phase, virtually all children were exposed to wild polioviruses at an early age. Large outbreaks were rare because large cohorts of nonimmune susceptible children rarely accumulated. Frequent exposure to wild polioviruses maintained population immunity and had the potentially important additional beneficial effect of boosting the immunity of women of childbearing age. Outbreaks were more likely to occur in smaller, more isolated populations than in large populations that could support continuous poliovirus circulation. The endemic phase was inevitably followed by an epidemic phase [1, 8, 9, 23, 28, 29, 33, 34, 66]. In the United States and Europe, outbreaks of increasing size and severity occurred for six decades until the mid-1950s and were halted only by the introduction of IPV [8, 9, 23, 29, 34]. Unfortunately, the vaccine era arrived unequally in the world, starting first with the most developed countries of Europe, North America, Australia, and New Zealand and progressing to Japan, the Soviet Union and Eastern Europe, and the countries of temperate South America [8, 9]. As the vaccine phase progressed in more developed countries, periodic epidemics appeared in less developed countries [8, 9]. Incomplete vaccine coverage in some developing countries had the perverse effect of reducing, but not eliminating, poliovirus circulation, potentially increasing the risk of explosive epidemics following the buildup of nonimmune susceptible persons in the population. The eradication phase has been permanent in most countries, but continued wild poliovirus circulation in a few areas carries ongoing global risks, and some countries have allowed immunity gaps to widen after eradication of indigenous wild polioviruses and suffered outbreaks from imported wild polioviruses [10, 12] (Sect. 10.6) or from the emergence and spread of cVDPVs (Sect. 10.8) [133]. The global post-eradication phase (Sect. 11.2) is far more complex than originally envisioned and is a key element of the current WHO strategic plan [22].

5.2 Geographic Distribution

Before the vaccine era, all three poliovirus serotypes had virtually a worldwide distribution. Virus circulated continuously in populous tropical areas, and the intensity of wild poliovirus circulation ("force of infection") was high, especially in areas with high population densities [180]. For example, up to the 1990s in Mumbai, India, all three serotypes of wild poliovirus could be found in the community, and children in high-risk urban slums were occasionally found to be concurrently infected with all three wild poliovirus serotypes [285, 286]. In recent years, some children in low-coverage endemic communities were coinfected with wild poliovirus types 1 and 3.

As with other enteroviruses, poliovirus circulation had a distinct seasonality in temperate zones. Paralytic cases peaked during summer and early autumn and could disappear altogether in winter. Sewage sampling could detect the presence of virus before and after the appearance of cases, but generally not throughout the year in smaller communities in cooler climates [103]. In both temperate zones and tropical areas, different serotypes predominated in different years. Poliovirus circulation would stop completely in small, rural populations, which would then be subject to outbreaks once poliovirus was reintroduced into the community [28].

Very isolated communities had no poliovirus infections for years. For example, age-stratified seroprevalence data have shown that Eskimo communities in Canada and the United States experienced sharp outbreaks covering a broad age distribution preceded and followed by many years with no serologic evidence of poliovirus circulation [85, 287]. A similar pattern of infrequent outbreaks also occurred in isolated tropical communities. Following introduction of poliovirus from the mainland, a large outbreak in the Andaman and Nicobar Islands in the Bay of Bengal in 1947–1948 affected a broad age distribution, with an overall paralytic attack rate of 10 % and a CFR of 14 % [288].

In the vaccine era, poliovirus type 1 had the widest geographic distribution, and poliovirus type 2 the most restricted [180]. It is difficult to separate out the effects of immunization, even at low rates of coverage, from the intrinsic biological properties of wild polioviruses. For example, type 1 is most frequently associated with large outbreaks and appears to be able to spread over wider geographic areas than type 3 [180] and (especially) type 2. Wild poliovirus type 2 was the first to be eradicated globally (Fig. 13.3) [13] but was also the first to disappear regionally. In the United States, for example, wild poliovirus type 2 disappeared long before type 3 and finally type 1. By the mid-1980s, no wild type 2 polioviruses could be found in several large countries, including Brazil and China, where the other two serotypes were still endemic [170]. Only wild poliovirus type 1 was found in the Caribbean during the 1970s and 1980s, whereas both types 1 and 3 could be found in the larger island populations of the Philippines and Indonesia until the mid-1990s [289, 290].

With the introduction of OPV, OPV-like viruses of all three serotypes became ubiquitous in areas of high coverage. Unlike wild polioviruses, vaccine-related strains usually do

not persist. For example, in Cuba, where OPV was administered only twice a year in campaigns, vaccine-related viruses disappeared from the environment within 4 months of the second campaign round [291].

5.3 Seasonality

In temperate zones, circulation of polioviruses, like that of all enteroviruses, is seasonal [23]. The summer-fall seasonality of poliomyelitis outbreaks was clearly described in the early reports of epidemics in Europe and the United States [41, 45]. Seasonality was most pronounced in temperate zones and gradually decreased toward the equator, where intense poliovirus circulation could occur throughout the year [9, 34, 56]. In the tropics, the residual poliomyelitis seasonality was variable and circulation tended to increase during the rainy season. The typical summer-autumn wild poliovirus seasonality peak was offset by 6 months between the northern and southern temperate zones [9, 292]. Poliomyelitis outbreaks have occurred on rare occasions during the winter months [110].

Poliovirus seasonality is a reflection of the fluctuation in the number of transmission chains during the year [168, 193]. However, the underlying mechanisms for poliovirus seasonality are unknown. Seasonal patterns of human association do not appear to be major factors because the peaks of the poliovirus and rotavirus (another non-enveloped enteric virus) seasons are offset by 6 months in the United States [34]. One hypothesis for poliovirus seasonality is based on the observation that poliovirus is more stable when relative humidity is above 40 % [293]. In temperate zones, indoor relative humidity is highest in the summer, and in the tropics humidity is high during the rainy season and throughout the year in coastal areas, a pattern closely correlated with the observed patterns of poliovirus seasonality. However, seasonal patterns of human migration can also facilitate poliovirus dissemination. For example, the spread of wild poliovirus from the tropics to more temperate zones was correlated with the seasonal migration of underimmunized farm worker families moving with the harvest season. Reaching underimmunized children in migrant populations remains a key eradication strategy in developing countries [22].

Seasonality has been an important facilitating factor for the eradication of wild polioviruses in developing countries. Mass immunization campaigns in the cooler months, when the transmission chains of poliovirus (and potentially competing enteroviruses) are at their seasonal low [193], have been a mainstay of eradication efforts [294–296]. In the northern Andean region, polio circulation ceased in cities in the temperate highlands years before it ceased in cities of the tropical coastlands [164]. In temperate Bolivia, OPV coverage rates of only 50 % were sufficient to eradicate polio [70]. Sequence data showed that wild poliovirus was imported into the Bolivian highlands from tropical coastal Peru [170]. In Brazil, polio was rapidly controlled in the south, but the reservoirs persisted in the northeast, with its more tropical climate as well as poor sanitation [70]. Polio had already been eradicated from the temperate Southern Cone (Argentina, Chile, Paraguay, Uruguay) before the launch of the PAHO Polio Eradication Initiative [18]. In China, polio persisted in the provinces of the southeast but not in the coastal northeast [172, 297, 298]. One caveat is that the level of immunization (and OPV efficacy) is usually higher in temperate zones than in tropical zones.

5.4 Age and Sex

In endemic areas, children are chiefly responsible for maintaining poliovirus circulation. The primary ages of first infection (as indicated by appearance of cases) was 2 years or younger in the pre-vaccine era [9, 23, 28, 29, 66]. Older individuals may have added to poliovirus transmission, but their contributions were likely to have been relatively small because most would have had prior exposure to poliovirus and the effect of reexposure would have been to boost mucosal immunity and thus limit poliovirus excretion. In both developing and developed countries, the hygiene of children <2 years of age favors enteric virus dissemination, but in the pre-vaccine era the likelihood of exposure to wild poliovirus was much higher in settings with poor sanitation [29].

The age distribution of poliomyelitis cases has shifted dramatically to older age groups over the past century [9, 23, 28, 29, 66]. During the 1916 epidemic in New York, 80 % of cases were among children <5 years of age. By the mid-1950s, peak cases were in children 5–9 years of age and two-thirds of deaths were in patients >15 years of age [23]. In developing countries, poliomyelitis remained a disease of younger children, with >75 % of cases <2 years of age and >95 % of cases <5 years of age [299, 300].

In the endemic phase, which represented all countries before the late nineteenth century, many children would be infected by the then-prevalent wild polioviruses while still protected by maternal antibody, and thus could become immune without risk of paralytic disease. As sanitation improved, the first exposures to poliovirus were delayed to later in life, when the risk of severe disease is increased, and after protective maternal antibodies had waned. Delayed infection also resulted in expansion of the pool of nonimmune susceptible individuals, increasing the potential for explosive outbreaks once wild poliovirus was reintroduced into the population. Paul had first proposed this process to explain the sudden appearance of epidemic poliomyelitis, first in the most developed parts of Europe and North America and then elsewhere [1]. Paul's hypothesis has been repeatedly confirmed [23, 34], and it predicted an irreversible shift from the endemic to the epidemic phase. This had been the pattern in all countries until poliomyelitis was finally brought under control through immunization.

It has been known for decades that males are more susceptible than females to paralytic poliomyelitis and to more severe forms of the disease [9, 23, 66, 301]. The reasons for this are unknown, although it has been suggested that greater physical exertion by boys might be a factor [23]. A majority of the paralytic cases occurred in males in recent outbreaks in Namibia (89 %) [80], Republic of Congo (68 %) [302], and Tajikistan (66 %) [178].

5.5 Occurrence in Families and Contact Groups

Polioviruses, like other enteroviruses, are highly communicable [23]. In the pre-vaccine era, virus was spread efficiently by young children to other family members, many of whom had prior exposure and were not susceptible to disease. In the vaccine era, most cases of contact vaccine-associated paralytic poliomyelitis (VAPP; Sect. 9.1.4) were within the family unit [303, 304]. More recently, concerns have focused on extended family units in developing countries, especially among groups that refuse immunization and who could represent an interconnected, socially defined reservoir of poliovirus transmission within an otherwise adequately immunized population. Attention is also being given to nomads and other mobile populations who could facilitate dissemination of poliovirus originating from fixed reservoir communities [22].

5.6 Epidemiologic Patterns of Poliomyelitis

5.6.1 Epidemiologic Patterns in Developed Countries in Temperate Zones

The shift from endemic to epidemic phase was first observed in countries with the highest standards of community sanitation and personal hygiene [23, 28, 29, 33, 34]. As outlined above, the epidemic phase appears to have been the consequence of delaying poliovirus exposure to later age groups who are prone to more severe paralytic disease and to the accumulation of nonimmune susceptible populations poised for large outbreaks. As sanitation conditions continued to improve, the poliomyelitis outbreaks steadily shifted in size and severity and peaked in increasingly older age groups. In some settings the shift was gradual; in others it was abrupt [23].

The mechanism proposed by Paul [1] for the shift from endemicity to epidemicity received further support by the observation that families with the highest socioeconomic advantages were at highest risk for severe poliomyelitis, whereas less advantaged families living in communities with poor sanitation were at reduced risk [23]. Indeed, the last outbreaks in the United States in the epidemic pre-vaccine era included parents in more advantaged families whose children carried wild poliovirus into the home [305].

5.6.2 Poliomyelitis in the Vaccine Era

The high-income countries of North America, Western Europe, and the southwest Pacific were quick to adopt widespread immunization with IPV. The impact was rapid and dramatic. In the United States, for example, the incidence of paralytic poliomyelitis fell from 13,850 in 1955 to 829 in 1961 (Fig. 13.7) [308]. With the availability of OPV in 1961, more countries adopted immunization against poliomyelitis, and the downward trend continued. In the United States, Canada, Australia, and New Zealand, combined cases fell >700-fold from 44,378 in 1951–1955 to 62 in 1968 [8], and in Europe cases fell >50-fold from 28,359 in 1951–1955 to 529 in 1968 [8]. Dramatic progress was also made in the Soviet Union as some republics reported no cases by 1968 [8]. Poliomyelitis cases in Japan, which did not use IPV and introduced OPV in 1961 [309], fell >120-fold from 2414 in 1951–1955 to 20 in 1968 [8]. Apart from Singapore, the picture for the rest of Asia was not encouraging. Several countries in Latin America (Costa Rica, Uruguay, Chile, and Argentina) reduced cases by 4- to 50-fold [8], and Cuba eradicated indigenous polioviruses in 1962 [8, 310], as did Jamaica in 1968 [8]. However, many other countries in Latin America and the Caribbean made little progress [8]. Only Israel in the Middle East made evident progress (>35-fold reduction by 1968), and poliomyelitis in Africa remained virtually uncontrolled [8].

The downward trends continued in the United States and other higher-income countries through the 1970s (Fig. 13.7) [9]. Circulation of wild polioviruses indigenous to the United States apparently ceased after the 1970 outbreak along the Texas-Mexico border [163, 311]. Subsequent sporadic poliomyelitis cases (and a small outbreak in 1972) were usually associated with wild type 1 polioviruses imported from Mexico [163]. An outbreak associated with type 1 virus originating in Turkey spread to underimmunized religious communities in the Netherlands and Canada, and to the Amish community in the United States in 1978–1979 [147], was followed in August 1979 by one last sporadic case associated with wild type 1 poliovirus imported from Mexico [163]. A similar picture emerged in Europe. Outbreaks associated with imported wild polioviruses occurred in the Netherlands in 1971 (type 1) [312, 313], 1978 (type 1) [163, 312, 313] and 1992 (type 3) [314, 315]; in Sweden in 1977 (type 2) [105]; in Spain in 1983 (type 3) [180]; in Finland in 1984 (type 3) [110, 316]; and repeatedly in the Balkans and the southern Republics of the Soviet Union (types 1 and 3) [180, 317]. Also, a large outbreak in Taiwan in 1982 (1,031 cases) from wild type 1 poliovirus (probably imported from Indonesia) highlighted the risk to poliomyelitis-free countries in Asia of reinfection by importation from neighboring countries [318].

In many developed countries using OPV, the only cases of poliomyelitis each year were from VAPP (Sect. 9.1.4) [24, 319–323]. However, the risks of importation of wild polioviruses

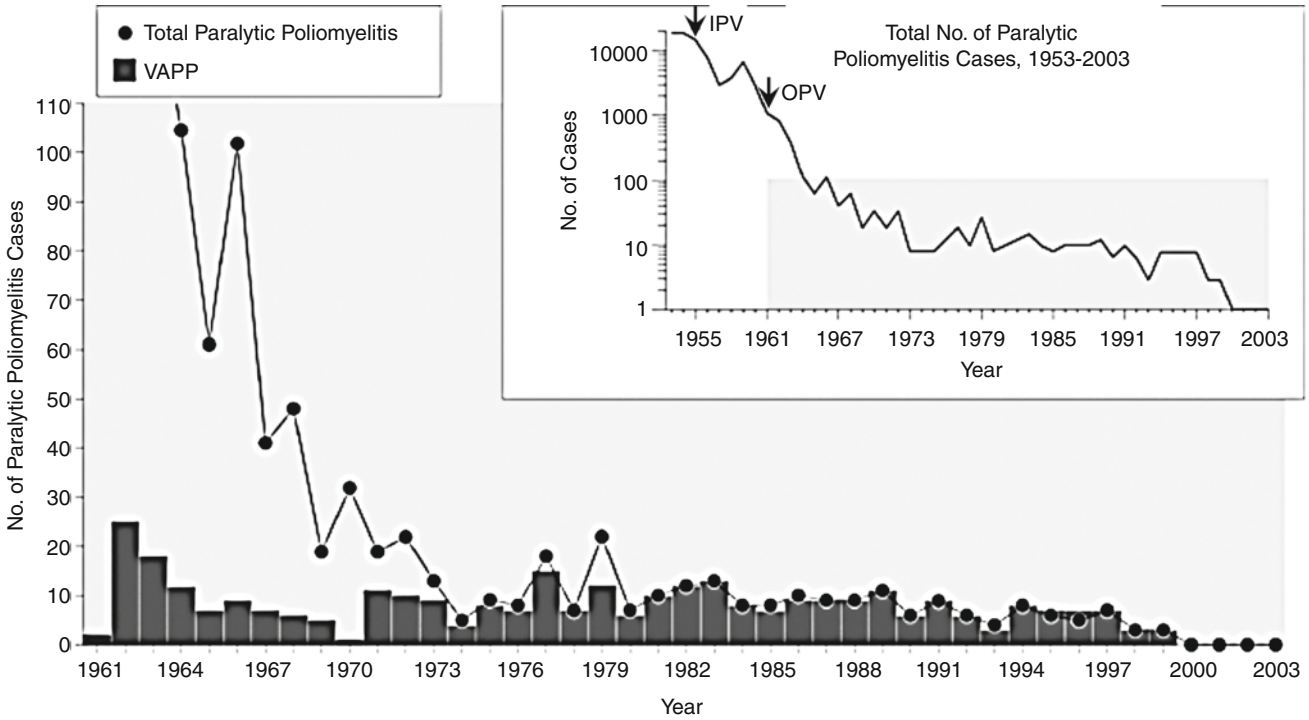

Fig. 13.7 Reported cases of poliomyelitis, United States, 1953–2003. Arrows indicate years of introduction of inactivated poliovirus vaccine (IPV; 1955) and oral poliovirus vaccine (OPV; 1961–62). The last wild poliovirus (type 1) case occurred in August 1979 [163, 306]. Bars indicate cases of vaccine-associated paralytic poliomyelitis (VAPP). New VAPP cases stopped after the shift to IPV in 2000 [307], but vaccine-derived poliovirus (VDPV) infections occurred in Minnesota in 2005 (type 1) [132] and a separate immunodeficiency-associated VDPV (iVDPV) case occurred in Minnesota in 2008 (type 2) [223]. Note that abscissa of inset is a logarithmic scale and abscissa of main graph is a linear scale (Source: Centers for Disease Control and Prevention (CDC). Reproduced from reference Alexander [307])

remained, and the sharp divergence between the poliomyelitis-free and poliomyelitis-endemic worlds was unsustainable.

5.6.3 Poliomyelitis in Developing Countries

By 1980, many developing countries had made little progress in controlling poliomyelitis in the nearly two decades since the widespread availability of OPV [9, 324]. With their growing populations, the problem of epidemic poliomyelitis in developing countries, well recognized by the mid-1950s [292], was increasing [98, 324]. In addition, lameness surveys revealed that the incidence of poliomyelitis in Africa and other developing countries was far higher than originally believed [97, 98, 319, 325]. Poliomyelitis cases in much of Africa and Asia had been grossly underreported [324], with the actual global incidence being tenfold greater than the officially reported counts. For example, lameness surveys in 1981–1982 suggested that India had half of the estimated world total of 400,000 poliomyelitis cases per year [326, 327]. Throughout the 1980s, the city of Mumbai alone reported ~1,000 cases per year [285, 286], more than 100 times the rate (from VAPP) for the United States over the same time period (Fig. 13.7) [303, 304]. Moreover, large outbreaks, some recurring, occurred in many developing countries as they steadily shifted to the epidemic phase [180]. Clearly, remedial action was urgently needed.

6 Mechanisms and Routes of Transmission

As with other enteroviruses, poliovirus is spread by person-to-person contact via two routes of transmission, fecal–oral and respiratory [23, 29]. The relative importance of these two routes varies by setting. Fecal–oral is the more efficient route because fecal shedding continues for up to 6 weeks, and the quantities of virus shed in stool may be as high as 300 million infectious particles per day [328]. Under experimental conditions, vaccine virus was shown to spread to contacts even when administered in gelatin capsules, thereby bypassing throat infection and respiratory transmission [329]. In areas of poor sanitation and hygiene, fecal–oral transmission (via contaminated fingers, food or water, utensils, or toys) is most likely the dominant route, as young children are continually exposed to unsanitary conditions and live in close proximity to contaminated soil and open sewers. Respiratory transmission probably played a more important role in developed countries with high standards of hygiene and sanitation, and where in the pre-vaccine era exposure to poliovirus in higher socioeconomic communities was typically delayed by several years. The effectiveness of IPV, which blocks oropharyngeal but not intestinal infection [330], to stop wild poliovirus transmission is evidence of the

importance of respiratory transmission in settings where fecal–oral transmission is less prominent.

There is no evidence for an extrahuman reservoir for poliovirus, apart from virus stored in laboratory freezers [331]. Sequence analyses show close genetic relatedness among the polioviruses obtained in the same locales from clinical cases, stool surveys, and sewage sampling [111, 113]. Virus imported over short or long distances can consistently be linked by molecular epidemiologic methods to infections recently occurring in the source communities [10, 11, 163, 193, 332].

On very rare occasions, poliomyelitis infections and outbreaks have started by mechanisms other than direct person-to-person transmission. In the spring of 1955, 204 vaccine-associated cases occurred in the United States following injection of children with IPV preparations which contained residual infectious wild poliovirus (the Cutter incident) [333, 334]. The majority of case isolates were derived from the neurovirulent type 1 Mahoney strain used in IPV production [1, 333, 334]. A small number of cases in Uttar Pradesh, India, in 2000 and again in 2002–2003 were found to be associated with the type 2 reference strain, MEF-1, found in contaminated lots of OPV [335, 336].

7 Pathogenesis and Immunity

7.1 Pathogenesis

Studies on the pathogenesis of poliomyelitis in humans date to the nineteenth century [1]. The availability of a primate model for pathogenesis in 1909 accelerated the pace of pathogenesis research up to the mid-1950s [1, 242, 243]. Interest in the pathogenesis of poliomyelitis waned with the availability of poliovirus vaccines and the increased focus on molecular virology, even though pathology studies continued to address key unanswered questions [337, 338]. Two major models of pathogenesis, one by Bodian [242] (Fig. 13.8) and the other by Sabin [243], were proposed in 1955 and 1956. The principal difference between the two models is whether the major primary sites of poliovirus replication are in lymphatic tissues (Bodian) or mucosal tissues (Sabin) [244]. According to Bodian's model, orally ingested poliovirus first replicates locally in lymphatic tissues at the sites of initial virus implantation (tonsils, intestinal microfold epithelial [M] cells of the Peyer's patches in the ileum). Within 1–2 days virus spreads via lymphatic pathways from the small intestine to the mesenteric lymph nodes and from the tonsils and adenoids to deep cervical lymph nodes.

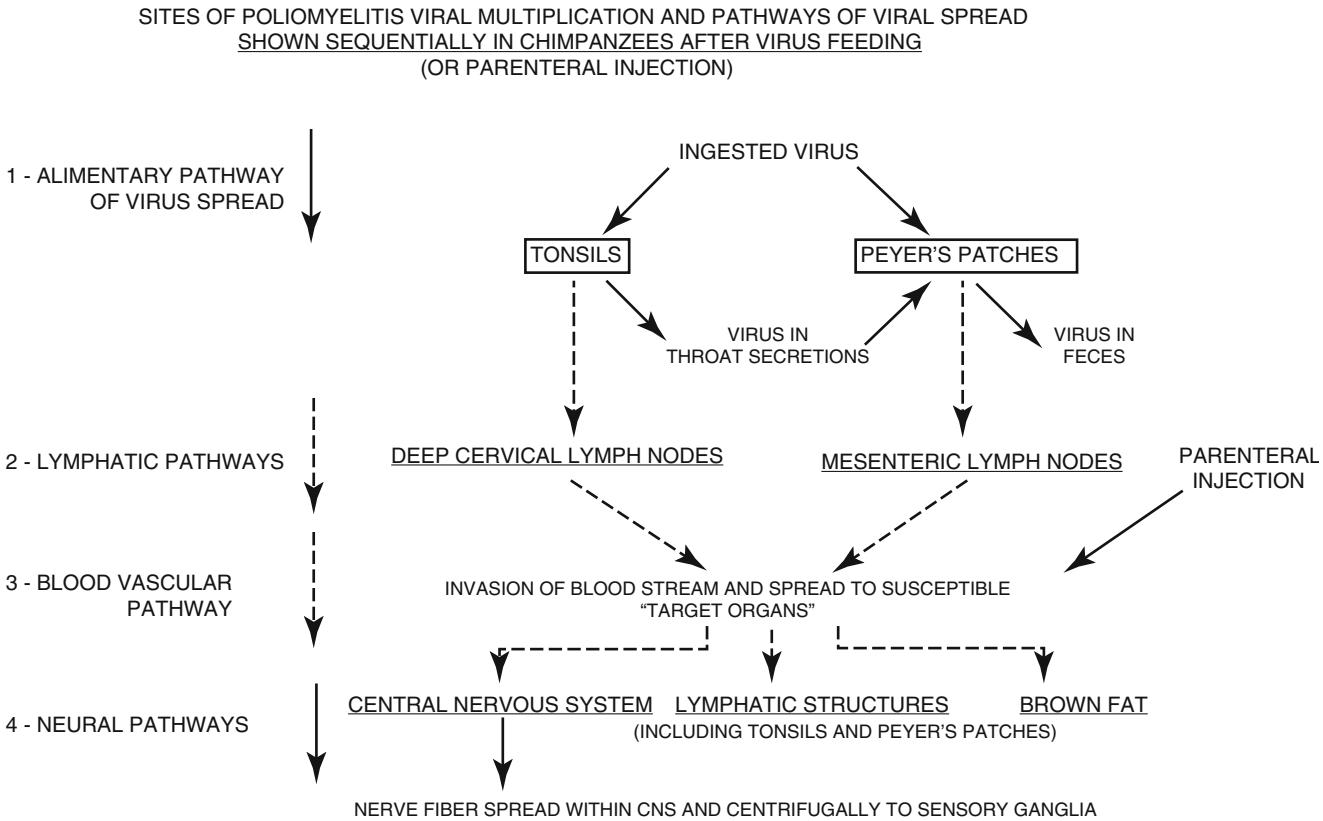

Fig. 13.8 David Bodian's scheme of the pathogenesis of poliovirus infection based on studies in monkeys, chimpanzees, and humans. Reproduced with permission from the original article by Bodian [241]. (Copyright 1955, American Association for the Advancement of Science (http://www.sciencemag.org).) *CNS* central nervous system

By days 1–3, virus appears in the feces and throat. In the next viremic phase, virus invades the bloodstream and infects other susceptible "target organs," including further spread to systemic lymph nodes, brown fat, and occasionally motor neurons of the CNS. The first 1–2 days of infection are asymptomatic. During viremia, all tissues are exposed to virus, and about 10 % of infected persons experience "minor illness" at days 3–4, with malaise and fever accompanying systemic viral infection. Clinical signs subside after day 4. Viremia ends with the appearance of antibody by day 6, and virus bound to antibody can be detected for a few days longer [320]. Incubation periods (time from exposure to disease onset) vary in different individuals, but they are usually from 7 to 17 days but range from 2 to 35 days [23]. Virus replicates in the oropharynx for 1–2 weeks and is shed in the stool for 3–6 weeks [23, 321].

Progression to "major illness," including nonparalytic poliomyelitis (aseptic meningitis) and paralytic poliomyelitis, occurs within 8–30 days of exposure [28]. The biphasic nature of the disease, with minor illness followed by major illness, has been termed the "Dromedary" form [322] to imply two humps (even though the dromedary is a one-humped camel) [1]. Invasion of the CNS may occur by either penetration of the blood–brain barrier or by retrograde axonal transport. Paralytic illness follows directly from the lytic infection of motor neurons, constituting the gray matter of the spinal cord. In fatal cases, virus replication in motor neurons rises sharply the day preceding paralysis, peaks to high titers at day 3, and disappears by day 7 [29]. In spinal poliomyelitis, motor neurons in the anterior horn of the spinal cord are rapidly destroyed, with the severity of paralysis correlated with the extent of neuronal destruction [29]. In bulbar poliomyelitis, motor neurons in the medulla in the brainstem are primarily affected. Lesions may also occur in the reticular formation in the brainstem and in the thalamus and hypothalamus just above the brainstem. Lesions in the brain occur in the roof nuclei of the cerebellum and in the precentral gyrus of the motor cortex [23, 29]. Other regions of the cortex are usually resistant to infection [264]. Some motor neurons may lose function because of edema, but the damage is temporary and motor function is restored once the inflammation subsides [23]. Local secretory IgA immunity can block poliovirus replication in tonsils and the intestinal tract, and neutralizing IgG and IgM antibodies can prevent virus spread to motor neurons of the CNS.

Poliovirus pathogenesis has enjoyed a renaissance [26, 232, 244, 323, 338] after cloning and identification of the PVR, CD155 [208], and the development of transgenic mice expressing CD155 [340–342]. The transgenic mice are susceptible to poliovirus infection upon intraspinal, intracerebral, intravenous, intranasal, intraperitoneal, or intramuscular inoculation and exhibit clinical signs and neural lesions typical of human poliomyelitis [232]. However, the transgenic mice are usually resistant to infection by the oral route [232, 340, 343]. It has been found in both humans and transgenic mice that CD155 RNA and protein are expressed in a wide variety of tissues, including tissues that do not support poliovirus replication [323]. Therefore, CD155 expression is necessary but not sufficient for poliovirus replication in vivo, and a stage in the replication cycle after initiation of translation determines tissue tropism [323]. One potential mechanism for the observed patterns of tissue tropism is that a barrier of innate immunity in extraneural tissues blocks poliovirus replication [232]. In support of this view, it was found that in PVR-transgenic mice deficient in the interferon-α/β receptor, poliovirus replication occurred not only in the CNS but also in extraneural tissues such as the liver, pancreas, and small intestine [343, 344].

The mechanism of axonal retrograde transport has been reexamined in transgenic mice [345]. At the molecular level, the cytoplasmic domain of CD155 was found to specifically bind to Tctex-1, a light chain of the dynein motor complex (a driver for retrograde transport), and that the rate of Tctex-1 transport is similar to the rate of poliovirus ascent along nerve fibers [232, 346].

New developments in the pathogenesis of poliomyelitis have been the subject of several recent reviews [26, 232, 244, 323, 338].

7.2 Immunity

Neutralizing antibody protects against paralytic disease [57]. Type-specific immunity from natural infection is lifelong [85], and immunity also appears to be permanent for individuals who have produced neutralizing antibodies after receipt of OPV or IPV. Infants are protected against disease during the first few months of life by maternal antibody, which has a half-life of approximately 28 days and falls to undetectable levels after 6 months [347]. In the endemic period, children <6 months of age rarely contracted paralytic disease, but could induce protective immunity when infected with wild poliovirus [28]. It has long been known that even low levels of circulating antibodies are protective [57], because virus titers during viremia are low [58].

Neutralizing IgM and IgG antibodies appear within a few days of exposure to virus. IgM titers decline after 10 days, but IgG titers continue to rise for at least 2 months [348]. Mucosal immunity in the gastrointestinal tract and oropharynx, in the form of secretory IgA, rises more slowly and persists for at least several months, but the duration of mucosal immunity to poliovirus has not been intensively studied [7, 349]. Children who have had tonsillectomy are more susceptible to poliomyelitis than children with intact oropharyngeal lymphoid tissue, a finding that points to the importance of oropharyngeal mucosal immunity in protection from disease [350–352]. OPV induces both serum antibody and intestinal immunity, whereas intestinal immunity from IPV is low (Sect. 9.1.5) [7, 348].

Additional evidence for the critical importance of neutralizing antibody to immunity is that individuals with primary

(B-cell) immunodeficiencies are at a 3,000-fold higher risk than immunocompetent individuals of contracting VAPP when exposed to OPV (Sect. 9.1.4) [353], and some may develop prolonged poliovirus infections [354]. Patients with primary immunodeficiencies are treated with intravenous immunoglobulin (IVIG), and preparations used in the United States must meet minimum neutralization titers to poliovirus [7]. However, IVIG neutralizing titers become undetectable after 3–5 weeks [23], and several immunodeficient individuals with chronic poliovirus infections have contracted poliomyelitis despite IVIG treatment, presumably after titers of neutralizing antibodies fell below protective levels (see Sect. 10.8.2) [186, 223]. Newly developed chimpanzee–human neutralizing monoclonal antibodies may hold promise for the availability of high-titer preparations for immunotherapy of immunodeficient patients with prolonged poliovirus infections [228].

8 Patterns of Host Response and Diagnosis

8.1 Clinical Features

The patterns of host response were described in Sect. 7.1 in the context of the pathogenesis of poliomyelitis. Paul recognized four categories of response to poliovirus infection in susceptible individuals: (1) inapparent (asymptomatic) infections, (2) abortive poliomyelitis, (3) nonparalytic poliomyelitis (aseptic meningitis), and (4) paralytic poliomyelitis [28]. Abortive poliomyelitis is described as minor illness, and nonparalytic and paralytic poliomyelitis constitute major illness. The proportion of each category of host response depends on the patient age, the poliovirus serotype, and other physiologic factors such as tonsillectomy, pregnancy, recent injections, trauma, or recent strenuous physical exertion [29].

8.1.1 Inapparent (Asymptomatic) Infections

Most (up to 95 %) wild poliovirus infections of nonimmune susceptible individuals are without fever or other symptoms. Infected individuals produce protective neutralizing antibodies and have permanent resistance to paralysis upon reinfection by the same poliovirus serotype. Infections that occur under the cover of maternal antibody are asymptomatic. Individuals with inapparent infections shed virus in their stool and can participate in the spread of infection.

8.1.2 Minor Illness: Abortive Poliomyelitis

Abortive poliomyelitis is the most frequent disease manifestation, occurring in about 8 % of poliovirus infections [23, 28]. Symptoms are indistinguishable from those of many other infections and include fever, malaise, drowsiness, headache, vomiting, and sore throat. Abortive poliomyelitis occurs during the viremic phase a few days after infection. Complete recovery occurs within a few days, although virus shedding may continue for up to 6 weeks postexposure.

8.1.3 Major Illness: Nonparalytic Poliomyelitis (Aseptic Meningitis)

Aseptic meningitis occurs in 1–2 % of infections. It may be preceded by minor illness and is characterized by signs of inflammation of the meninges, including severe headache, and stiffness and pain in the back and neck. Aseptic meningitis lasts 2–10 days and is usually followed by complete recovery. In rare instances, the disease progresses to paralytic poliomyelitis. Other viral infections, including those of many nonpolio enteroviruses (Chap. 11), may cause aseptic meningitis [23].

8.1.4 Major Illness: Paralytic Poliomyelitis

Paralytic poliomyelitis occurs in <1 % of wild poliovirus infections (Sect. 3.4) and is characterized by acute flaccid paralysis (AFP) usually accompanied by fever. It may be preceded by minor illness with apparent recovery followed a few days later by rapid onset of paralysis. Minor illness preceding paralysis may be absent in adolescents and adults, but pain in the affected extremities during onset of paralysis may be severe. Paralysis progresses rapidly and is descending (i.e., moving proximally to distally), with loss of deep reflexes but no sensory loss. Paralysis is usually asymmetric and affects the legs more frequently than the arms, especially in young children. Residual paralysis persists beyond 60 days. The affected muscles and severity of paralysis depends on the location and concentration of neuronal lesions [29]. Paralytic poliomyelitis is further divided into three categories based on the CNS lesions and the corresponding affected muscle groups.

Spinal Poliomyelitis

Spinal poliomyelitis (~79 % of paralytic poliomyelitis cases) occurs when lesions are localized to the spinal cord and cause weakness in the legs, arms, back, or abdominal muscles. Spinal poliomyelitis may also paralyze the diaphragm and intercostal muscles, causing respiratory failure.

Bulbar Poliomyelitis

Bulbar poliomyelitis (~2 % of paralytic poliomyelitis cases) occurs when lesions form in the cranial nerve or medulla and results in paralysis of the pharynx, vocal cords, and facial nerves. Bulbar poliomyelitis may rapidly progress to respiratory failure when the respiratory centers of the medulla are attacked.

Bulbospinal Poliomyelitis

Bulbospinal poliomyelitis (~19 % of paralytic poliomyelitis cases) is a combination of the above two forms and is most frequently seen in adults [29].

8.1.5 Post-Polio Syndrome

Post-polio syndrome is a recrudescence of weakness, pain, fatigue, and atrophy in the muscles originally affected by acute paralytic poliomyelitis [355]. Symptoms progress over a period of years and first appear on average 30–35 years after recovery from the initial acute attack [356]. It is estimated that

25–50 % of patients with acute paralytic poliomyelitis will develop some signs of post-polio syndrome, the risk being highest among survivors with more severe paralysis from the original attack. In the United States, over 440,000 survivors of paralytic poliomyelitis were recognized in 1994–1995, but no updated counts have been obtained. Post-polio syndrome is a neurologic condition whose underlying mechanisms are unknown. Current evidence indicates that the syndrome results from the denervation of the peripheral neuromuscular junctions that expanded during recovery to compensate for neuronal damage from the original infection [355]. It is thought that the neurologic dysfunction progresses subclinically for many years until a critical threshold is reached beyond which the remaining motor neurons cannot maintain the extended number of neuromuscular junctions [355]. Post-polio syndrome does not appear to result from persistence of the original poliovirus infection [357]. Polio eradication is the most effective way to prevent post-polio syndrome.

8.2 Diagnosis

Most clinicians trained since the late 1960s in the United States and other developed countries have never seen a case of poliomyelitis. Similarly, clinicians in most developing countries no longer see poliomyelitis, apart from rare sporadic cases of VAPP (Sect. 9.1.4). Nonetheless, the risk of importation of wild poliovirus (or cVDPVs; Sect. 10.8.1) and the potential for outbreaks remain as long as poliovirus circulation continues anywhere in the world [10, 12, 80–82, 132, 175–178, 181]. Diagnosis of paralytic poliomyelitis is primarily based on three criteria: (1) clinical signs and course, (2) virologic testing, and (3) residual neurologic deficit at 60 days after onset of paralysis [7].

8.2.1 Clinical Signs and Course

The most obvious sign of poliomyelitis is the appearance of AFP. However, AFP has multiple etiologies, including Guillain–Barré syndrome, transverse myelitis, enterovirus infections (especially EV71; Chap. 11), other viral infections, traumatic neuritis, Bell's palsy, hypokalemia, and toxin exposure [7]. Studies in the Americas comparing clinical findings with the isolation of wild poliovirus from stool specimens reported a sensitivity of 75 % and specificity of 73 % for poliomyelitis when the diagnostic criteria for AFP cases were age <6 years and fever at onset of paralysis [68]. Inclusion of progression to complete paralysis in <4 days increased sensitivity [68], whereas inclusion of specific patterns of paralysis (descending, asymmetric, absence of paralysis in all limbs) increased specificity but reduced sensitivity [7]. In India, which achieved AFP rates >25 per 100,000 children <15 years of age, residual paralysis at 60 days was most strongly correlated with wild poliovirus infection [358].

8.2.2 Virologic Testing

Virologic testing has high sensitivity provided that adequate specimens are collected soon after onset of paralysis and received by the laboratory in good condition (section "Clinical specimens"). Because wild polioviruses can be accurately identified by molecular methods, and contaminants ruled out by sequence analysis, specificities approach 100 %. Classification of cases of VAPP requires the inclusion of clinical, epidemiologic, and laboratory findings [303, 304, 307, 359]. In countries using OPV, most isolations of OPV-like virus from patients with AFP are independent events and do not signal the occurrence of VAPP. Laboratory detection of genetically divergent vaccine-derived polioviruses (VDPVs; Sect. 10.8) indicate either prolonged replication in an immunodeficient individual or community circulation of VDPVs [133]. The distinguishing genetic properties of the more divergent VDPVs, coupled with information about the rates of poliovirus vaccine coverage in the source community, are strong predictors of the VDPV category [133].

8.2.3 Residual Neurologic Deficit

The clinical case definition for paralytic poliomyelitis includes residual paralysis at 60 days after onset of paralysis [100, 133]. In less severe cases of paralytic poliomyelitis, residual paralysis may be difficult to discern because of functional compensation by intact muscles [7].

9 Control and Prevention of Poliomyelitis

9.1 Poliovirus Vaccines

Attempts to inactivate poliovirus infectivity through formalin treatment date to 1911, but when the results from leading investigators were consistently disappointing, interest in immunization against poliomyelitis waned [1]. Interest was briefly rekindled during the field trials of 1935, conducted by two groups who took different approaches to experimental poliovirus vaccines, one applying formalin inactivation [360] and the other using live virus that was claimed to be "attenuated" through treatment with detergent [361]. However, the appearance of poliomyelitis cases—including fatalities—linked to the field trials [362] cast a long shadow on further attempts to develop a poliovirus vaccine. The experimental poliovirus vaccines of 1935 were developed before the advent of cell culture, before the identification of three distinct poliovirus serotypes, before the outlines of poliovirus pathogenesis were firmly established, and, in the case of one investigator, a failure to distinguish between chemical inactivation and attenuation of neurovirulence through genetic selection [361]. Prospects for a poliovirus vaccine again brightened in 1948 with the successful immunization of

monkeys with formalin-inactivated poliovirus [363]. Moreover, the rising incidence of paralytic polio in developed countries in the postwar years greatly increased the urgency of developing and deploying effective poliovirus vaccines.

9.1.1 Inactivated Poliovirus Vaccine (IPV)

The IPV of Salk and colleagues was the first poliovirus vaccine to be licensed [4, 5]. Its development followed several key advances in virology, pathology, and immunology [5]: (1) the cultivation of poliovirus in nonneural cells [53], (2) the identification of three poliovirus serotypes [54, 55], (3) the finding that viremia precedes paralysis [58], and (4) the demonstration that administration of immune globulin protected against paralytic poliomyelitis [57]. IPV is prepared by formalin inactivation of three wild, virulent reference strains: Mahoney (type 1), MEF-1 (type 2), and Saukett (type 3). A less virulent type 1 strain, Brunenders [364], is used in IPV production in Sweden and Denmark. Although antigenic sites 1 (Fig. 13.5) of types 2 and 3 are modified by formalin inactivation [365], immunization with IPV can induce high titers of neutralizing antibodies protective against all poliovirus strains. After exposure of some IPV recipients with live wild poliovirus contained in production lots of incompletely inactivated vaccine (the Cutter incident) [333, 334], conditions for IPV manufacture were modified, resulting in a reduction in the immunogenicity of IPV preparations [23]. However, improvements in cell culture technology in the 1970s led to development of an enhanced-potency IPV (eIPV), similar in immunogenicity to the original product [5, 35, 366], which has replaced the second-generation IPV.

IPV was licensed for use in the United States, Canada, and Western Europe in 1955 and was the only poliovirus vaccine available until licensure of OPV in 1961–1962. IPV use in the United States declined after the introduction of OPV, but it has been used continuously by some countries in Western Europe (Finland, Iceland, Sweden, and the Netherlands) and some provinces of Canada [5, 35]. In 1997, in response to the eradication of wild polioviruses in the Americas and the continuing occurrence of cases of VAPP (Sect. 9.1.4), the United States shifted from an all-OPV immunization schedule to a sequential IPV/OPV schedule in 1997, which was replaced in 2000 by an all-IPV schedule [5, 307].

9.1.2 Oral Poliovirus Vaccine (OPV)

Early attempts to produce live-virus vaccines date to the work of Jenner, who "vaccinated" with cowpox virus to protect against smallpox in the 1790s, and Pasteur, who developed an "attenuated" rabies vaccine in the 1880s [35]. Experimental live poliovirus vaccines were tested in monkeys as early as 1910 [29]. In the 1930s, Theiler demonstrated that an effective attenuated yellow fever vaccine could be produced by serial passage of virus in chick embryo tissues [367]. Theiler applied this basic approach to develop an experimental attenuated variant of the poliovirus type 2 Lansing strain in 1946 [368]. In 1952, Enders, Weller, and Robbins developed the attenuated poliovirus type 1 Brunenders strain by serial passage in cultured cells [364]. Two years later, Li and Schaeffer developed a highly attenuated derivative of the neurovirulent type 1 Mahoney strain, LS-c, by passage at 35 °C in cultured rhesus or cynomolgus monkey kidney cells and in monkey skin [369]. The LS-c strain was further treated by three consecutive single-plaque passages by Albert Sabin to produce the LS-c, 2ab strain, generally known as Sabin type 1 (Sabin 1) [370]. Sabin also developed attenuated strains for serotypes 2 and 3. The Sabin 2 OPV strain (P712, Ch, 2ab) was derived from virus (P712) that was isolated from a healthy child and shown to have low neurovirulence [370]. In contrast, the attenuated Sabin 3 strain (Leon 12 a_1b) was produced by rapid passage in monkey kidney cell culture of a highly neurovirulent strain isolated from the spinal cord of a child who had died of bulbospinal poliomyelitis [370]. In addition to Sabin, two other groups, led by Koprowski [329] and Cox [371], developed attenuated poliovirus vaccine strains for each serotype. All three sets were carefully evaluated for low neuropathogenicity for monkeys, immunogenicity, genetic stability on human passage, safety (inability to cause paralysis in man), and restricted capacity to spread [372, 373]. The Sabin strains, which had the lowest neuropathogenicity and an excellent safety record from large-scale field trials [6, 60], were approved for worldwide distribution. In the United States, OPV was licensed sequentially (Sabin 1, August, 1961; Sabin 2, October, 1961; Sabin 3, March, 1962). Licensure of Sabin 3 was delayed because of concerns about its undesirable genetic instability and relatively low immunogenicity [1]. OPV was initially administered serially in monovalent form, frequently in mass immunization campaigns (SOS; "Sabin on Sunday"), but successful tOPV field trials in Canada led to licensure of a trivalent formulation in 1963 [7]. OPV and its application to poliomyelitis eradication has been recently reviewed [7].

OPV was developed in the 1950s using the well-established empirical approach of rapid passage of virus at suboptimal temperatures in cells and tissues of nonhuman origin. It would be another two decades after licensure of OPV that the molecular basis of OPV attenuation became amenable to systematic investigation.

9.1.3 Genetic Determinants of Attenuation of the Sabin OPV Strains

Identification of the genetic determinants of attenuation of the Sabin OPV strains has been comprehensively reviewed [207, 209, 268, 374]. The first reports of the sequences of complete poliovirus genomes in the early 1980s [375, 376] and the development of infectious poliovirus cDNA clones

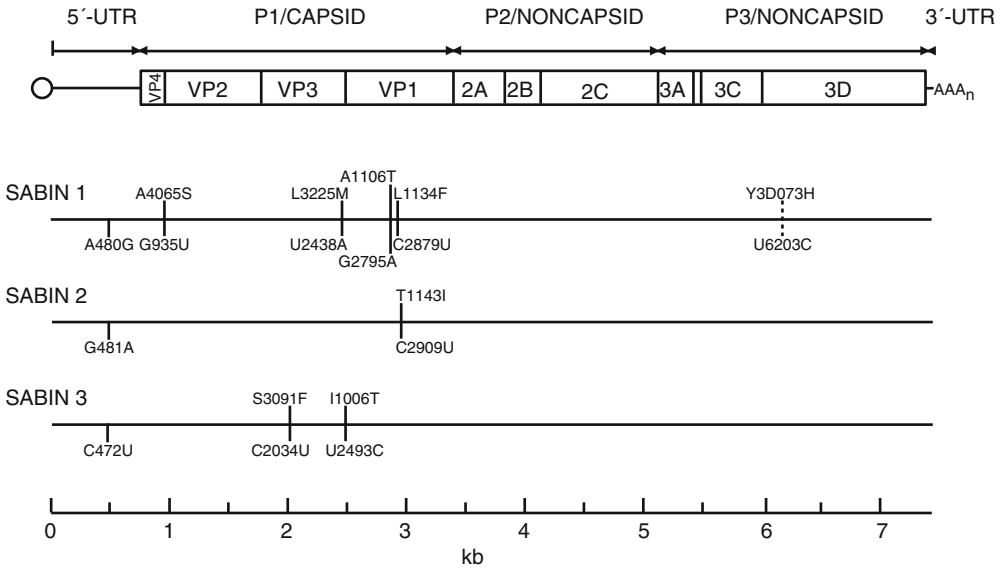

Fig. 13.9 Location of principal attenuating nucleotide (*lower bars*) and amino acid (*upper bars*) substitutions in each of the three Sabin OPV strains. Abbreviations of nucleotide residues: *A* adenine, *C* cytosine, *G* guanine, *U* uracil. Abbreviations of amino acid residues: *A* alanine, *C* cysteine, *F* phenylalanine, *H* histidine, *I* isoleucine, *L* leucine, *M* methionine, *S* serine, *T* threonine, *Y* tyrosine. Substitutions are shown as nonattenuated parent–position–Sabin strain; nucleotide positions are numbered consecutively from residue 1 of the RNA genome; amino acid positions are indicated by the abbreviated name of the viral protein (4, VP4; 2, VP2; 3, VP3; 1, VP1; 3D, 3D-polymerase) and numbered consecutively from residue 1 of each protein. For example, a guanine (Mahoney) → uracil (Sabin 1) substitution at RNA position 935 (G935U) encodes an alanine (Mahoney) → serine (Sabin 1) replacement at residue 65 of VP4 (A4065S). The Y3D073H substitution in Sabin 1 and S3091F substitution in Sabin 3 are important determinants of temperature sensitivity (Figure summarizes findings from references [379–384] (Redrawn from reference Kew et al. [94])

[377] opened the way for systematic investigation of the critical mutations responsible for the attenuated and temperature-sensitive phenotypes of the Sabin OPV strains. A common feature of the Sabin strains is the presence of nucleotide substitutions in the IRES, which in serotypes 1 and 3 have been clearly shown to be critical attenuating mutations. Additional mutations encoding amino acid substitutions in the capsid region contribute to and stabilize the attenuated phenotype.

Sabin 1

The 57 nucleotide substitutions distinguishing the Sabin 1 strain from its neurovirulent parent, Mahoney, are scattered throughout the genome [144]. Six map to the 5′-UTR, 49 map to the coding region (21 of which encode amino acid substitutions), and 2 map to the 3′-UTR. Infectious cDNA constructs containing different combinations of blocks of Sabin 1 and Mahoney sequences were tested for neurovirulence in monkeys or transgenic mice expressing the CD155 receptor, for temperature sensitivity, and for other phenotypic properties distinguishing the two strains [378, 379]. The single most important determinant of the attenuated phenotype of Sabin 1 was the A → G substitution at position 480 (abbreviated A480G) in the IRES [380]. Four other substitutions contributing to the attenuated phenotype mapped to the capsid region (one in VP4, one in VP3, and two in VP1), and a substitution contributing to the temperature-sensitive phenotype (but not to the attenuated phenotype) mapped to the $3D^{pol}$ region encoding the RdRP (Fig. 13.9) [7, 378, 379, 385].

Sabin 2

Only two nucleotide substitutions (G481A in the IRES and C2909U encoding a threonine → isoleucine substitution at position 143 of VP1) appear to be main determinants of the attenuated phenotype of Sabin 2 (Fig. 13.9) [381, 382]. Because P712 has inherently low neurovirulence [370], identification of critical attenuating sites in Sabin 2 involved determination of the effects of introduction of sequences derived from a minimally divergent neurovirulent revertant of Sabin 2 (obtained from a case of VAPP) into infectious cDNA constructs derived from Sabin 2 [381, 382]. The neurovirulence of Sabin 2 is remarkably stable in routine tests of OPV lots in monkeys, because the G481A substitution (not found in the IRES sequences of wild type 2 polioviruses) does not increase neurovirulence in monkeys, as it does in transgenic mice [386].

Sabin 3

Detailed analysis of the attenuated phenotype of Sabin 3 has been possible because the neurovirulent parental strain, Leon, differs from Sabin 3 by only ten nucleotide substitutions

[387]. In addition, numerous neurovirulent revertants of the Sabin 3 strain have been isolated from patients with VAPP [383] and from healthy OPV recipients [388]. Only three substitutions (C472U in the IRES, C2034U encoding a serine → phenylalanine substitution at position 91 of VP3, and U2493C encoding an isoleucine → threonine substitution at position 6 of VP1) appear to be the main determinants of the attenuated phenotype (Fig. 13.9) [207, 383, 388].

In all three Sabin strains, the attenuated phenotype is determined by multiple substitutions. The substitutions in the IRES, which alter stem-loop structures [207, 389–391] and reduce the efficiency of initiation of translation of the poliovirus RNA template [390, 392], contribute most to the attenuated phenotype of the Sabin 1 and Sabin 3 strains. Mutations restoring the original stem-loop structure in the IRES (Sabin 1, G480A [back mutation] or U525C [suppressor]; Sabin 2, A481G; Sabin 3, U472C) are frequently found in vaccine-related isolates from healthy OPV recipients [389, 393] and patients with VAPP [207] as well as from the environment [109]. In Sabin 3, the critical C472U substitution reduces the efficiency of binding of the polypyrimidine tract-binding protein (PTB), required for initiation of translation, to the IRES [394]. The translational deficit for Sabin 3 is moderate in intestinal cells, where PTB levels are high, but severe in neurons, where PTB levels are low. The precise mechanisms by which the capsid mutations contribute to the attenuated phenotype are less clear. Impairment of the efficiency of binding to the CD155 receptor [379] and reductions in the stability of the capsid [215] may play a role.

Sabin and other developers of OPV strains struck a balance between low neuropathogenicity, good immunogenicity, and acceptable levels of genetic stability [373]. The high genetic stability of the Sabin type 1 strain is probably attributable to the greater number of substitutions contributing to the attenuated phenotype. This property is especially important for the Sabin 1 vaccine strain, because wild type 1 polioviruses typically have high paralytic attack rates and can spread over wide geographic areas in explosive outbreaks [10, 170, 175–177, 180, 181]. Sabin 2 may revert more rapidly, but its immunogenicity is very high [7, 373] and the paralytic attack rates of wild type 2 polioviruses are low (Sect. 3.4) [34, 101]. Sabin 3 is associated with the highest rates of VAPP (Sect. 9.1.4), which is probably a consequence of low genetic stability of the critical attenuating substitution [275], relatively low immunogenicity [395], and an intermediate paralytic attack rate for type 3 polioviruses [34, 101]. Nonetheless, all three Sabin strains normally have very low pathogenic potentials, and incidence of VAPP in countries with high rates of OPV coverage [304] are several orders of magnitude lower than the incidence of paralytic poliomyelitis in areas with circulating wild polioviruses.

9.1.4 Vaccine-Associated Paralytic Poliomyelitis (VAPP)

After over 40 years of use and many billions of doses distributed worldwide, OPV has been associated with few adverse events. The most commonly recognized adverse event is VAPP, which is clinically indistinguishable from poliomyelitis caused by wild polioviruses. The first cases of VAPP were recognized within a year of licensure of OPV, and most of the early cases were associated with the Sabin 3 strain [396]. The Sabin 3 association was unambiguous because OPV had been delivered in monovalent form [7, 301, 396]. VAPP rates are very low and similar worldwide [7, 359, 397–400]. In the United States, the risk of VAPP in first-dose OPV recipients is about 1 case per 1.4 million children immunized [7, 301, 303, 304, 307, 401]. In immunologically normal recipients, the risk of VAPP decreases sharply (>tenfold) for subsequent doses. VAPP cases are sporadic and occur in both OPV recipients and their unimmunized household and non-household contacts. A small proportion (~7 %) of VAPP cases in the United States are classified as "community-acquired," indicating no known exposure to OPV. VAPP in OPV recipients and household contacts is most frequently associated with Sabin 3 (71 % of cases), followed by Sabin 2 (26 % of cases) [304]. VAPP in non-household contacts and in community-acquired cases is most frequently associated with Sabin 2 (50 % of cases), followed by Sabin 3 (33 % of cases) [304]. Sabin 1 is rarely associated with VAPP in immunocompetent individuals when administered in tOPV [303, 304, 398].

Poliovirus isolates from immunocompetent VAPP case-patients show only limited genetic divergence from the parental OPV strains, although the key substitutions conferring the attenuated phenotype have frequently reverted [393, 402]. Many isolates from VAPP cases, AFP cases with incidental isolation of vaccine-related virus (much more frequent than VAPP cases), and healthy OPV recipients are vaccine/vaccine recombinants [161, 279, 403, 404]. The biological and genetic properties of viruses isolated from healthy OPV recipients/contacts are often indistinguishable from viruses isolated from patients with VAPP [393, 405].

Persons with primary B-cell immunodeficiencies (Sect. 10.8.3) [406] should not be given OPV because they are at a much higher (~3,000-fold) risk for VAPP [353, 354]. However, some children have received OPV before their immunodeficiency was recognized. Immunodeficiency-associated VAPP (iVAPP) differs markedly from VAPP in immunocompetent individuals, as it is rarely associated with Sabin 3 (14 % of cases), and is more frequently associated with Sabin 2 (72 % of cases) and Sabin 1 (31 % of cases) (some patients were infected with more than one serotype) [354]. On the other hand, persons with T-cell immunodeficiencies, including those infected with HIV, do not appear to be at elevated risk for VAPP [304, 353, 354, 407].

Each case of VAPP is an independent event. It is estimated that 250–500 cases of VAPP occur worldwide, most in countries free of circulating wild poliovirus (Fig. 13.2a) [7]. VAPP is a direct clinical consequence of the genetic instability of the Sabin OPV strains, and the most effective means to prevent VAPP is to stop OPV use after cessation of wild poliovirus circulation [22, 307].

9.1.5 Sequential Use of IPV and OPV

The relative merits of IPV and OPV have been compared for many years [23, 408]. Key advantages of IPV are (1) it efficiently induces serum immunity protective against paralytic disease, (2) it is unaffected by interference by other enteroviruses or among vaccine components, (3) it can be used in combination with other injectable vaccines, and (4) it presents no risk for reversion to virulence. The main disadvantages to IPV are (1) it provides much reduced levels of intestinal immunity; (2) until recently, most preparations had been produced from virulent wild poliovirus strains; and (3) its costs of production and delivery are higher than OPV [24, 409]. Key advantages of OPV are (1) it is easily administered, (2) it confers both serum immunity and intestinal immunity (the latter important to limiting poliovirus transmission), (3) it can be easily used in mass campaigns in developing country settings, (4) it is suitable for use in outbreak control, and (5) it has low costs of production and delivery. Disadvantages to OPV include (1) it presents a continuous, low-level risk of VAPP, (2) it may have low per-dose efficacy rates of seroconversion in some high-risk settings, (3) it is subject to interference by other enteroviruses and among OPV strains (especially by the type 2 strain), (4) it has the potential to establish chronic infections in persons with primary immunodeficiencies, and (5) it has the potential to spread person-to-person and cause poliomyelitis outbreaks when used at low rates of coverage [23, 94, 133, 408, 410]. A sequential IPV/OPV immunization schedule can combine the major advantages of each vaccine and mitigate some disadvantages. A sequential schedule can virtually eliminate VAPP in OPV recipients and reduce it in OPV contacts, because shedding of OPV-related viruses is reduced. This was observed in the United States when it shifted to a sequential IPV/OPV schedule between 1997 and 2000, as no cases of VAPP were observed in persons using the sequential IPV/OPV schedule (Fig. 13.7) [307]. Mucosal immunity induced by an IPV/OPV schedule is much better than with IPV alone [411]. However, global implementation of any schedule using IPV will require much higher rates of routine immunization coverage than currently exist (Sect. 11.2.1) [22, 412].

9.2 Impact of Immunization

As described in Sect. 5.6.2, the high-income countries of North America, Western Europe, and the southwest Pacific were quick to adopt widespread immunization with IPV, with dramatic impact. In the United States, for example, the incidence of paralytic polio fell from 13,850 in 1955 to 829 in 1961 [308]. Although transition to OPV in the United States was not complete until the mid-1960s, the last peak year (>100 cases) for polio was 1966 (Fig. 13.7), and all domestic reservoirs for poliovirus circulation apparently had been eliminated after 1970 [163, 413]. Similar highly favorable results were achieved in other developed countries [8, 9, 309, 414, 415]. IPV was not widely used in developing countries (and some developed countries, such as Japan [309]), partly because of cost and also because of the challenges of administering an injectable vaccine to large populations. Thus, the possibility of widespread immunization against polio had to await the availability of OPV, where its key advantages were decisive. The synchronous induction of intestinal immunity through mass OPV campaigns efficiently blocks person-to-person transmission of wild poliovirus, thereby protecting both individual vaccine recipients and the wider community. Despite its advantages, OPV coverage in most developing countries of Asia, Africa, and the Americas remained low, and by 1970 a widening divide separated high-income countries where wild poliovirus circulation had stopped from most low-income countries where wild poliovirus circulation continued unabated [8, 9]. By 1985, poliomyelitis had all but disappeared in developed countries, while poliomyelitis crippled a child on average every 90 s in developing countries (Figs. 13.2 and 13.3).

10 The Global Polio Eradication Initiative (GPEI)

10.1 Prelude to the GPEI

A compelling case for global poliomyelitis eradication has been advanced for decades [294] and receives continued support from the perspectives of social equity [17, 416], economic benefit [417], and technical feasibility [12, 418]. However, the launch of a vertical program to eradicate poliomyelitis was controversial in the early 1980s [30] and remained so nearly two decades later [419]. Several key factors described below set the stage for the 1988 WHA resolution establishing the GPEI [30].

10.1.1 Poliomyelitis Eradication in Cuba, 1962

Sabin advocated mass OPV campaigns as the most effective means of polio control [294]. Cuba adopted his approach in 1962 and within a year had stopped all wild poliovirus circulation [295, 310], becoming the first country to eradicate poliomyelitis. The experience in Cuba conclusively demonstrated that mass campaigns were effective in a tropical developing country setting.

10.1.2 Smallpox Eradication, 1977

Smallpox was the first infectious disease to be eradicated. Global smallpox eradication was launched in 1966 following a WHA resolution to eradicate the disease by 1976. The last natural case of smallpox was reported in Somalia in 1977,

and global eradication was certified in 1980 [284]. In addition to the complete disappearance of a universally feared viral disease, the successful smallpox program accrued many other public health benefits. The program forged strong international cooperation in public health, trained a generation of international public health professionals who would lead the way to polio eradication, and set an irreversible precedent that infectious diseases could be controlled on a global scale [30]. In light of the smallpox eradication initiative, the Expanded Program on Immunization (EPI) was established by the WHO in 1974 to make immunization against six diseases (diphtheria, pertussis, tetanus, poliomyelitis, measles, and tuberculosis) available to every child in the world by 1990 [420]. The core EPI strategy was high routine immunization coverage, which was to prove insufficient to control poliomyelitis in some developing country settings.

10.1.3 National Immunization Days (NIDs) in Brazil, 1980

Brazil began routine immunization with OPV in the early 1960s [296]. The impact of immunization was initially unclear because poliomyelitis was not a notifiable disease until 1968. Although mass campaigns in 1971–1973 reduced poliomyelitis cases, they were replaced by routine immunization in 1974. Cases sharply increased until 1980, when Brazil reestablished mass campaigns in the form of biannual NIDs, targeting all children <5 years of age (regardless of prior immunizations) for immunization with OPV in a single day. Poliovirus circulation dropped sharply in Brazil after implementation of NIDs and set Brazil on the path to polio eradication [18, 296].

10.1.4 The PAHO Regional Polio Eradication Initiative, 1985

The dramatic success of mass campaigns in Cuba, Brazil, and Mexico led PAHO to resolve in 1985 to eradicate polio from the Americas by 1990 [14]. PAHO modeled their regional initiative on Brazil's successful strategy of NIDs and used poliomyelitis eradication as the vanguard of efforts to revitalize childhood immunization in the Americas. The PAHO approach of coordinated NIDs to supplement improved rates of routine OPV coverage eradicated indigenous wild polioviruses within 6 years [18]. PAHO set the standard for global efforts and built an infrastructure for the subsequent elimination of indigenous measles and rubella viruses from the Americas [421–423].

10.1.5 Rotary PolioPlus, 1985

Rotary International launched their global PolioPlus campaign in 1985, with the goal of helping eradicate poliomyelitis worldwide by 2005, the centennial of Rotary's founding. In their initial drives for support of PolioPlus, Rotarians more than doubled their target goal of $120 million for OPV and have raised more than $1.2 billion since 1985. Rotarians have been active in all phases of polio eradication, including active participation in mass campaigns, training of health workers, communications, regular engagement of national leaders, infrastructure development, support for surveillance and the GPLN, and support for research (http://www.endpolio.org/) [17, 30, 424].

10.2 The 1988 WHA Declaration to Eradicate Polio Worldwide

In view of the rapid progress attained in the Americas, the WHA resolved in 1988 to eradicate polio worldwide by the year 2000 [15], launching the WHO GPEI. At the time of the World Health Assembly resolution, wild polioviruses were circulating unabated in much of the developing world (Figs. 13.2 and 13.3). The Western Pacific Region set a goal of eradication of indigenous wild polioviruses by 1995, a goal reached in 1997 and certified in 2000 [425]. China, after years of outbreaks [426], adopted the strategy of NIDs and Subnational Immunization Days (SNIDs) which, when coupled with strengthened routine immunization, stopped wild poliovirus transmission by early 1994 [297, 427]. Other developing countries soon followed.

10.3 Biological Principles of Poliovirus Eradication

The key biological requirements for poliovirus eradication are the following: (1) the absence of a persistent carrier state, (2) virus is spread by person-to-person transmission, (3) immunization interrupts virus transmission, (4) the absence of any nonhuman reservoir hosts for the virus, and (5) finite virus survival time in the environment [230]. An additional important nonbiological requirement for any disease eradication effort is political will, arising from the perceived benefits of eradication, and expressed internationally through resolutions passed by the WHA. Essential to the success of the GPEI has been the strong alliance among the four "spearheading" partners, WHO (http://www.polioeradication.org/), UNICEF (http://www.unicef.org/health/index.html), Rotary International (http://www.endpolio.org/), and the United States Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/polio/), as well as strong support from national governments and new partners, including the Gates Foundation (http://www.gatesfoundation.org/What-We-Do/Global-Development/Polio) and the UN Foundation (http://www.unfoundation.org/news-and-media/press-releases/2013/fight-against-polio.html).

10.4 Basic Strategy for Global Polio Eradication

The four pillars of the GPEI strategy are (1) high routine immunization coverage of infants with OPV, (2) supplementary OPV immunization activities in the form of NIDs and

SNIDs, (3) targeted door-to-door "mop-up" OPV immunization in areas of focal transmission, and (4) sensitive surveillance for poliovirus [102]. Very high rates of routine OPV immunization are required to block poliovirus circulation in areas where the risk factors converge, conditions under which routine OPV coverage rates exceeding 90 % may be insufficient to block poliovirus circulation [7, 180, 296, 428]. Such rates are currently unattainable through routine immunization in the least developed countries. Supplementary immunization is the mainstay of polio eradication in developing countries and has been instrumental in raising population immunity rates above the thresholds required to block poliovirus transmission [429]. Supplementary immunization strategies are driven by poliovirus surveillance, which is used to guide the intensified SNIDs and mop-up campaigns to the reservoir communities where the chains of poliovirus transmission continue to survive and propagate.

10.4.1 AFP and Poliovirus Surveillance

Surveillance for circulating polioviruses has two arms: (1) AFP case investigations and (2) virologic studies of polioviruses obtained from clinical specimens or the environment. Even though most wild poliovirus infections are inapparent, over time, all effectively performing AFP surveillance systems can detect endemic poliovirus circulation. In suspected high-risk areas lacking effective AFP surveillance, supplementary surveillance activities, such as sampling community contacts of AFP cases, stool surveys of healthy children, or environmental sampling, have been implemented to increase sensitivity for detecting wild polioviruses [104, 106, 107, 111, 113, 116, 315].

10.5 Increasing Momentum Toward Eradication, 1988–2001

The global incidence of poliomyelitis was on a virtually unbroken downward trend between 1987 and 2001 (Figs. 13.2 and 13.3). Three WHO regions reported their last indigenous cases before 2000: the Americas (last indigenous case: Peru, 1991) [430], the Western Pacific Region (last indigenous case: Cambodia, 1997) [425], and the European Region (last indigenous case: Turkey, 1998) (Fig. 13.3) [431]. Wild poliovirus type 2 was last detected in October 1999 in Uttar Pradesh, India [13]. By 2001, the tide of global polio eradication, although delayed in some places, seemed unstoppable [432].

10.5.1 Polio Eradication in Conflict Countries

The sense of momentum was reinforced by control of poliomyelitis in countries with armed conflict such as Colombia, Peru, the Philippines, Sri Lanka, Cambodia, Angola, the Democratic Republic of the Congo, and Somalia [433, 434]. In many countries, conflict was suspended during "Days of Tranquility" allowing children to be immunized during NIDs and SNIDs. For example, Colombia and Peru were reservoirs for all three wild poliovirus serotypes in the early 1980s. Active cross-border migration of high-risk populations among the northern Andean countries facilitated wide dissemination of wild poliovirus [164, 170] and required close synchronization of NIDs among the affected countries [18]. A guerilla movement in the interior of Peru targeting health clinics and health personnel required active engagement by public health leaders to ensure access to unimmunized children. The final chains of wild poliovirus transmission in the Americas were broken in the interior of Peru in 1991 [18]. In Sri Lanka, major military campaigns in a devastating civil war were suspended during the NIDs, and wild poliovirus circulation stopped after 1993. In the 1990s, Vietnam and Cambodia were still recovering from decades of conflict. Detailed aerial mapping of the migratory at-risk populations in the water courses linking Cambodia and Vietnam (with active cross-border poliovirus transmission) set the stage for house-to-house and boat-to-boat immunization campaigns that stopped wild poliovirus transmission in 1997 [290, 435]. The Democratic Republic of the Congo and Angola have experienced shattering civil conflicts over the past two decades. Nonetheless, both countries eradicated their indigenous wild polioviruses in 2000 and 2002, respectively [11]. Both countries appear to have eradicated polio twice, the second time was a widely disseminated wild type 1 poliovirus imported from India around 2002 [177]. Similarly, Somalia eradicated the indigenous wild polioviruses in 2002 [436], and again eradicated wild poliovirus type 1 imported from Nigeria in 2005 [177, 437].

10.5.2 Polio Eradication in Very High-Risk Settings

Successful control of poliomyelitis in settings of intrinsically high biological risk demonstrated the feasibility of global polio eradication. However, conditions varied widely, and creative local solutions repeatedly had to be found and implemented [434]. The critical test case for the PAHO polio eradication initiative was Brazil, the most populous country in Latin America. Control of wild poliovirus transmission was more difficult in the tropical northeast (with its lower levels of hygiene and sanitation), and migration from the endemic northeast to the rapidly growing megacities of Rio de Janeiro and São Paulo presented the continuous risk of reinfection with wild polioviruses. Nonetheless, steady pressure from NIDs, SNIDs, and mop-up campaigns stopped all wild poliovirus transmission by early 1989 [75]. Success in the Americas did not necessarily presage success elsewhere because the Americas were often seen as having several key advantages: (1) greater resources and infrastructure than many developing countries elsewhere, (2) a tradition of strong public health leadership in many countries, (3) geographic isolation protective against re-importation of wild polioviruses, and (4) a cultural unity conducive to a common approach.

China, comprising 22 % of the world population, presented challenges of scale not encountered in the Americas [426]. Climate varied widely from the temperate north to the tropical south, and levels of infrastructure and sanitation also varied widely in this rapidly developing country. Population size and density across wide areas favored efficient wild poliovirus transmission, and massive economic migration from the countryside into the increasingly prosperous cities presented additional challenges of reaching unimmunized children. However, China had already established good coverage through routine immunization with tOPV, and a series NIDs and SNIDs in 1992–1993 broke the remaining chains of indigenous wild poliovirus transmission by early 1994 [297, 427].

Other successes in high-risk settings in Asia and Africa followed. Indonesia and the Philippines have humid tropical climates and very high population densities in their most populous islands. A combination of strengthened routine immunization and NIDs at high rates of coverage stopped wild poliovirus types 1 and 3 transmission in both countries by 1995 [289, 290]. Bangladesh has one of the highest population densities in the world, and wild poliovirus was deeply entrenched in multiple endemic reservoirs with poor infrastructure. High-quality NIDs built upon a solid national program of routine immunization stopped wild poliovirus transmission in 2000 [425]. Neighboring India has more children <5 years of age than any other country. Despite the biological challenges of high population densities, tropical conditions, and poor infrastructure in many areas, the southern states stopped indigenous wild poliovirus transmission through a combination of high rates of routine immunization coverage and mass campaigns. Progress was much slower in the large and populous states of Uttar Pradesh and Bihar (with a combined monthly birth cohort >500,000), but all wild poliovirus transmission stopped in India in 2011 [12, 91]. Eradication was especially challenging in these two Indian states because the per-dose efficacy of OPV was low, as many children with poliomyelitis had received multiple OPV doses. Mass campaigns (NIDs, SNIDs, and large-scale mop-ups) overcame weaknesses in routine immunization [438]. The most populous parts of Afghanistan (northern provinces) [184], Pakistan (Punjab province) [184], and Nigeria (southern states) [185] stopped wild poliovirus circulation several years ago, only to be subject to reinfection primarily from domestic endemic reservoirs.

10.5.3 Genotypic Indicators of Progress

Progress in polio eradication has been accompanied by a steady reduction in the genetic diversity of circulating wild polioviruses. Of the 20 wild poliovirus type 1 genotypes, 5 wild poliovirus type 2 genotypes, and 17 wild poliovirus type 3 genotypes found in 1988 [439], only 4 (or fewer) wild poliovirus genotypes remain (two type 1 and two type 3) (Figs. 13.10 and 13.11). Diversity within the surviving genotypes continues to decline as genetic clusters steadily disappear (Sect. 10.7), and wild poliovirus type 3 may be very near eradication in both Asia and Africa.

10.6 Resurgence of Wild Polioviruses, 2002–2011

After years of steady decline, poliomyelitis cases increased sharply from 483 cases in 2001 to 1918 cases in 2002 (Fig. 13.2) [436]. This increase was the result of a large epidemic in India (1,600 cases), primarily associated with wild poliovirus type 1, centered in Uttar Pradesh (78 % of total cases) [409], and a rise in reported cases in Nigeria [440]. Global cases fell to 784 in 2003, as the epidemic in India was controlled by large-scale NIDs and SNIDs [441], but wild poliovirus type 1 was beginning to spread from northern Nigeria to neighboring countries [442]. The upsurge in Nigeria followed suspension of NIDs and SNIDs in several northern states in 2003 and 2004 in response to false rumors about OPV safety [443]. By 2006, wild poliovirus type 1 had spread from northern Nigeria to 18 countries, across a wide importation belt in Africa from Guinea in the west to Somalia in the Horn of Africa, and into Asia from Saudi Arabia and Yemen in the west to Indonesia at the southeastern rim, sparking large outbreaks (>100 cases) in Sudan, Somalia, Yemen, and Indonesia [10, 176]. Wild poliovirus type 1 introduced into Angola from Uttar Pradesh caused cases that were first reported in 2005 [176], and Nepal experienced repeated importations from northern India [176]. The majority of cases in 2005 were associated with imported virus, but most of the outbreaks outside of the core endemic reservoirs of Nigeria, India, Pakistan, and Afghanistan were largely controlled by the end of 2005 (Fig. 13.2d, e) [176], except for Kenya and Uganda where virus originating from Nigeria persisted until 2011 [10]. By 2006, most wild type 1 poliovirus cases occurred in the core reservoir countries, but virus of Nigerian origin continued to circulate in Niger, Ethiopia, Yemen, and Kenya [444], and virus of Indian origin spread to Bangladesh and from Angola to Namibia [80] and the Democratic Republic of Congo [444]. Instrumental in rolling back the wave of imported wild poliovirus type 1 was the widespread use in NIDs and SNIDs of monovalent type 1 OPV (mOPV1), which has a higher type-specific per-dose efficacy than tOPV because interference from the robust type 2 component of OPV is eliminated [196, 410]. However, exclusive use of mOPV1 in successive campaigns led to the development of growing immunity gaps to poliovirus types 2 and 3.

In India, as wild poliovirus type 1 cases declined 87 % from 646 in 2006 to 87 in 2007, wild poliovirus type 3 cases surged from 28 in 2006 to 787 in 2007 [445]. Widespread

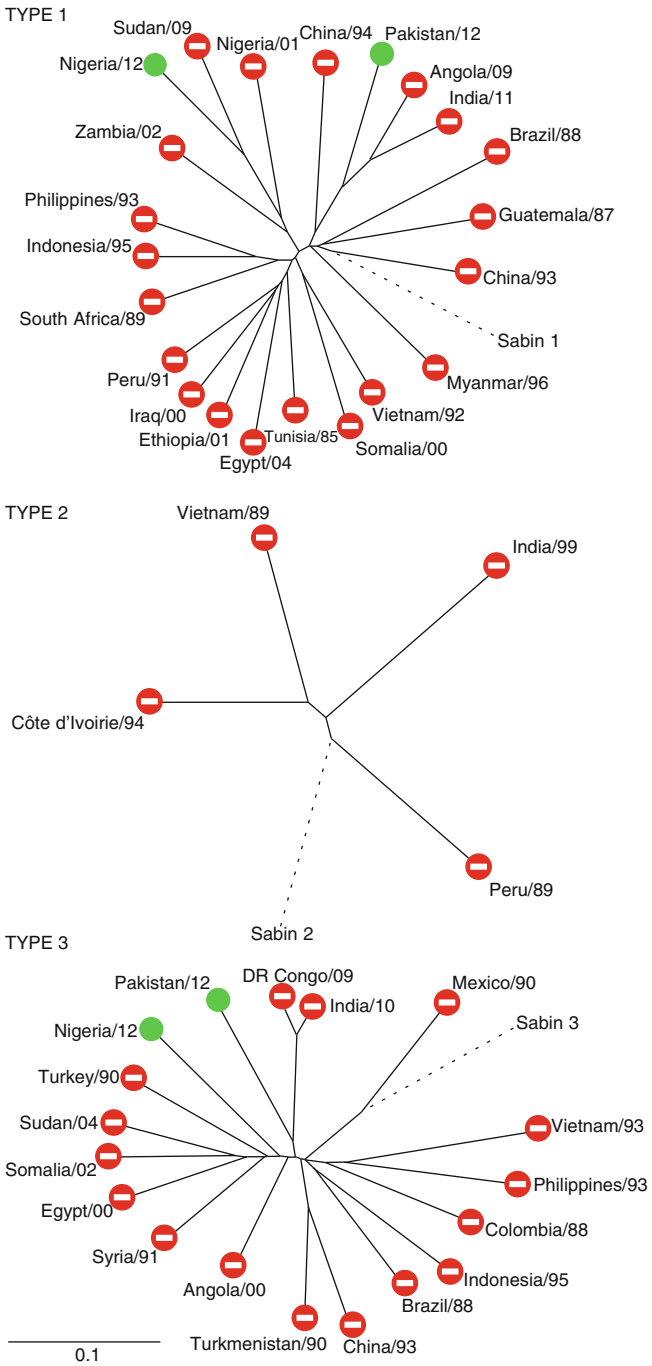

Fig. 13.10 Radial neighbor-joining trees of VP1 sequence relationships of representatives of wild poliovirus genotypes detected since the launch of polio eradication activities in the Americas in 1985. Genotypes believed to be extinct are represented by stop signs at the branch tips. Sequences representing the extinct genotypes usually are from the last known isolate of that genotype (Source: WHO Global Polio Laboratory Network. Modified from reference Chumakov and Kew [439])

circulation of wild poliovirus type 3 continued in Uttar Pradesh and Bihar through 2009 [438]. Wild poliovirus type 3 imported from Uttar Pradesh into Angola caused 24 cases in 2008 and spread to the Democratic Republic of Congo in 2008 and 2009 [10, 177]. During 2007–2011, wild poliovirus type 3 from Nigeria spread east to Chad, Cameroon, and the Central African Republic and north and west to Niger, Mali, Côte d'Ivoire, and Guinea [10, 177].

A second wave of wild poliovirus type 1 from Nigeria began in 2008 and spread to 14 countries, reinfecting many of the same countries that experienced the 2003–2006 outbreaks [177]. Wild poliovirus type 1 imported into Chad from northeastern Nigeria in 2010 caused a large outbreak that continued into 2012, resulting in reestablished transmission in Chad and further export of wild poliovirus type 1 into the Central African Republic [10]. However, effective NIDs and SNIDs in the countries of West Africa again rolled back the imported Nigerian virus [10, 16].

While wild poliovirus type 1 was disappearing from India, virus originating from Uttar Pradesh was associated in 2010 with a large outbreak (458 cases) in Tajikistan [178], with further spread to Turkmenistan, Kazakhstan, the Russian Federation, and probably Uzbekistan [181]. During 2010–2011, wild poliovirus type 1, originally imported several years earlier into Angola from Uttar Pradesh, sparked a large outbreak (~441 cases) in the Republic of the Congo with a very high CFR (Sect. 3.1) [81]. These outbreaks were controlled by aggressive mass immunization campaigns, and all wild polioviruses of Indian origin appear to have been eradicated worldwide.

Wild poliovirus type 1 circulation continued at much reduced levels in Pakistan and Afghanistan [184]. However, in 2011 wild poliovirus type 1 from southern Pakistan was imported into Xinjiang in western China, causing an outbreak of 21 cases [10, 82]. Sensitive surveillance in China detected the outbreak in its early stages, and a series of intensive mass immunization campaigns halted poliovirus circulation. Although China and the Western Pacific Region are again free of wild poliovirus circulation, the risk remains of re-importation from neighboring Pakistan even as poliomyelitis is in decline in that country.

10.7 Wild Poliovirus in Retreat, 2011–2012

A major milestone in global polio eradication was the eradication of indigenous wild poliovirus in India, once the world's most intense wild poliovirus reservoir [326, 327] and the source of repeated dissemination of wild poliovirus into countries in four continents [11, 170, 176, 177, 179, 315, 415, 446]. The last wild poliovirus type 3 in India was isolated in October 2010 and the last wild poliovirus type 1 was isolated in January 2011 (Figs. 13.10 and 13.11) [12, 91]. In neighboring Pakistan and Afghanistan, which constitute a common epidemiologic block, the diversity of wild poliovirus types 1 and 3 has been in steady decline, despite fluctuations in case counts (Fig. 13.12) [12].

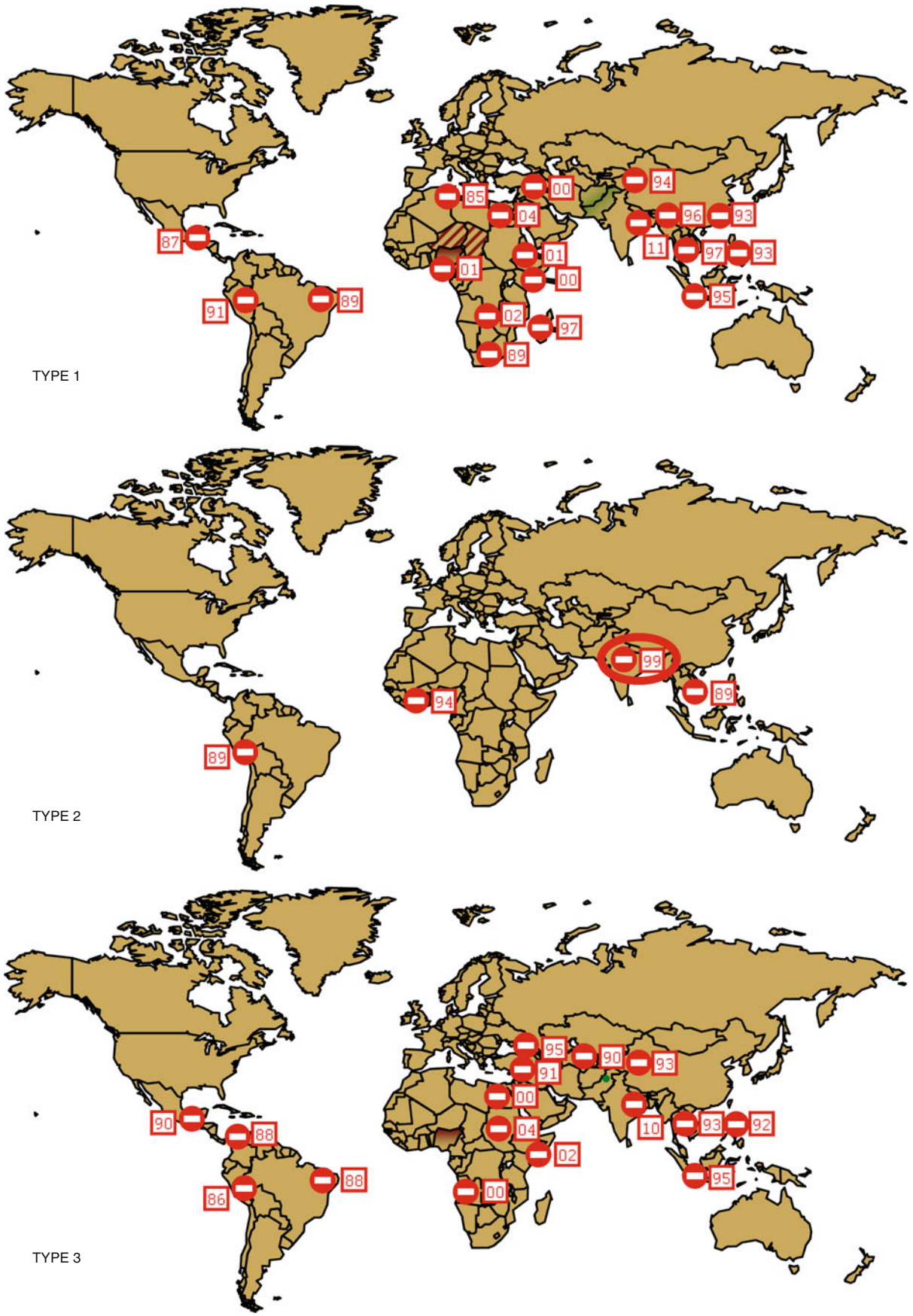

Fig. 13.11 Progressive eradication of wild poliovirus genotypes, 1986 to 2012. Stop signs indicate the year and location where the last isolate was obtained for each extinct genotype. Surviving type 1 and type 3 poliovirus genotypes are color-coded; hatch and shading patterns indicate, respectively, countries with reestablished transmission and sporadic importation of West Africa genotype viruses (Source: WHO Global Polio Laboratory Network. Modified from reference Kew and Pallansch [11])

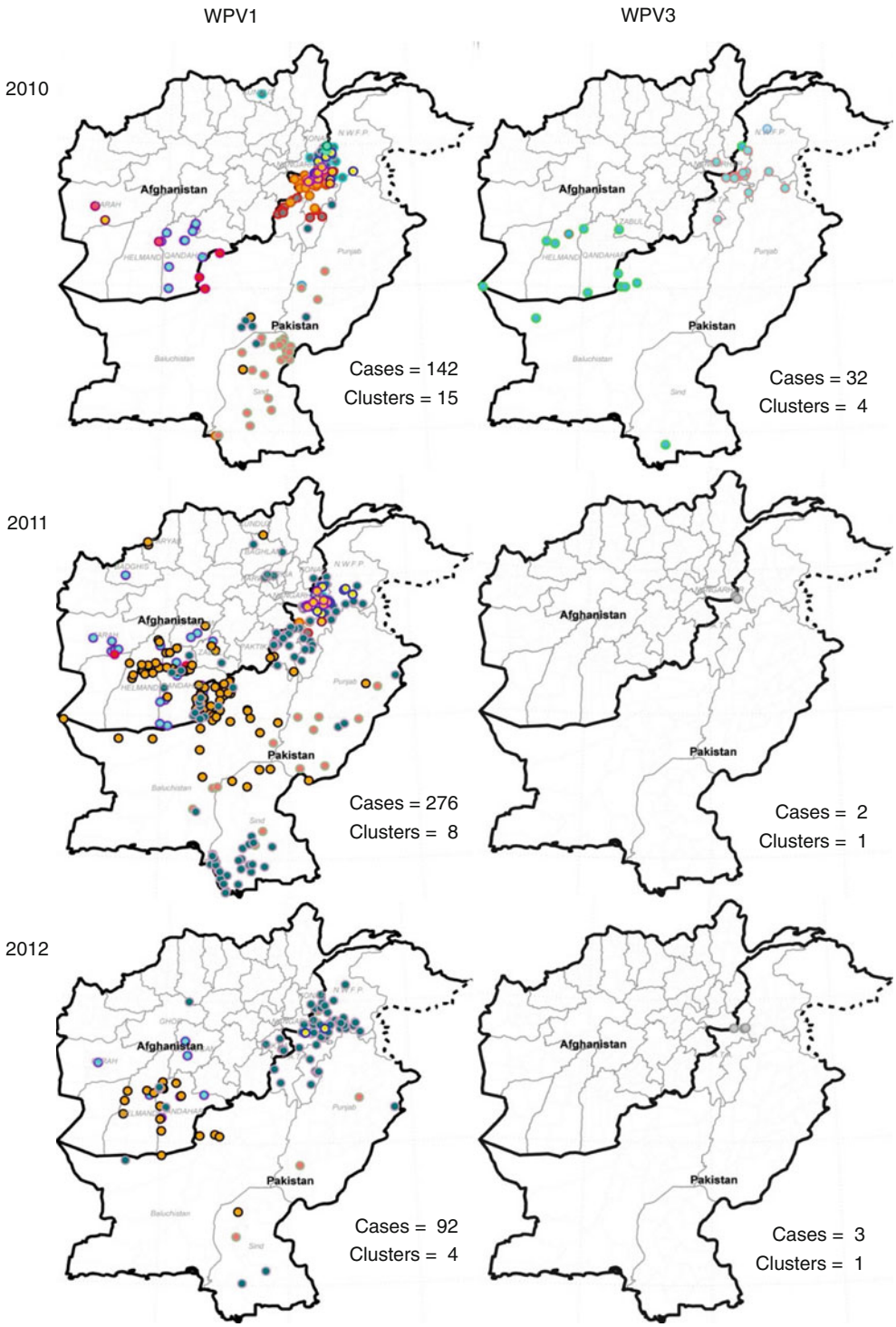

Fig. 13.12 Spot maps of genetic clusters based upon VP1 sequence relationships among isolates of wild poliovirus type 1 (*WPV1* left column) and wild poliovirus type 3 (*WPV3* right column), endemic to Pakistan and Afghanistan, 2010–2012. Isolates within a genetic cluster share ≥95 % VP1 nucleotide sequence identity, and the cluster count provides an indication of the genetic diversity of circulating wild polioviruses. Genetic clusters are color-coded to facilitate visualization of endemic reservoirs, transmission pathways (including cross-border transmission), and decline in genetic diversity. In 2011, poliovirus type 1 from southern Pakistan was imported into Xinjiang, China, and caused an outbreak of 21 cases [82]. (Map prepared by Elizabeth Henderson [CDC] based on sequence data produced by the Regional Polio Reference Laboratory at the National Institute of Health, Islamabad, Pakistan, and by CDC). As of April 2014, the last wild poliovirus type 3 isolate in Asia was from a child in the Federally Administered Tribal Area of Pakistan who had onset of AFP in April 2012

One measure of genetic diversity is the number of genetic "clusters," defined as isolates sharing ≥95 % VP1 nucleotide sequence identity (isolates within a genotype share ≥85 % VP1 nucleotide sequence identity [163]). Distinct clusters originate from different local endemic reservoirs, and in areas of sensitive surveillance, the disappearance of clusters within a genotype is an indication that source reservoirs within geographic area are being cleared of wild polioviruses. Just as the stepwise disappearance of genotypes is a measure of global progress toward eradication (Figs. 13.10 and 13.11), the stepwise disappearance of clusters is a measure of regional and local progress (Figs. 13.12, 13.13, and 13.14). Poliovirus genetic clusters are color-coded and mapped to facilitate visualization of patterns of circulation and to monitor progress toward eradication.

The number of wild poliovirus type 1 clusters in Pakistan and Afghanistan fell from 15 in 2010 to 4 in 2012 (Fig. 13.12).

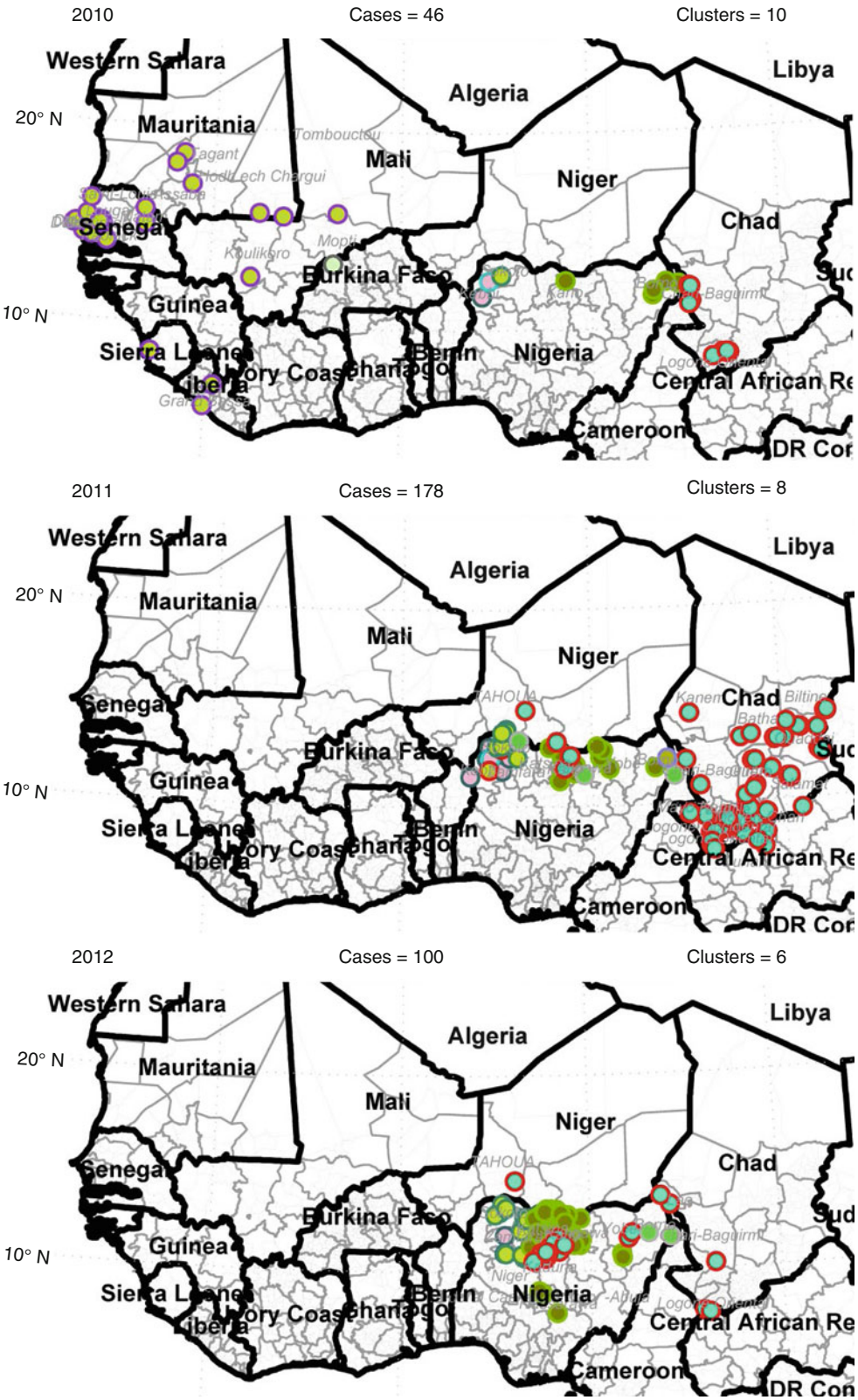

Fig. 13.13 Spot maps showing spread and retreat of genetic clusters of wild poliovirus type 1 from Nigerian reservoirs to neighboring countries in West and Central Africa, 2010–2012 (Map prepared by Elizabeth Henderson (CDC) based on sequence data produced by the Regional Polio Reference Laboratory at the National Institute for Communicable Diseases, Johannesburg, South Africa, and CDC). As of April 2014, the last wild poliovirus type 3 isolate in Asia was from a child in the Federally Administered Tribal Area of Pakistan who had onset of AFP in April 2012

Wild poliovirus type 3 clusters decreased from 4 in 2010 to 1 in 2012 (Fig. 13.12), and the last case associated with wild poliovirus type 3 in Pakistan (and in Asia; Fig. 13.2c) was reported in April 2012 [12]. By the end of 2012, reservoirs within Afghanistan appear to have been cleared of wild polioviruses.

Although progress in Nigeria has been less dramatic than in Pakistan and Afghanistan, wild poliovirus type 1 clusters

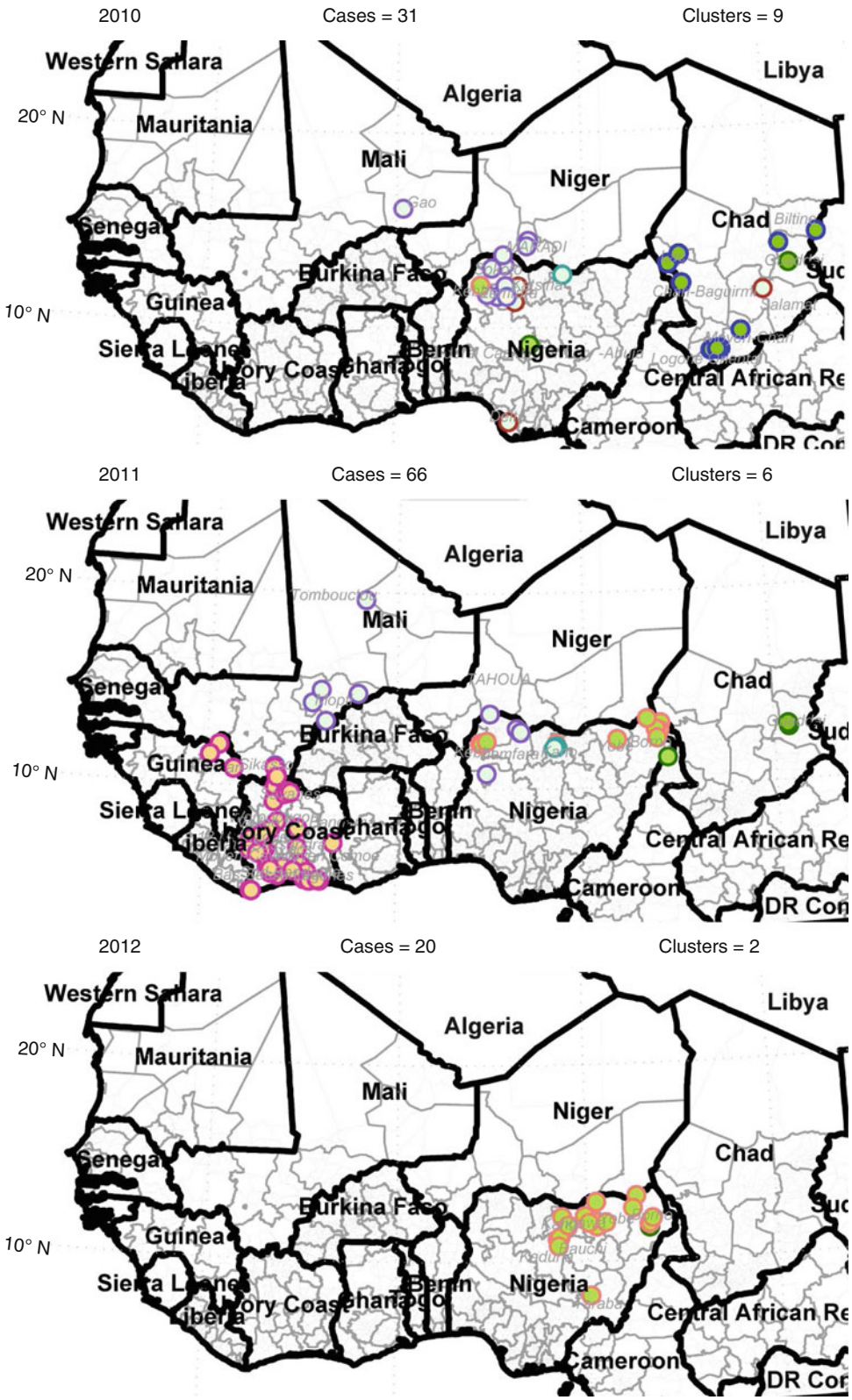

Fig. 13.14 Spot maps showing spread and retreat of genetic clusters of wild poliovirus type 3 from Nigerian reservoirs to neighboring countries in West and Central Africa, 2010–2012 (Map prepared by Elizabeth Henderson based on sequence data produced by the Regional Polio Reference Laboratory at the National Institute for Communicable Diseases, Johannesburg, South Africa, and CDC). As of April 2014, the last wild poliovirus type 3 isolate in the world was from a child in northeastern Nigeria who had onset of AFP in November 2012

Table 13.2 AFP and wild poliovirus cases by WHO region and globally, 2001–2012

	AFP cases (virologically confirmed wild poliovirus cases) reported[a]						
	WHO region[b]						
Year	AFR	AMR	EMR	EUR	SEAR	WPR	Global
2001	8,542 (69)	2,207 (0)	3,865 (143)	1,764 (3)[c]	10,612 (268)	6,529 (0)	33,519 (483)
2002	8,587 (208)	2,168 (0)	4,625 (110)	1,717 (0)	12,900 (1,600)	6,835 (0)	36,832 (1,918)
2003	8,181 (446)	2,229 (0)	5,290 (113)	1,529 (0)	11,289 (225)	6,397 (0)	34,915 (784)
2004	9,719 (934)	2,309 (0)	6,176 (187)	1,516 (0)	16,270 (134)	6,521 (0)	42,511 (1,255)
2005	11,683 (879)	2,213 (0)	8,849 (727)	1,479 (0)	31,530 (373)	6,680 (0)	62,434 (1,979)
2006	12,472 (1,189)	2,151 (0)	8,739 (107)	1,481 (0)	36,665 (701)	7,011 (0)	68,519 (1,997)
2007	12,080 (367)	2,111 (0)	9,394 (58)	1,449 (0)	46,124 (894)	6,237 (0)	77,395 (1,315)
2008	14,256 (912)	2,063 (0)	10,799 (174)	1,360 (0)	50,509 (565)	6,417 (0)	85,404 (1,651)
2009	15,127 (691)	1,873 (0)	10,611 (176)	1,363 (0)	54,962 (741)	6,291 (0)	90,227 (1,604)
2010	16,500 (657)	2,006 (0)	11,338 (169)	2,087 (478)[d]	60,456 (48)	6,401 (0)	98,788 (1,352)
2011	16,636 (350)	1,704 (0)	11,742 (278)	1,544 (0)	65,331 (1)	7,303 (21)[e]	104,260 (650)
2012	18,110 (128)	2,437 (0)	11,100 (95)	1,529 (0)	66,176 (0)	7,585 (0)	106,937 (223)

Source: WHO (http://apps.who.int/immunization_monitoring/en/diseases/poliomyelitis/afpextract.cfm). Starting in 2001, all wild poliovirus case counts were based on virologic confirmation by the GPLN

[a]cVDPV outbreaks not included

[b]WHO regions: *AFR* Africa, *AMR* Americas, *EMR* Eastern Mediterranean, *EUR* Europe, *SEAR* South-East Asia, *WPR* Western Pacific

[c]Importations of wild poliovirus type 1 into Bulgaria (from Pakistan) and Georgia (from India) [11]

[d]Outbreaks in Tajikistan, Turkmenistan, Kazakhstan, and Russian Federation following importation of wild poliovirus type 1 from India [178]

[e]Outbreak in Xinjiang, China, following importation of wild poliovirus type 1 from Pakistan [82]

fell from 10 in 2010 to 6 in 2012 (Fig. 13.13) and wild poliovirus type 3 clusters fell from 9 in 2010 to 2 in 2012 (Fig. 13.14). In addition, the surge of wild polioviruses from Nigeria was again rolled back between 2010 and 2012 (Figs. 13.13 and 13.14). In 2012, only Chad (5 cases in 2012 compared with 132 cases in 2011) and Niger (1 case in 2012 compared with 5 cases in 2011) had residual circulation of the wild poliovirus type 1 imported from Nigeria [12]. Although wild poliovirus transmission in Africa is localized to northern Nigeria, the risk for resurgence remains until immunization activities in key northern states are intensified [12].

Important factors in improving OPV coverage have been intensified community engagement, targeting mobile and migrant populations for immunization, and high-resolution mapping of underserved communities [12, 22]. An additional critical factor for eradication of wild polioviruses has been the widespread use of monovalent OPV type 1 (mOPV1) and mOPV3 in NIDs, SNIDs, and mop-ups. However, in countries where both wild poliovirus types 1 and 3 were co-circulating, balancing the use of mOPV1, mOPV3, and tOPV in NIDs and SNIDs was difficult. Priority was usually given to the use of mOPV1 because of the higher risk of wild poliovirus type 1 spread compared with wild poliovirus type 3 [10, 176, 177, 180, 181] and the greater risk of large outbreaks [181]. Routine OPV immunization exclusively uses tOPV, but coverage rates in the core reservoirs remain low [12], such that immunity gaps to types 2 and 3 could develop with near exclusive use of mOPV1 [447, 448]. The introduction of bivalent OPV (bOPV; types 1 and 3) [92, 449, 450] in late 2009 and early 2010 sharply reduced wild poliovirus type 3 circulation in areas where coverage rates in mass campaigns were high (Figs. 13.2c, 13.12, and 13.14) [12, 16, 82, 451]. However, a remaining challenge is to prevent widening immunity gaps to poliovirus type 2.

In 2012, more than two billion doses of OPV were administered to 448 million people, primarily to children <5 years of age in NIDs and SNIDs in 46 countries [12]. Only 223 cases of poliomyelitis associated with wild poliovirus infection were reported in 2012, an historic low, from a total of 106,937 reported cases of AFP, the most ever investigated (Table 13.2) [12]. The number of wild poliovirus cases in 2012 fell below the estimated number of worldwide VAPP cases (250–500) (Fig. 13.2a). For the first time, the risk of poliomyelitis from the use of OPV exceeded the annual case burden from wild poliovirus infection.

10.8 Emergence of Vaccine-Derived Polioviruses (VDPVs), 2000–2012

In principle, all clinical and environmental vaccine-related poliovirus isolates are VDPVs. However, as described in section "Categories of poliovirus isolates", for the purposes of poliovirus surveillance, vaccine-related isolates have been classified into two broad categories: (1) OPV-like isolates, which have limited divergence from their parental OPV strains and are ubiquitous wherever OPV is used, and (2) VDPV isolates, whose higher level of VP1 sequence divergence from their parental OPV strains (>1 % [types 1 and 3] or >0.6 % [type 2]) indicates prolonged replication (or

transmission) of the vaccine virus. VDPVs are further categorized as (1) circulating VDPVs (cVDPVs), (2) immunodeficiency-associated VDPVs (iVDPVs), and 3) ambiguous VDPVs (aVDPVs) [133].

Although the vast majority of vaccine-related isolates are OPV-like, VDPVs are of particular interest because of their implications for current and future strategies for global polio eradication [22, 94, 133, 428, 450]. VDPVs can cause paralytic polio in humans and have the potential for sustained circulation. The clinical signs and severity of paralysis associated with VDPV and wild poliovirus infections are indistinguishable. VDPVs resemble wild polioviruses phenotypically [133] and differ from the majority of vaccine-related poliovirus isolates by having genetic properties consistent with prolonged replication or transmission. Because poliovirus genomes evolve at a rate of approximately 1 % per year (Sect. 3.6.3), vaccine-related viruses that differ from the corresponding OPV strain by >1 % of VP1 nucleotide positions are estimated to have replicated for at least 1 year in one or more persons after administration of an OPV dose. This is substantially longer than the normal period of 4–6 weeks of vaccine virus replication in an OPV recipient [321]. The demarcation for type 2 VDPVs was lowered to 0.6 % to increase sensitivity for early detection of type 2 cVDPV outbreaks [133, 168, 452].

It seems likely that many OPV-like isolates have recovered the capacity for higher neurovirulence and possibly increased transmissibility. The small number of substitutions controlling neurovirulence (Sect. 9.1.3; Fig. 13.9) was found to have reverted among many OPV-like isolates, especially among isolates of types 2 and 3 [382, 388, 393]. Because the critical attenuating mutations in the 5′-UTRs of the Sabin strains also affect fitness for virus replication in the human intestine [393], it appears possible that revertants at these sites would have a higher fitness for person-to-person spread. However, spread is normally limited by high OPV coverage, and the VDPVs represent viruses whose potentials for prolonged replication or transmission have been clearly actualized, as demonstrated by their genetic prioperties.

The three categories of VDPVs differ in their public health importance. Circulating VDPVs pose the same public health threat as wild polioviruses because they have recovered the biological properties of wild polioviruses, have the potential to circulate for years in settings where poliovirus vaccination coverage to prevent that particular type is low, and require the same control measures. Immunodeficiency-associated VDPVs may be excreted by persons with certain primary immunodeficiencies for many (>10) years with no apparent paralytic signs [223, 453]. Persons infected with iVDPVs without paralysis are at risk of developing paralytic poliomyelitis [186, 223, 454] and may infect others with poliovirus, presenting the potential risk of outbreaks in areas with low poliovirus vaccine coverage [132]. Ambiguous VDPVs are heterogeneous: some represent the initial isolates from cVDPV outbreaks, whereas others, such as highly divergent aVDPVs detected in sewage, are probably iVDPVs from inapparent chronic infections.

10.8.1 Outbreaks of Circulating VDPVs (cVDPVs)

Among the two well-defined categories of VDPVs, cVDPVs are of the greatest current public health concern [22, 450, 455]. Since 2000, cVDPV outbreaks have occurred in 18 countries, with the large majority (86.6 %) of reported cases associated with type 2 (Fig. 13.15; Table 13.3). Type 1 cVDPVs were associated with outbreaks in Hispaniola in 2000–2001 and Indonesia in 2005 [166, 183]. In contrast, type 3 cVDPV outbreaks are rare, accounting for only 1.7 % of known cVDPV cases, an unexpected finding because the type 3 OPV strain is a major contributor to VAPP in OPV recipients [94]. Because the case/infection ratio for poliovirus type 2 infections is low (Sect. 3.4), the number of type 2 cVDPV infections worldwide since 2000 is estimated at nearly one million [168, 461].

During 2005–2012, a polio outbreak of 411 cases associated with cVDPV2 was reported from 11 northern and 3 north central states of Nigeria (Fig. 13.16; Table 13.3) [447, 448, 455]. The outbreak peaked at 153 cases in 2009, but 27 cases were detected in 2010, 35 cases in 2011, and 7 cases in 2012. Genetic analysis resolved the outbreak into >20 independent type 2 VDPV emergences that occurred during 2004–2012, at least 7 of which established circulating lineages [168, 448]. The outbreak occurred in states where routine immunization coverage with tOPV is low and NIDs and SNIDs using tOPV were infrequent [447, 448]. Spread of the type 2 cVDPV from northern Nigeria has been very limited, with only six cases in Niger from multiple importations and one case in Chad from an importation in 2010 (Fig. 13.16; Table 13.3). The limited spread may indicate that surrounding areas have higher levels of population immunity to poliovirus type 2 (because of the high immunogenicity of Sabin 2 and its higher tendency to infect OPV contacts) and that poliovirus type 2 may have an intrinsically lower capacity to spread than types 1 and 3 (Figs. 13.13 and 13.14).

Most of the other recent outbreaks have also been associated with type 2 cVDPVs (Table 13.3), several of which (e.g., Afghanistan, the Democratic Republic of Congo, India, Pakistan, and Yemen) were associated with multiple independent emergences [133]. Other type 2 cVDPVs have emerged successively in the same geographic area [459]. In several settings, this reflects the continued weakness in routine immunization with tOPV and the extensive use of mOPV1 and bOPV in mass campaigns. However, it also reflects the greater tendency of the Sabin 2 OPV strain to revert and spread to contacts [94]. Three separate type 3 cVDPV emergences were detected in Ethiopia in 2009–2010, and a single type 1 cVDPV emergence was detected in Mozambique in 2011 (Table 13.3).

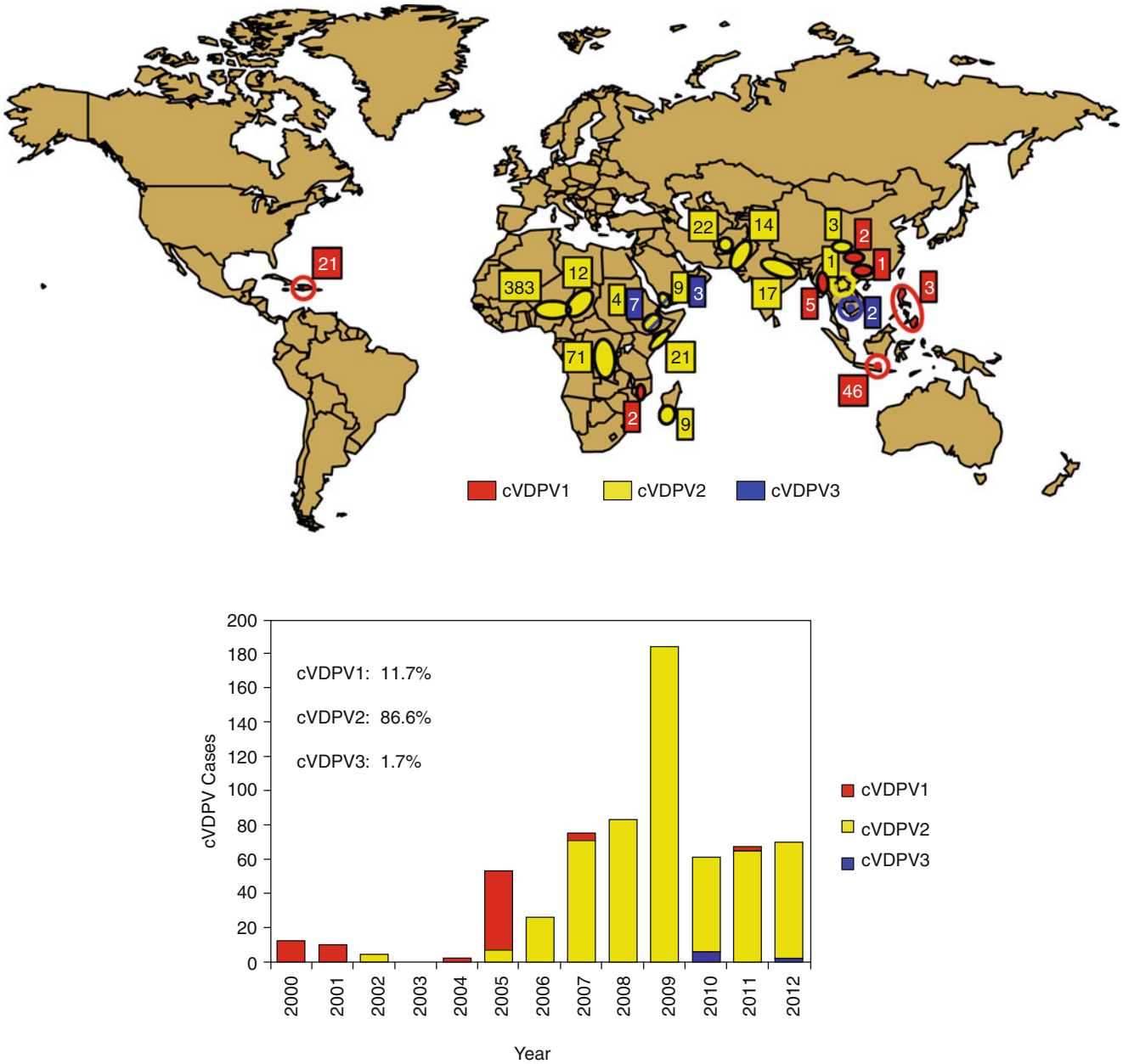

Fig. 13.15 Circulating vaccine-derived poliovirus (cVDPV) outbreaks, 2000–2012. Map: Location of cVDPV outbreaks, color-coded by serotype (*red* cVDPV type 1 [cVDPV1], *yellow* cVDPV2, *blue* cVDPV3). The independent emergences of cVDPV2 and cVDPV3 in Ethiopia and Yemen are indicated by *yellow* and *blue* diagonal patterns. Apart from the 2000–2001 cVDPV1 outbreak on the island of Hispaniola (Haiti and the Dominican Republic) and the limited spread of the cVDPV2 from Nigeria to Niger and Chad, all other outbreaks are independent events. Some countries had successive (e.g., Madagascar) or concurrent (e.g., Nigeria and D. R. Congo) cVDPV2 outbreaks. *Bar chart*: cases associated with cVDPV outbreaks, 2000–2012, color-coded by serotype (Modified from reference Kew [10])

In 2013, cVDPV2 from the outbreak in Chad continued with 4 new cases and spread to Cameroon (4 cases), Niger (1 case), and Nigeria (4 cases). The cVDPV2 outbreak in Pakistan continued with 47 new cases and spread to Afghanistan (4 cases in 2012–2013).

Key risk factors for cVDPV emergence and spread are (1) development of immunity gaps arising from low OPV coverage, (2) prior elimination of the corresponding wild poliovirus serotype, (3) emphasis on use mOPV and bOPV in NIDs and SNIDs [133, 447], and (4) insensitive AFP surveillance. Many of these factors exist in areas of insecurity and where rates of routine tOPV coverage remain low, such as in parts of northern Nigeria, Somalia, and Yemen. In this context, type 2 VDPVs present the greatest threat for emergence [133], and routine immunization should be strengthened and, for the immediate future, whenever possible regular

Table 13.3 Circulating vaccine-derived poliovirus (cVDPV) outbreaks, 2000–2012

Country	Sero-type	Years	Reported cases	% VP1 divergence	Estimated independent emergences	References
Haiti/Dominican Republic	1	2000–2001	21	1.9–2.6	1	[166]
Madagascar	2	2001–2002	4	2.5–3.0	1	[187]
Philippines	1	2001	3	3.1–3.5	1	[189]
China	1	2004	2	1.0–1.2	1	[456]
Laos	2	2004–2005	1 (2 contacts)	1.1–1.2	1	[94]
Cambodia	3	2005–2006	2	1.9–2.4	1	[457]
D R Congo	2	2005–2009	7	1.0	>10	[458]
Indonesia	1	2005	46	1.1–3.0	1	[183]
Madagascar	2	2005	5	1.1–2.7	1	[459]
Nigeria[a]	2	2005–2012	385	0.7–7.2	>20	[168, 447, 448]
China	1	2006	1 (6 contacts)	1.4–2.2	1	[190]
Myanmar	1	2006–2007	5	1.5–2.2	1	[457]
D R Congo	2	2008–2012	64	0.7–3.5	>10	[133, 455]
Ethiopia	2	2008–2009	4	1.3–3.1	2	[458]
Somalia[b]	2	2008–	18	0.7–4.0	4	[133, 455]
Ethiopia	3	2009–2010	7	1.1–1.2	1	[455]
India	2	2009–2010	17	1.3–1.6	5	[455]
Afghanistan	2	2010–	22	0.9–5.5	2	[455]
Madagascar	2	2011	0 (2 contacts)	3.3–3.7	1	[133]
Mozambique	1	2011	2	3.0–4.3	1	[133, 455]
China	2	2011–2012	3	0.7–1.8	1	[133]
Yemen	2	2011	9	0.6–1.6	4	[133]
Yemen	3	2011–	3	2.0–3.0	1	[460]
Chad	2	2012–	12	0.7–2.1	2	[460]
Pakistan[c]	2	2012–	14	0.7–2.9	2	[460]

[a]cVDPV2 from Nigeria was exported to Niger (2006, 2009–2012; 7 total cases) and Chad (2010 and 2012; 2 cases)
[b]cVDPV2 from Somalia was exported to Kenya (2012; 3 cases)
[c]cVDPV2 from Pakistan was exported to Afghanistan (2012; 3 cases)

immunization campaigns using tOPV should be conducted to close any immunity gaps.

All of the cVDPVs shown in Table 13.3 except those from China have vaccine/non-vaccine recombinant genomes, which are very rare among OPV-like and iVDPV isolates [94]. Recombination with other species-C enteroviruses frequently occurs during wild virus circulation and may be interpreted as an indication of person-to-person transmission. Whether recombination facilitates cVDPV emergence is unclear, because type 1 cVDPVs from China had nonrecombinant genomes [456] and recombination continues after emergence, similar to what is observed with wild polioviruses [94].

10.8.2 Immunodeficiency-Associated VDPVs (iVDPVs)

It has long been recognized that patients with primary B-cell immunodeficiencies (defects in antibody production) could become chronically infected when exposed to OPV [353]. Unambiguous demonstration that vaccine-related poliovirus isolates from immunodeficient patients had unusual sequence properties awaited the application of molecular tools, such as oligonucleotide fingerprinting [186, 462] and genomic sequencing [165, 186, 454], to poliovirus diagnostics. The extent of sequence divergence is correlated with the duration of the prolonged infection [165, 173, 186, 223, 454]. Not all isolates from immunodeficient patients are classified as iVDPVs. Some isolates are from specimens taken early in the prolonged infection and no subsequent specimens were taken. In other situations, either the prolonged infections had resolved spontaneously or the patient died from complications of the immunodeficiency (including fatal poliomyelitis) [354]. Prolonged iVDPV infections are independent events, and the isolates obtained from such infections trace separate pathways of divergence from the original OPV strains.

Since the introduction of OPV in 1961, >70 persons with primary immunodeficiencies have been found worldwide to be excreting iVDPVs; the majority of these immunodeficiencies were detected only after onset of AFP (Table 13.4). The extent of sequence divergence is correlated with the duration of the prolonged infection [165, 173, 186, 223, 454]. Four of the iVDPV isolates are highly divergent (>10 % VP1 sequence divergence from the parental OPV strain),

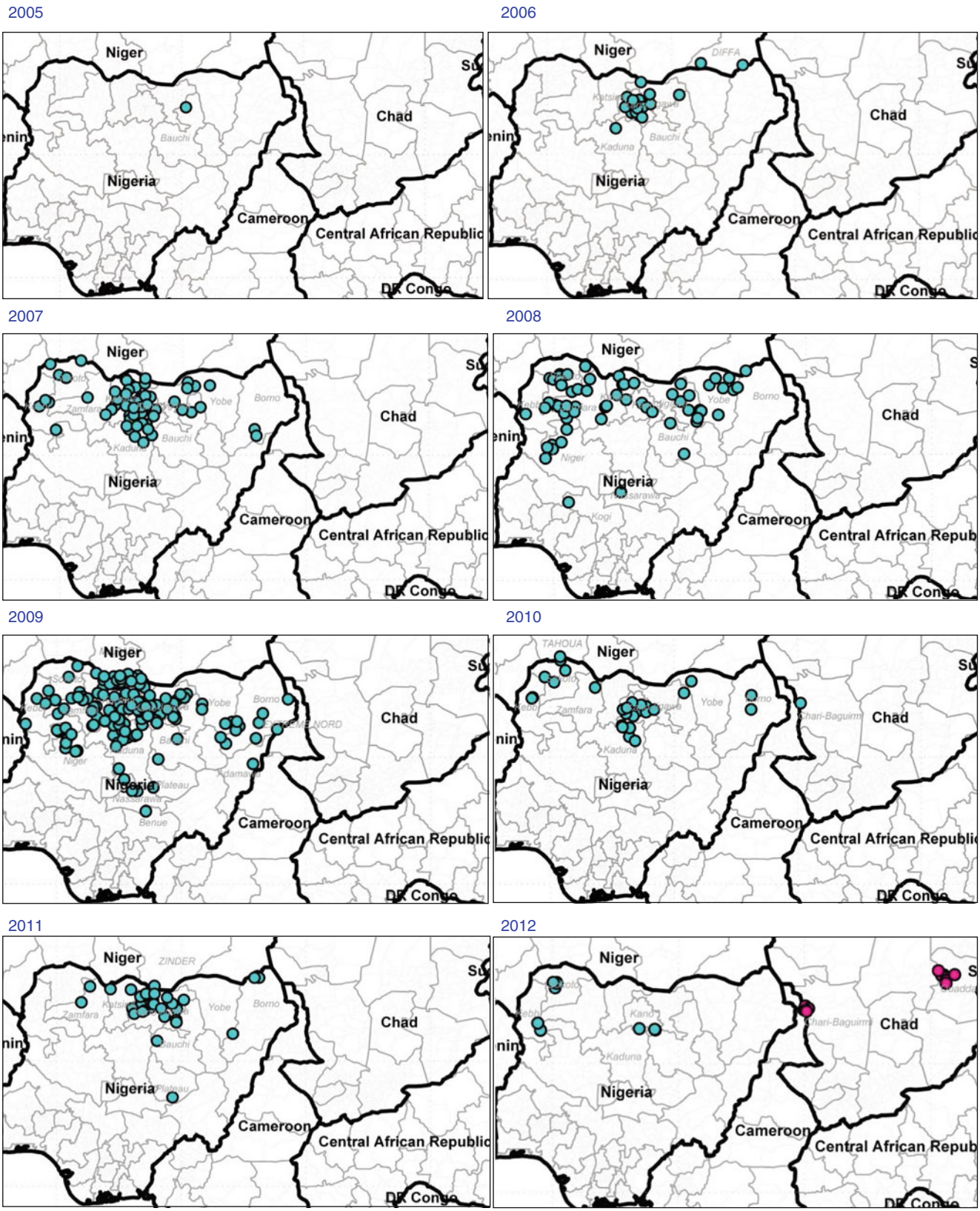

Fig. 13.16 Emergence and spread of type 2 cVDPV in Nigeria, 2005–2012. At least 20 independent emergences of type 2 cVDPVs (*green dots*) occurred in Nigeria between 2005 and 2012. Independent type 2 cVDPV emergences that occurred in Chad in 2012 are represented by *purple dots* (Map prepared by Elizabeth Henderson based on sequence data produced by CDC)

Table 13.4 Documented prolonged iVDPV excretors, 1962–2012

Country	Year detected	Immune deficiency[a]	Paralysis	Serotype	Maximum %VP1 divergence from Sabin	References
UK	1962	HGG	No	1	2.5	[463–465]
UK	1962	HGG	No	3	2.3	[165, 466]
Japan	1977	XLA	Yes	2	–	[462]
USA	1980	AGG	Yes	2	1.3	[354]
USA	1981	**CVID**	Yes	1	**10.0**	[186]
USA	1986	XLA	Yes	2	2.0	[467]
USA	1986	**CVID**	No	1	**5.4**	[467]
	1992			2	**11.8**	
UK	1987	**CVID**	No	2	**4.1**	[464, 468]
USA	1989	AGG	Yes	1	1.1	[354]
Germany	1990	**CVID**	Yes	1	**8.3**	[454]
USA	1990	SCID	Yes	2	1.9	[354]
USA	1991	**CVID**	Yes	2	**1.4**	[354]
Iran	1995	HGG	Yes	2	2.2	[469]
UK	1995	**CVID**	No	2	**12.9**	[453, 464]
USA	1995	SCID	Yes	2	2.1	[354]
Argentina	1998	XLA	Yes	1	2.8	[470]
Germany	2000	**CVID**	Yes	1	**3.5**	[467]
UK	2000	**CVID**	No	2	**6.3**	[464]
Taiwan	2001	**CVID**	Yes	1	**3.5**	[173]
Kazakhstan	2002	HGG	Yes	2	2.3	[467]
Kuwait	2002	MHC II def	No	2	2.0	[467]
UK	2002	ICF syndrome	No	2	2.5	[464]
Peru	2003	AGG	Yes	2	1.2	[467, 471]
Thailand	2003	HGG	Yes	2	2.2	[472]
China	2005	XLA	Yes	2; 3	4.2; 3.9	[457, 467]
Iran	2005	MHC II def	Yes	1; 2	1.1; 1.4	[469, 473]
Morocco[b]	2005	SCID	Yes	2	4	[467]
Syria	2005	HGG	Yes	2	1.3	[457, 467]
USA	2005	SCID	Yes	1	>2.3	[132]
Iran	2006	SCID	Yes	2	1.7	[469]
Iran	2006	XLA	Yes	3	2.1	[192, 469]
Kuwait	2006	SCID	Yes	3	1.2	[457]
Syria	2006	HCI	Yes	2	2.2	[457]
Tunisia[c]	2006	SCID	No	2	2.0	[457, 467]
Belarus	2007	HGG	Yes	2	1.9	[195]
Egypt	2007	SCID	Yes	3	1.1	[457]
Iran	2007	SCID	Yes	1; 2	1.7; 1.7	[469]
Iran	2007	XLA	Yes	3	2.0	[469]
Russia	2007	HGG	Yes	1	1.0	[195]
Iran	2008	XLA	Yes	2	1.2	[195]
Argentina	2009	HGG	Yes	1	3.8	[458]
Colombia	2009	AGG	Yes	2	1.5	[455]
India	2009	**CVID**	Yes	1	**6.2**	[455]
USA	2009	**CVID**	Yes	2	**12.3**	[223]
Algeria	2010	HLA-DR	Yes	2	1.8	[455]
China	2011	PID	Yes	3	2.0	[455]
China	2011	PID	Yes	2	1.9	[455]
India	2010	PID	Yes	2	1.6	[455]
Iraq	2010	PID	Yes	2	1.2	[455]

Table 13.4 (continued)

Country	Year detected	Immune deficiency[a]	Paralysis	Serotype	Maximum %VP1 divergence from Sabin	References
Sri Lanka	2010	SCID	No	2	1.3	[455, 474]
Egypt	2011	PID	No	2	1.4	[133]
Egypt	2011	AGG	Yes	1	2.1	[133]
Egypt	2011	PID	Yes	3	4.2	[133]
Iran	2011	SCID	Yes	2	2.0	[133]
Iran	2011	XLA	Yes	2	2.7	[133]
Iran	2011	PID	Yes	1; 2	2.7; 3.3	[133]
South Africa	2011	AGG	Yes	3	1.9	[475]
Sri Lanka	2011	**CVID**	Yes	3	**1.9**	[133, 474]
Turkey	2011	PID	No	2	1.8	[455]
West Bank	2011	SCID	No	2	1.2	[133]
China	2012	**CVID**	Yes	2; 3	**1.4**; **1.6**	[133]
Egypt	2012	PID	No	2	1.0	[460]
India	2012	HGG	Yes	2	2.8	[133]
Iran	2012	PID	Yes	2	1.4	[133]
Iran	2012	PID	Yes	2	1.1	[460]
Iraq	2012	PID	Yes	2	1.0	[460]

[a]*Ab deficiency* antibody deficiency, *AGG* agammaglobulinemia, *CVID* common variable immunodeficiency (**CVID** is shown in **bold font** because it is most frequently associated with chronic iVDPV excretion and highly divergent iVDPV isolates), *HCI* humoral and cellular immunodeficiency, *HGG* hypogammaglobulinemia, *HLA-DR* HLA-DR-associated immunodeficiency, *ICF* immunodeficiency-centromeric instability-facial abnormalities, *MHC II def* major histocompatability complex class II molecule deficiency, *SCID* severe combined immunodeficiency, *XLA* X-linked agammaglobulinemia
[b]Patient treated in Spain
[c]Patient treated in France

suggesting that the chronic poliovirus infections had persisted for ~10 years (Table 13.4). Patients with the most divergent isolates and the most prolonged (chronic) infections have common variable immunodeficiency (CVID), a group of late-onset immunodeficiencies that have multiple etiologies [476]. CVID is the most prevalent (~1 in 50,000) [476] of the primary immunodeficiencies, but only a small proportion of CVID patients exposed to OPV become chronically infected with iVDPVs.

Unlike cVDPV outbreaks, prolonged iVDPV infections cannot be prevented by high OPV coverage. Persons with prolonged iVDPV infections can transmit poliovirus to others, raising the risk for VDPV circulation in settings of low population immunity to the corresponding poliovirus serotype. So far, all reports of persistent iVDPV infections have been from countries with high to intermediate levels of development, where the rates of vaccine coverage are high and where the survival times of immunodeficient patients may be extended by treatment with intravenous immune globulin. The survival rates for persons with primary immunodeficiencies are probably very low in developing countries at highest risk for poliovirus spread. The population of prolonged iVDPV excretors is declining in developed countries because some patients have died (Table 13.4), some have cleared their infections, and no new iVDPV infections have been found in countries that have shifted to IPV. However, the prevalence of long-term iVDPV excretors might be higher than suggested by existing surveillance of persons with primary immunodeficiencies, and several aVDPVs have properties closely resembling iVDPVs (Sect. 10.8.3). The development of treatment for prolonged iVDPV infections might facilitate detection of and access to those with prolonged infections [133, 477].

Type 2 iVDPVs are the most prevalent (63 %), followed by type 1 (22 %) and type 3 (15 %). Some patients have heterotypic iVDPV infections, with the isolates having similar degrees of divergence from the parental OPV strains, consistent with a common OPV dose initiating the infections (Table 13.4). In addition to the occasional heterotypic infections, iVDPV isolates generally have distinguishing genetic properties, including heterogeneity at sites of nucleotide variability within serotypes (indicative of mixed virus populations), extensive antigenic variability, and either nonrecombinant or vaccine/vaccine recombinant genomes [94, 133].

10.8.3 Ambiguous VDPVs (aVDPVs)

Unlike the well-defined cVDPV and iVDPV categories, aVDPVs are a heterogeneous collection of isolates (Tables 13.5 and 13.6). Some aVDPVs have diverged by just over 1 % from the parental OPV strains, have no detected progeny, and may reflect the extremes of the usual distribution of

Table 13.5 Other vaccine-derived poliovirus (VDPV) with genetic evidence of community circulation, 1965–2012

Country	Serotype	Years	Reported cases (contacts)	% VP1 divergence	Estimated independent emergences	Reference
Byelorussia	2	1965–1966	0 (9)	1.6–2.1	3	[156]
Romania	1	2002	1 (7)	1.1–1.3	1	[479]
Laos	2	2004	1 (1)	1.1	1	[94]
United States	1	2005	0 (5)	2.3–2.6	1	[132]
China	1	2006	1 (6)	1.4–2.2	1	[190]
Madagascar	2	2011	0 (2)	3.3–3.7	1	[133]

Table 13.6 Highly divergent aVDPVs from the environment, 1998–2012

Country	Years isolated	Serotype	% VP1 divergence	Reference
Israel	2009–2012	1	8.0–13.8	[117, 133, 455, 460]
	1998–2012	2[a]	6.6–16.2	
	2006–2011	2[b]	10.7–11.2	
Finland	2008–2012	1	12.4–14.0	[133, 455, 460, 480]
	2008–2012	2	13.0–15.5	
	2008–2010	3	13.7–14.6	
Slovakia	2003–2004	2	13.4–15.0	[119]
Estonia	2008–2012	2	13.5–16.2	[455, 460]
	2002–2008	3	12.6–14.9	

[a]Group 1 aVDPV2 isolates from Israel are genetically distinct from Group 2 isolates, and are probably derived from two different chronic excretors
[b]Group 2

vaccine-related variants in countries using OPV [133]. Detection of other aVDPVs have foreshadowed subsequent cVDPV outbreaks [133, 478], others indicate limited person-to-person spread of OPV virus in small communities with gaps in OPV coverage [94, 132, 156, 190, 479], whereas others appear to indicate more prolonged circulation (Table 13.5) [133]. Most of these latter aVDPVs have vaccine/non-vaccine recombinant genomes typical of cVDPVs.

Many of the aVDPVs are isolated from the environment, and some have genetic properties (mixed VDPV populations, extensive antigenic changes, and nonrecombinant or vaccine/vaccine recombinant genomes) typical of iVDPVs. Highly divergent aVDPVs have been detected in Israel [117], Estonia [455], Slovakia [119], and Finland [120], countries with high rates of polio vaccination coverage that are unlikely to have extensive VDPV transmission (Table 13.6). The sequence divergence of some of these isolates is consistent with more than 15 years of replication from the original initiating OPV dose. It is likely that the sources of these viruses are immunodeficient persons with asymptomatic VDPV infections, although the infected persons remain to be identified.

10.9 Certification of Polio Eradication

The WHO GPEI has established a formal process for the certification of polio eradication to ensure that future immunization policy decisions are based on the best available evidence [481]. The certification process for polio is modeled on the International Smallpox Certification Commission, which certified in December 1979 that smallpox had been eradicated [482]. However, polio certification differs in important ways from the smallpox model. PAHO established its Regional Certification Commission (RCC) in 1990 and certified regional eradication of indigenous wild polioviruses in 1994 [112, 430], setting the standard for certification by individual regions and implicitly considering genetic evidence to distinguish indigenous wild polioviruses from imported virus. Because of the high proportion of inapparent wild poliovirus infections, regional certification requires a period of at least 3 years of sensitive surveillance since the last reported case associated with indigenous wild poliovirus. Small outbreaks from imported virus occurred on the eve of certification in the Western Pacific (in China in 1999 [483, 484]) and in Europe (in Bulgaria [485] and Georgia [11, 431] in 2001), but they were quickly controlled and followed by intensive surveillance. The certification of eradication of indigenous wild polioviruses by three WHO regions—the Americas in 1994 [112, 430], the Western Pacific in 2000 [486], and Europe in 2002 [431]—has stood the test of time. No indigenous wild poliovirus circulation has been detected in these regions post-certification. Cases and outbreaks associated with imported wild polioviruses and cVDPVs (in addition to cases of VAPP) have occurred subsequently (Sects. 10.6 and 10.8.1), but circulation has been promptly stopped by effective outbreak control measures [12, 82, 166, 178, 189, 190, 456]. The South-East Asia Region was certified by the RCC as polio-free in March 2014

(http://www.searo.who.int/entity/campaigns/polio-certification/en/). Key to certification was the observation that >3 years have passed since the last wild poliovirus case in India where surveillance has been maintained at high levels of sensitivity [12]. Similarly, documentation in all countries except Pakistan and Afghanistan has been accepted by the Eastern Mediterranean RCC. Several countries in the African Region have also prepared detailed documentation of polio-free status [22].

10.9.1 The Certification Process

Review of documentation to assess polio-free status is organized on three tiers [481]. National Certification Committees (NCCs) meet to critically assess national data and prepare detailed reports to Regional Certification Commissions (RCCs), who in turn report to the Global Certification Commission (GCC). The NCCs, RCCs, and GCC meet on a regular basis, and in the regions already certified as free of poliovirus circulation, the RCCs working with NCCs are primarily responsible to oversee and support activities to maintain the polio-free status. The NCC reports contain current information on the following: (1) national demographic data, including high-risk populations, (2) poliovirus vaccine coverage and any changes in vaccine policy, (3) surveillance data and indicators of surveillance sensitivity, (4) descriptions of any poliomyelitis cases, (5) information on the performance of laboratories serving each country, and (6) progress toward poliovirus containment. NCC, RCC, and GCC members are charged to serve as independent experts. Following certification of all WHO regions, the GCC will review all RCC reports and other relevant information to determine when the world can be declared free of all poliovirus circulation [481]. Special provisions are planned to recognize the global eradication of wild poliovirus type 2 (Sect. 11.2) [22]. An important prerequisite for certification is poliovirus containment.

10.9.2 Laboratory Containment of Polioviruses

Containment is integral to poliovirus eradication [487, 488]. Unlike smallpox, where very few facilities stored materials containing (virus stocks) or potentially containing (clinical specimens) smallpox virus (variola), many facilities worldwide store materials containing polioviruses. The purpose of containment is to minimize, as much as possible, the risk of reintroduction into the community of polioviruses stored in laboratories and vaccine production facilities. In 1998, the GCC approved the *WHO Global Action Plan for Laboratory Containment of Wild Polioviruses* [487] and subsequently established that satisfactory completion of containment activities be a requirement for regional certification. Containment will be implemented in three phases. During the Laboratory Inventory and Survey Phase (Phase 1) countries will (1) conduct national surveys of all biomedical laboratories to identify those storing materials containing (poliovirus stocks) or potentially containing (clinical specimens or environmental samples taken at locations during times of known poliovirus circulation) wild and vaccine-derived polioviruses, (2) encourage destruction of all unneeded materials, (3) develop inventories of laboratories retaining these materials and report to the RCC, (4) encourage implementation of biosafety level 2 (BSL-2) procedures in all enterovirus laboratories, and (5) plan for Phase 2. The Global Eradication Phase (Phase 2) will begin 1 year after detection of the last wild poliovirus or cVDPV at which time countries will (1) notify biomedical laboratories that wild poliovirus and cVDPV transmission has been stopped; (2) require laboratories on national inventories to select among the three options to (a) render materials noninfectious, (b) transfer all materials to high containment facilities, or (c) implement the appropriate laboratory procedures (BSL-2/polio or BSL-3/polio); and (3) document that all containment requirements have been met for global certification [487]. The Post-Global Certification Phase (Phase 3) was developed in response to the WHO goal of stopping all OPV use post-certification and proposes to minimize facility-associated poliovirus risk by destruction of both wild and vaccine-related polioviruses in all facilities, except for an absolute minimum needed for vaccine production, quality control, reference, and research [22].

Phase 1 containment was completed in the Americas several years after certification of polio-free status. In the United States, among the 105,356 laboratories surveyed, 180 laboratories in 122 institutions were found to be storing materials containing or potentially containing wild poliovirus; 87 held wild poliovirus stocks, 56 stored materials potentially containing wild polioviruses, and 37 stored both [489]. Europe completed Phase 1 containment in 2006 [96]. Among the 55,748 biomedical facilities surveyed, 265 laboratories in 164 institutions were found to be storing materials containing or potentially containing wild poliovirus; 116 held wild poliovirus stocks and 149 stored materials potentially containing wild polioviruses. In 20 European countries, 1 or more laboratories reported destroying all previously stored wild poliovirus materials during the survey. The Western Pacific Region completed Phase 1 containment in 2009 [490]. Of the 77,260 biomedical facilities surveyed, 45 laboratories (27 of which are members of the GPLN) retained materials containing or potentially containing wild polioviruses. Surveys have been completed in 155 of the 194 WHO member states, with the remaining surveys to be completed in countries in sub-Saharan Africa (these countries are thought unlikely to have many facilities with wild poliovirus materials). Worldwide ~550 facilities, including 6 IPV manufacturers, have been found to be storing wild poliovirus-infectious materials or potentially infectious materials [22]. VDPVs are stored in GPLN laboratories and a small number of collaborating laboratories.

11 Unresolved Problems

The immediate and crucial unresolved problem is to finish the task of eradicating wild polioviruses from the last remaining reservoir communities. The second unresolved problem is implementation of the "polio endgame" to ensure that the promise of polio eradication is forever secured. The third challenge is to build upon the legacy of polio eradication.

11.1 Eradication of Wild Polioviruses in Last Global Reservoirs

In May 2012, the WHA declared that polio eradication was a "programmatic emergency for global public health" and called for intensification of eradication activities in the remaining reservoir areas [22]. A Global Emergency Action Plan and National Emergency Action Plans (NEAPs) were developed and implemented in the remaining three reservoir countries [22]. All three countries face challenges of conflict and insecurity, as well as underperformance of immunization activities in high-risk areas. Afghanistan and Pakistan made clear improvements in 2012, but the pace of eradication has been slower in northern Nigeria.

11.1.1 Afghanistan

Polio has been controlled in most of Afghanistan except for the southern region, where intense armed conflict continues. The last case associated with wild poliovirus type 3 had onset in April 2010. Wild poliovirus type 1 had spread from the southern region provinces of Kandahar and Helmand in 2012 (Fig. 13.12), but the only 2013 cases were associated with wild poliovirus type 1 imported across the northeastern border from reservoirs in the insecure Tribal Areas of Pakistan. Only 2–5 % of children in southern Afghanistan were inaccessible in 2012 compared with 6–21 % in 2010 and 2011 [184]. In 2012, 65 % of case-patients were underimmunized and 35 % had received no OPV dose. The Afghanistan NEAP calls for reaching missed children through (1) improved detailed planning of mass campaigns; (2) better selection, training, monitoring, and support of vaccination teams; (3) enhanced community engagement; (4) robust response to poliomyelitis outbreaks; (5) expanded field staff in high-risk areas; and (6) use of permanent polio teams of trusted local people in insecure areas who visit homes and immunize children on a regular basis [22, 184].

11.1.2 Pakistan

Pakistan made dramatic progress in 2012 with implementation of the NEAP. Wild poliovirus type 3 was highly localized to the tribal areas in 2012 and was last detected in April 2012 from an AFP case, with no subsequent detections in the environment (Fig. 13.12) [184]. Several wild poliovirus type 1 reservoirs have been cleared and genetic diversity continues to decline. In recent years, >70 % of cases have been in the Pashtun minority, many of whom migrate widely within Pakistan and into Afghanistan. The NEAP focuses on (1) the implementation of an aggressive polio campaign schedule in the remaining reservoirs, (2) immunizing children of migrant and mobile Pashtun communities, (3) closer integration of immunization campaigns with community engagement, (4) closer civil–military cooperation in insecure areas, and (5) increased (>1,350) human resources for deployment in high-risk areas [22, 184]. Campaigns in some highest-risk areas (with ~165,000 children) have been suspended because of repeated fatal attacks on young women vaccinators from the community [184]. However, the attacks have been met by the young vaccinators with courage and resolve, and campaigns have continued elsewhere, with large numbers of children receiving OPV in the key reservoir areas.

Polio cases in Pakistan increased from 58 in 2012 to 93 in 2013; all cases in 2013 were associated with wild poliovirus type 1. Virus spread from Pakistan to Syria (33 cases in 2013) (http://www.polioeradication.org/), and to Egypt and Israel where wild poliovirus type 1 was detected in sewage [491].

11.1.3 Nigeria

All three poliovirus serotypes circulated in northern Nigeria in 2012: wild type 1, type 2 cVDPV, and wild type 3. In 2011–2012, 25 % of poliomyelitis case-patients had never received a dose of OPV [185]. Progress has been gradual in the northern states, and although polio campaign quality has improved, serious gaps remain in rural and hard-to-reach communities. In addition to insecurity in the northeast, 18 % of missed children are because their parents refuse them to be vaccinated because of anti-OPV rumors propagated by some clerics [22]. The Polio Emergency Action Plan calls for the following: (1) improved detailed planning of mass campaigns; (2) better selection, training, monitoring, and support of vaccination teams; (3) enhanced community engagement; (4) increased (~2,500) human resources for deployment in high-risk areas; (5) real-time data monitoring; (6) focused interventions in hard-to-reach rural and nomadic communities; (7) GIS mapping of settlements and GPS tracking of vaccination teams; and (8) strengthened surveillance [22, 185]. Full implementation of this plan should break all remaining chains of poliovirus transmission, as had been achieved a decade earlier in the more densely populated and tropical southern states. As in Pakistan, fatal attacks on young women vaccinators have been an impediment to complete implementation of the plan across the northern Nigerian states.

Polio cases in northern Nigeria decreased from 122 in 2012 to 53 in 2013; all cases in 2013 were associated with wild poliovirus type 1. Wild poliovirus type 1 spread from Nigeria to Somalia (194 cases in 2013), and from Somalia to Kenya (14 cases) and Ethiopia (9 cases). Wild poliovirus type 1 of Nigerian origin spread from Chad to Cameroon in 2013 (http://www.polioeradication.org/).

11.2 Implementation of the Polio Eradication Endgame

Compared with the straightforward smallpox eradication endgame, the polio eradication endgame is much more complex and includes the following elements, some operating on parallel tracks: (1) cessation of wild poliovirus transmission, (2) outbreak response (especially cVDPVs), (3) strengthened routine immunization, (4) IPV introduction and shift from tOPV to bOPV, (5) sequential IPV/bOPV in routine immunization, (6) completion of poliovirus containment (ultimately including vaccine-related isolates), (7) global certification, (8) cessation of bOPV use, and (9) mainstreaming of polio activities into national and global disease alert and response systems. Several of these activities are discussed above. A key new element is the worldwide withdrawal of use of the type 2 component of OPV.

11.2.1 Withdrawal tOPV

Since 1999, all poliomyelitis cases associated with poliovirus type 2 (apart from a few cases in India associated OPV contaminated with MEF-1 [335, 336]) have been associated with the continued use of tOPV. The rising incidence of type 2 cVDPV outbreaks (Sect. 10.8.1; Fig. 13.15), representing nearly 90 % of all reported cVDPV cases, prompted the WHO GPEI to plan for the logistically challenging step of worldwide withdrawal of tOPV and replacement with bOPV [22]. The tOPV–bOPV switch, targeted for 2016, would be predicated on the complete cessation of type 2 cVDPV transmission and will require continued sensitive AFP and poliovirus surveillance. Under the new strategic plan, by the end of 2014, polio-funded field staff will devote >50 % of their time assisting countries to strengthen routine immunization. Large stockpiles of mOPV2 (500 million doses; in addition to 300 million doses each of mOPV1 and mOPV3) will be maintained, and a robust surveillance and response capacity established. In addition, steps will be taken to secure affordable IPV and introduce at least one dose of IPV into the routine immunization programs of all countries by the end of 2015. Before tOPV withdrawal, the GCC must "validate" (certification will be for all poliovirus serotypes) the eradication of type 2 wild polioviruses and the elimination of type 2 cVDPV transmission. Also, Phase 2 containment of type 2 wild polioviruses and cVDPVs must be completed, along with Phase 1 containment of type 2 vaccine-related isolates. When all prerequisites are met, coordinated global cessation of tOPV use in routine immunization and mass campaigns will occur over a short time period, replaced with bOPV, the remaining tOPV stocks will be withdrawn worldwide, and the process carefully documented [22]. After the switch, the WHO will stockpile "stand-alone" IPV (IPV is usually formulated as a component of multivalent injectable vaccines) for emergency deployment to areas peripheral to outbreak communities. The tOPV–bOPV switch will precede the global bOPV cessation and withdrawal projected for 2019.

11.2.2 Transition to IPV

Many previously OPV-using countries, including the United States, have transitioned to exclusive use of IPV. Several countries have maintained a sequential IPV/OPV schedule (Fig. 13.17), similar to what the United States employed in 1997–1999 (Sect. 9.1.5). The WHO has obtained extensive experience in the introduction of new vaccines into low- and middle-income countries. However, worldwide use of IPV will be greatly facilitated by reducing the cost of IPV, which is substantially higher than OPV. Steps to reduce IPV costs could include (1) the use of intradermal fractional (1/5th) IPV doses [492], (2) development of new adjuvanted intramuscular IPV products, and (3) replacement of conventional IPV (using neurovirulent wild strains as seeds) with IPV based on the Sabin OPV strains [493–495], more suitable for production in developing countries [22]. New-generation biotechnology tools are being explored to develop genetically stable IPV seeds that would substantially enhance biological containment in IPV production facilities [496–499]. However, the many options for immunization products and schedules present increasingly complex policy decisions for national public health authorities [500].

11.3 The Legacy of Polio Eradication

The GPEI is the largest public health initiative in history, engaging communities, governments, health professionals, and private donors throughout the world. During the past quarter century, the GPEI has trained millions of volunteers, social mobilizers, and health workers to reach and immunize children in the most marginalized and vulnerable communities in the world [22]. In all but a few places, poliomyelitis, increasingly a disease of the poorest of the poor, has gone the way of smallpox. Moreover, other health interventions have directly benefited from polio immunization campaigns. For example, vitamin A supplements delivered during OPV campaigns are estimated to have prevented more than one million childhood deaths [22], and the administration of

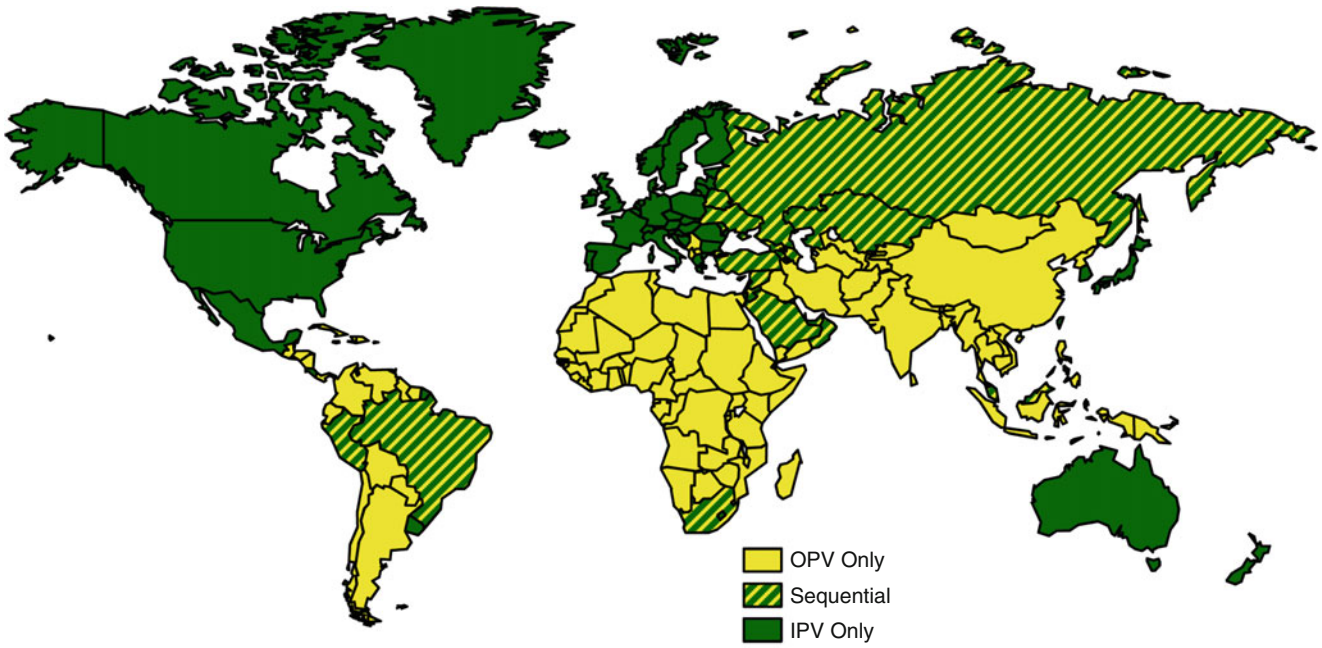

Fig. 13.17 Countries using exclusively oral poliovirus vaccine (*yellow* OPV), exclusively inactivated poliovirus vaccine (*green* IPV), or a combination of IPV and OPV (hatched pattern) (Modified from reference Sutter [7])

anti-helminthics and the distribution of bed nets have spared many more of disease and early death.

As the century-long struggle against poliomyelitis draws to a close, a new generation of highly experienced public health professionals—epidemiologists, virologists, and program managers—from around the world have built on the solid foundations of the GPEI to help guide the control of other vaccine-preventable diseases, including measles, rubella, rotaviral gastroenteritis, tetanus, invasive bacterial diseases, yellow fever, Japanese encephalitis, and hepatitis B [22, 422, 423, 501–503]. The model of close integration of field and laboratory surveillance with programmatic action has been replicated in other vaccine-preventable disease programs. The GPLN, the first laboratory network with global reach, established the blueprint for other vaccine-preventable disease laboratory networks [130, 131], some of which have grown to be more extensive than the GPLN [129]. These new networks are guided by laboratory network coordinators who are experienced in ensuring high technical performance and working in close cooperation with epidemiologists. Another important legacy of the GPEI, building on the models of smallpox eradication and the EPI, is a permanent worldwide change in public perception and expectation. Outbreaks of vaccine-preventable diseases, and the huge costs they inflict on individuals and communities, are no longer acceptable, and public demand for their control has steadily grown. As poliomyelitis fades into history, the legacy of its eradication will help shape global efforts to control infectious diseases for many years to come.

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Filoviruses: Marburg and Ebola

14

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1 Historical Overview

Because of the inordinate media attention given to the filoviruses, particularly Ebola virus, a brief historical overview is in order.

1.1 Marburg Virus

In 1967 the first known appearance of the filoviruses occurred in Germany (Marburg and Frankfurt) and Yugoslavia (Belgrade) [1, 2]. The disease was associated with the import of green monkeys (*Cercopithecus aethiops*, now *Chlorocebus aethiops*) from Uganda as a source of monkey kidney cells for the manufacture of polio virus vaccine. There were 29 cases who had direct contact with the monkeys or tissues from the monkeys and an additional 6 cases among individuals who had contact with the initial patients. Seven fatalities occurred, all among the individuals with exposure to the primates or their tissues. The disease was initially called green monkey disease and the virus named after the town, Marburg, in which the initial cases were noted.

Over the ensuing months the virus was isolated by serial passage in guinea pigs and also produced disease in two species of monkey (*Chlorocebus aethiops* and *Macaca mulatta*) [3]. Interestingly, early reports suggested that there were no cytopathic effects in a variety of cell cultures, while there was suggestion of replication in the cultures by inoculation of guinea pigs with supernatant fluids of the cultures. Later reports do indicate the presence of cytopathic effect in a number of cell lines.

The virus had a distinct, long pleomorphic appearance in electron micrographs; this trait would eventually give the name to the family to which the virus belongs, the *Filoviridae*, because of the filamentous appearance of the virus.

Marburg virus appeared again in 1975 when a young Australian man, touring with his female companion in Southern Africa, became ill in South Africa after visiting Rhodesia (now Zimbabwe). He was hospitalized in Johannesburg 4 days after onset of illness and died there with rash and significant bleeding after 4 days of hospitalization. His companion, who remained at the side of the initial case constantly throughout his early illness and hospitalization, became ill 2 days after the death of the index case as did a nurse caring for him, 8 days after the death of the initial case. The companion and the nurse survived their infections [4]. The exact origin of the infection of the index patient was not clearly elucidated in the subsequent investigations [5].

2 The Dawn of Ebola Virus

In 1976 two outbreaks of a disease with similar signs and symptoms occurred more or less simultaneously in north central Zaire (now the Democratic Republic of the Congo or DRC) and in southern Sudan [6–8]. The outbreaks were larger than the initial Marburg incidents and were clearly comprised of chains of person-to-person transmission following the introduction of the infection into medical care facilities. The reuse of needles and syringes without proper sterilization was eventually thought to play a role in much of the transmission from patient to patient, at least within medical care facilities, in these outbreaks.

Material from patients was sent to a number of international laboratories, and it was soon discovered to be related to Marburg virus by its similar appearance in electron micrographs. In spite of the similarity of the appearance between Marburg virus and this new virus, there was no serological cross-reaction when reference sera from Marburg virus were reacted with this newer one or laboratory animals were cross-challenged [9].

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Field investigations of the Zaire and Sudan outbreaks did not yield evidence of a direct link between the outbreaks at the two locations. The virus was given the name Ebola virus after a river that is near the site of the original outbreak in Zaire. There was a single case in Zaire in 1977 [10] and another small outbreak in southern Sudan in 1979 [11, 12]. The viruses causing the outbreaks in Zaire and the outbreaks in Sudan are in fact quite distinct; however, they were not determined to be separate viruses until the early 1980s when a radioimmunoassay suggested that antibodies to the two viruses could distinguish between them [13] and then molecular biology had reached some ability to distinguish the two viruses from each other [14]. Following the 1979 outbreak in southern Sudan, there were no reported African outbreaks until 1995 when the outbreak in Kikwit, Zaire, occurred.

In 1989 Ebola virus came to the USA in, similar to the circumstances for Marburg virus in 1967 in Europe, in monkeys (cynomolgus macaques, *Macaca fascicularis*) in quarantine in Reston, Virginia, that were being imported for biomedical research into the USA from the Philippines. The monkeys were found to be dying of Ebola virus, although a second virus, Simian hemorrhagic fever virus, was also present [15]. By 1989, the ability of molecular biology to sequence and determine the relationships of viruses more easily indicated that this was a novel Ebola virus, given the name Ebola Reston, related but distinct from the two strains which had been the etiology of the Zaire and Sudan outbreaks in the 1970s, Ebola Zaire and Ebola Sudan. The outbreak was traced back to a facility in the Philippines where the imported monkeys had originated [16, 17]. There were to be further instances of monkeys from this same facility being infected with Ebola Reston, including 1992 into Italy [18, 19] and 1996 into the USA once more [20].

In 1994, a fourth Ebola virus, Ebola Ivory Coast, emerged when a female scientist studying mortality among chimpanzees in the Tai Forest National Park in Ivory Coast became ill following a postmortem exam that she had performed upon a chimp that had died. Her illness was a dengue-like syndrome, and the virus was isolated from her blood [21]. Serological testing at the Institute Pasteur in Paris and sequencing soon demonstrated that this was another novel Ebola virus.

Starting in the mid-1990s the number of Ebola virus outbreaks was more frequent and precludes individual discussion. Some of these outbreaks will be discussed in the context of their contribution to our knowledge of the diseases and viruses, but a complete listing of the occurrence of known outbreaks can be found in Table 14.1 (Marburg) and Table 14.2 (Ebola viruses). The relative size of each of these outbreaks and its location can also be seen in maps provided in Fig. 14.1 (Marburg) and Fig. 14.2 (Ebola).

Briefly, a fifth Ebola virus was discovered in late 2007 when an outbreak in western Uganda turned out to be caused by another novel and distinct virus, now known as Ebola Bundibugyo, after the Ugandan town in which the outbreak it caused was centered. Although next-generation sequencing sped up the characterization of this new virus, its novelty was indicated when targeted PCRs for the epidemic-prone viruses Ebola Zaire and Ebola Sudan failed to react, but an antigen detection assay reacted with initial patient bloods submitted to the Special Pathogens Branch at CDC in Atlanta [61]. As is common in Ebola outbreaks, much of the transmission occurred within medical facilities, where some of the initial patients sought care, and many of those subsequently infected included health-care workers [62]. In addition, the mortality associated with infection with this virus appears to be less than the Zaire and Sudan Ebola viruses [48].

Another filovirus has been associated with deaths in insectivorous bats in Spain, but the virus has not been isolated [63]. Its genetic signature appears to be between the existing Ebola viruses and Marburg virus, but its taxonomic status remains unsettled, and the host range and pathogenicity of the virus remain unknown and will probably remain so until a virus is either isolated or reconstructed from the sequence derived from the tissues of the dead bats.

3 The Virus

The filoviruses are negative-sense single-stranded RNA viruses. They are members of the family, *Filoviridae*, so named after the long filamentous appearance of the virions in electron micrographs. They belong to the order *Mononegavirales*. Marburg virus comprises one genus of the family (*Marburgvirus*), while the Ebola viruses comprise the other genus (*Ebolavirus*). The genomes are the largest of the viruses in this order having a genome of just over 19 kilobases. The order of the seven genes is similar to other members of the *Mononegavirales*, the rhabdoviruses and the paramyxoviruses. The nucleoprotein is located at the 3' end of the genome and is followed by VP35, VP40, glycoprotein, VP30, VP24, and the L (polymerase) open reading frames [64]. Several of these proteins have the ability to interfere with the immune response of the host and are believed to play a role in the high pathogenicity of the filoviruses [65–67].

4 The Disease

Infection with Marburg and Ebola viruses leads to similar diseases which are marked by inappropriate innate immune responses which both downregulate the useful antiviral effects expected in the early postinfection period and induce vigorous responses of certain cytokines and chemokines, inducing a sepsis-like syndrome with high mortality. The mortality ranges between a high of approximately 90 % seen with Ebola Zaire virus infection, followed by approximately 55 % seen with Ebola Sudan, and approximately

Table 14.1 Marburg outbreaks through 2012

Year(s)	Country	Apparent or suspected origin	Reported number of human cases	Reported number (%) of deaths among cases	Situation
1967	Germany and Yugoslavia	Uganda	31	7 (23 %)	Simultaneous outbreaks occurred in laboratory workers handling African green monkeys imported from Uganda [22]. In addition to the 31 reported cases, an additional primary case was retrospectively serologically diagnosed [23]
1975	Johannesburg, South Africa	Zimbabwe	3	1 (33 %)	A man with a recent travel history to Zimbabwe was admitted to a hospital in South Africa. Infection spread from the man to his traveling companion and a nurse at the hospital. The man died, but both women were given vigorous supportive treatment and eventually recovered [24]
1980	Kenya	Kenya	2	1 (50 %)	Recent travel history included a visit to Kitum Cave in Kenya's Mount Elgon National Park. Despite specialized care in Nairobi, the male patient died. A doctor who attempted resuscitation developed symptoms 9 days later but recovered [25]
1987	Kenya	Kenya	1	1 (100 %)	A 15-year-old Danish boy was hospitalized with a 3-day history of headache, malaise, fever, and vomiting. Nine days prior to symptom onset, he had visited Kitum Cave in Mount Elgon National Park. Despite aggressive supportive therapy, the patient died on the 11th day of illness. No further cases were detected [26]
1998–2000	Democratic Republic of Congo (DRC)	Durba, DRC	154	128 (83 %)	Most cases occurred in young male workers at a gold mine in Durba, in the northeastern part of the country, which proved to be the epicenter of the outbreak. Cases were subsequently detected in the neighboring village of Watsa. This is actually a series of small epidemics originating with miners who were working in a galleried gold mine [27]
2004–2005	Angola	Uige Province, Angola	252 [28]	227	Outbreak believed to have begun in Uige Province in October 2004. Most cases detected in other provinces have been linked directly to the outbreak in Uige [29]
2007	Uganda	Lead and gold mine in Kamwenge District, Uganda	2	2 (50 %)	Small outbreak, with 2 cases of young males working in a mine. To date, there were no reported cases among health workers [30]
2008	USA ex Uganda	Cave in Maramagambo forest in Uganda, at the southern edge of Queen Elizabeth National Park	1	0 (0)	A 44-year-old woman who resides in Colorado returned on January 1, 2008, from a Safari in Uganda. She became ill on January 4 and consulted her physician on January 6 and 8 and was hospitalized on January 8 with a diagnosis of acute hepatitis. She was discharged on January 19 with no serious sequelae. Testing of a sample drawn 10 days post-onset was initially negative by serology, virus isolation, and real-time RT-PCR. After the Dutch case, a convalescent serum was submitted which was found to be Marburg IgG positive by ELISA. A nested RT-PCR of the original sample was found to be positive for Marburg RNA [31]
2008	Netherlands ex Uganda	Cave in Maramagambo forest in Uganda, at the southern edge of Queen Elizabeth National Park	1	1 (100 %)	A 40-year-old Dutch woman with a recent history of travel to Uganda was admitted to a hospital in the Netherlands. Three days prior to hospitalization, the first symptoms (fever, chills) occurred, followed by rapid clinical deterioration. The woman died on the 10th day of the illness [32, 33]

(continued)

Table 14.1 (continued)

Year(s)	Country	Apparent or suspected origin	Reported number of human cases	Reported number (%) of deaths among cases	Situation
2012	Uganda	Kabale, Ibanda, Kampala, and Mbarara Districts	23	8 (35 %)	As of November 29, 2012, the Ugandan Ministry of Health reported 15 confirmed and 8 probable cases of Marburg virus infection, including 15 deaths, in the Kabale, Ibanda, Mbarara, and Kampala Districts of Uganda. Testing of samples by CDC's Viral Special Pathogens Branch is ongoing at the Uganda Virus Research Institute in Entebbe. Working with the Ministry's National Task Force, a CDC team is assisting in the diagnostic and epidemiologic aspects of the outbreak. Note that Kabale District, on the border with neighboring Rwanda, is distinct from Kibaale District, the site of the recently ended Ebola outbreak; both districts are in Uganda's Western Region [34]

Adapted from CDC. http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/marburg/marburgtable.htm

Table 14.2 Outbreaks caused by Ebola viruses through 2012

Year(s)	Country	Ebola subtype	Reported no. of human cases	Reported no. (%) of deaths among cases	Situation
1976	Zaire [Democratic Republic of Congo(DRC)]	Ebola Zaire	318	280 (88 %)	Occurred in Yambuku and surrounding area. Disease was spread by close personal contact and by use of contaminated needles and syringes in hospitals/clinics. This outbreak was the first recognition of the disease [8]
1976	Sudan	Ebola Sudan	284	151 (53 %)	Occurred in Nzara, Maridi, and the surrounding area. Disease was spread mainly through close personal contact within hospitals. Many medical care personnel were infected [7]
1976	England	Ebola Sudan	1	0 (0 %)	Laboratory infection by accidental stick of contaminated needle [35]
1977	Zaire	Ebola Zaire	1	1 (100 %)	Noted retrospectively in the village of Tandala [10]
1979	Sudan	Ebola Sudan	34	22 (65 %)	Occurred in Nzara, Maridi. Recurrent outbreak at the same site as the 1976 Sudan epidemic [36]
1989	USA	Ebola Reston	0	0 (0 %)	Ebola Reston virus was introduced into quarantine facilities in Virginia, Texas, and Pennsylvania by monkeys imported from the Philippines [15]
1990	USA	Ebola Reston	0	0 (0 %)	Ebola Reston virus was introduced once again into quarantine facilities in Virginia and Texas by monkeys imported from the Philippines. Four humans developed antibodies but did not get sick [37]
1989–1990	Philippines	Ebola Reston	4 (asymptomatic)	0 (0 %)	High mortality among cynomolgus macaques in a primate facility responsible for exporting animals in the USA [17]. Three workers in the animal facility developed antibodies but did not get sick [16]
1992	Italy	Ebola Reston	3 (asymptomatic)	0 (0 %)	Ebola Reston virus was introduced into quarantine facilities in Sienna by monkeys imported from the same export facility in the Philippines that was involved in the episodes in the USA. No humans were infected [18]
1994	Gabon	Ebola Zaire	52	31 (60 %)	Occurred in Mékouka and other gold-mining camps deep in the rain forest. Initially thought to be yellow fever; identified as Ebola hemorrhagic fever in 1995 [38]
1994	Ivory Coast	Ebola Ivory Coast	1	0 (0 %)	Scientist became ill after conducting an autopsy on a wild chimpanzee in the Tai Forest. The patient was treated in Switzerland [21]

Table 14.2 (continued)

Year(s)	Country	Ebola subtype	Reported no. of human cases	Reported no. (%) of deaths among cases	Situation
1995	Democratic Republic of the Congo (formerly Zaire)	Ebola Zaire	315	250 (81 %)	Occurred in Kikwit and surrounding area. Traced to index case patient who worked in the forest adjoining the city. Epidemic spread through families and hospitals [38, 39]
1996 (Jan–April)	Gabon	Ebola Zaire	37	21 (57 %)	Occurred in Mayibout area. A chimpanzee found dead in the forest was eaten by people hunting for food. Nineteen people who were involved in the butchery of the animal became ill; other cases occurred in family members [38]
1996–1997 (July–Jan)	Gabon	Ebola Zaire	60	45 (74 %)	Occurred in Booué area with transport of patients to Libreville. Index case patient was a hunter who lived in a forest camp. Disease was spread through close contact with infected persons. A dead chimpanzee found in the forest at the time was determined to be infected [38]
1996	South Africa	Ebola Zaire	2	1 (50 %)	A medical professional traveled from Gabon to Johannesburg, South Africa, after having treated Ebola virus-infected patients and thus having been exposed to the virus. He was hospitalized, and a nurse who took care of him became infected and died [40]
1996	USA	Ebola Reston	0	0 (0 %)	Ebola Reston virus was introduced into a quarantine facility in Texas by monkeys imported from the Philippines. No human infections were identified [20]
1996	Philippines	Ebola Reston	0	0 (0 %)	Ebola Reston virus was identified in a monkey export facility in the Philippines. No human infections were identified; one animal handler has Ebola antibody [41]
2000–2001	Uganda	Ebola Sudan	425	224 (53 %)	Occurred in Gulu, Masindi, and Mbarara Districts of Uganda. The three most important risks associated with Ebola virus infection were attending funerals of Ebola hemorrhagic fever case patients, having contact with case patients in one's family, and providing medical care to Ebola case patients without using adequate personal protective measures [42]
2001–2002 (Oct 1–March 2)	Gabon	Ebola Zaire	65	53 (82 %)	Outbreak occurred over the border of Gabon and the Democratic Republic of the Congo [43]
2001–2002 (Oct 1–March 2)	Democratic Republic of Congo	Ebola Zaire	57	43 (75 %)	Outbreak occurred over the border of Gabon and the Democratic Republic of the Congo. This was the first time that Ebola hemorrhagic fever was reported in the Democratic Republic of the Congo [43]
2002–2003 (Dec 2–April 03)	Democratic Republic of Congo	Ebola Zaire	143	129 (89 %)	Outbreak occurred in the districts of Mbomo and Kéllé in Cuvette Ouest Département [44]
2003 (Nov–Dec)	Democratic Republic of Congo	Ebola Zaire	35	29 (83 %)	Outbreak occurred in Mbomo and Mbandza villages located in Mbomo District, Cuvette Ouest Département [45]
2004	Sudan	Ebola Sudan	17	7 (41 %)	Outbreak occurred in Yambio county of southern Sudan. This outbreak was concurrent with an outbreak of measles in the same area, and several suspected EHF cases were later reclassified as measles cases [46]
2007	Democratic Republic of Congo	Ebola Zaire	264	187 (71 %)	Outbreak occurred in Kasai Occidental Province. The outbreak was declared over November 20. Last confirmed case on October 4 and last death October 10 [47]
Dec 2007–Jan 2008	Uganda	Ebola Bundibugyo	131	42 (32 %)	Outbreak occurred in the Bundibugyo District in western Uganda. First reported occurrence of a new strain [48, 49]

(continued)

Table 14.2 (continued)

Year(s)	Country	Ebola subtype	Reported no. of human cases	Reported no. (%) of deaths among cases	Situation
Nov 2008	Philippines	Ebola Reston	6 (asymptomatic)	0 (0 %)	First known occurrence of Ebola Reston in pigs. Strain closely similar to earlier strains. Six workers from the pig farm and slaughterhouse developed antibodies but did not become sick [50, 51]
Dec2008–Feb 2009	Democratic Republic of the Congo	Ebola Zaire	32	15(47 %)	Outbreak occurred in the Mweka and Luebo health zones of the Province of Kasai Occidental [52]
May 2011	Uganda	Ebola Sudan	1	1(100 %)	Single case in Luwero District, Uganda [53]
July 2012	Uganda	Ebola Sudan	24	7(29 %)	On July 28, 2012, the Uganda Ministry of Health reported an outbreak of Ebola hemorrhagic fever in the Kibaale District of Uganda. A total of 24 human cases (probable and confirmed only), 17 of which were fatal, have been reported since the beginning of July. Laboratory tests of blood samples, conducted by the Uganda Virus Research Institute (UVRI) and the US Centers for Disease Control and Prevention (CDC), confirmed Ebola virus in 11 patients, four of whom have died [54]
2012	Democratic Republic of the Congo	Ebola Bundibugyo	77	36 (47 %)	The DRC Ministry of Health has declared an end to the most recent Ebola outbreak in DRC's Province Orientale. The November 26 press release reports a final total of 77 cases, including 36 laboratory-confirmed cases, 17 probable, and 24 suspect cases, with a total of 36 deaths. CDC assisted the Ministry of Health in the epidemiologic and diagnostic aspects of the investigation. Laboratory support was provided both through CDC's field laboratory in Isiro and through the CDC/UVRI lab in Uganda. The Public Health Agency of Canada (PHAC) also provides diagnostic support through its field lab in Isiro. The outbreak in DRC has no epidemiologic link to the near-contemporaneous Ebola outbreak in the Kibaale district of Uganda [55–57]
2012	Uganda	Ebola Sudan	7	4 (57 %)	As of December 2, 2012, the Ugandan Ministry of Health reported 7 cumulative cases (probable and confirmed) of Ebola virus infection, including 4 deaths, in the Luweero District of central Uganda. CDC is assisting the Ministry of Health in the epidemiologic and diagnostic aspects of the outbreak. Testing of samples by CDC's Viral Special Pathogens Branch is taking place at the Uganda Virus Research Institute in Entebbe [58]
2014	Guinea and Liberia	Ebola-Zaire	(See situation)	(See situation)	Guinea [59] As of 18:00 on 26 April 2014, the Ministry of Health (MOH) of Guinea has reported a cumulative total of 224 clinical cases of Ebola Virus Disease (EVD), including 143 deaths. To date, 202 patients have been tested for ebolavirus infection and 121 cases have been laboratory confirmed, including 74 deaths. In addition, 41 cases (34 deaths) meet the probable case definition for EVD and 62 cases (35 deaths) are classified as suspected cases. A revised number of 25 health care workers (HCW) have been affected (19 confirmed), with 16 deaths (12 confirmed); the number of HCW was previously reported as 26.
					Liberia [60] From 13 March, the date of onset of the first laboratory confirmed case in Liberia, to 24 April, the Ministry of Health and Social Welfare (MOHSW) of Liberia has reported a total of 35 clinically compatible cases of EVD; 6 confirmed cases, 2 probable cases and 27 suspected cases. The date of onset of the most recent confirmed case was 6 April. The MOHSW has started to reclassify suspected cases against their laboratory test results. Most of the suspected cases are expected to be discarded at the end of this process

Adapted from CDC. http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/ebola/ebolatable.htm

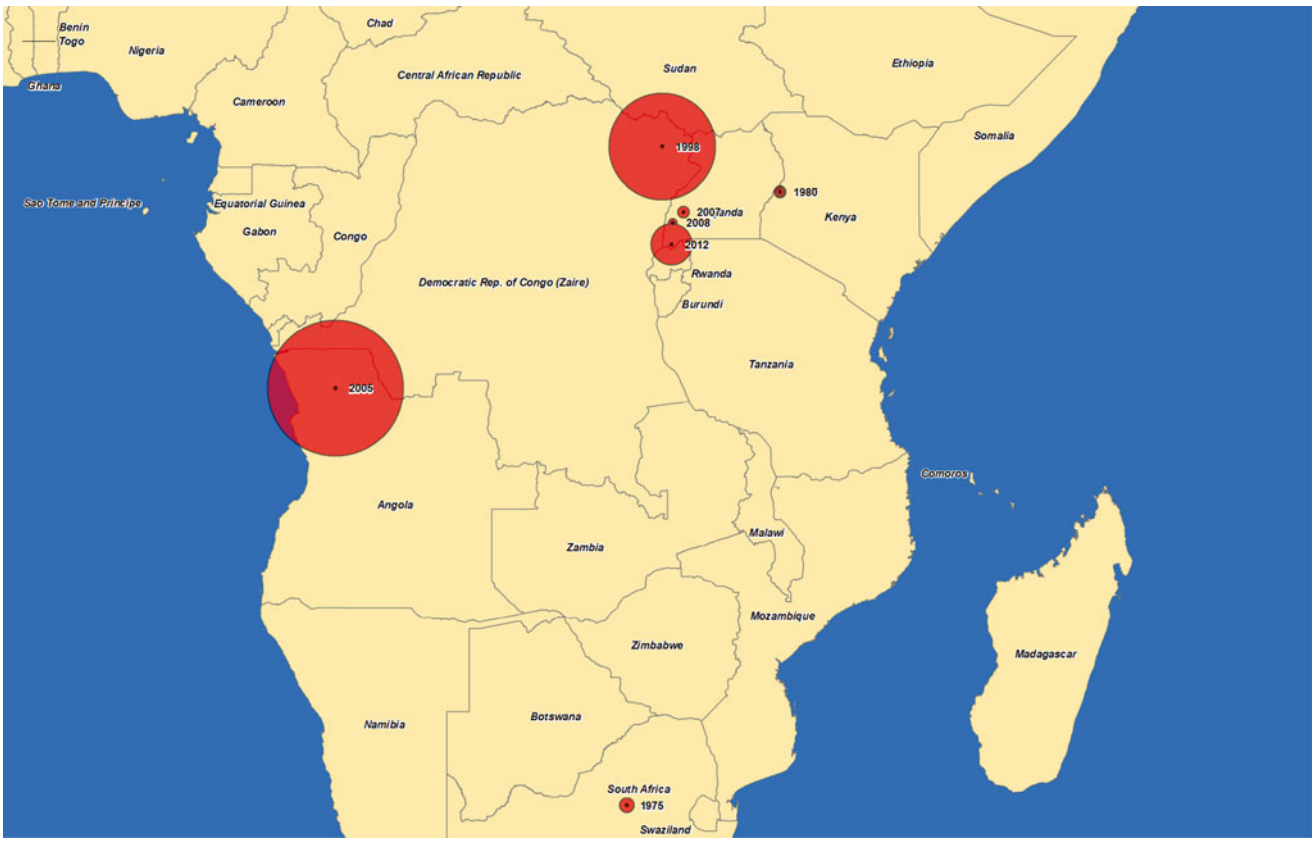

Fig. 14.1 Map of African Marburg virus outbreaks and year in which they occurred. The circles are proportional to the number of cases in the outbreak (see Table 14.1 for the number of cases). Note that the 1980 date obscures the date of the 1987 case, both of which originated at the same location, Kitum Cave on Mt. Elgon. The 1998 outbreak in DRC was actually a series of small outbreaks associated with a mine near the village of Durba which continued through 2000

35 % seen with Ebola Bundibugyo, the three viruses that seem to have epidemic potential. Ebola Ivory Coast has had too few infections, one or perhaps two, to allow a useful estimate of the mortality, while Ebola Reston has so far not caused mortality in the 10s of persons that have been infected. Marburg virus outbreaks have tended to be smaller, often with only one or two individuals infected, but there also may be strain differences in the mortality. Among the three outbreaks with significant numbers infected, the initial outbreak in Germany produced an overall mortality of approximately 23 % (7 of 30 or 31) [22, 23], while the small clusters constituting the 1998–2001 composite cases in the DRC outbreak had an overall mortality of 82 % (123/149) [27], and the 2005 outbreak in Uige, Angola, had a mortality of 90 % (227/252) [29].

In human cases, there is an incubation period of 5–7 days followed by onset of fever, weakness and often muscle pains, and abdominal discomfort. Rash is also a common feature, but in dark-skinned patients, it is not always apparent. Bleeding is not the common manifestation that many believe is the hallmark of the disease. In the Kikwit outbreak of Ebola Zaire in 1995, less than 50 % of the lab-confirmed cases had bleeding signs. The viruses are considered to be pantropic, infecting many tissues and also infecting many cell lines from a great many mammalian species [68]. Among those with bleeding signs, petechiae, ecchymoses, and gastrointestinal bleeding along with failure of venipuncture sites to clot are the most common forms of bleeding. Nevertheless the disease is severe and vascular permeability, loss of fluids from the GI tract from diarrhea and vomiting, and diminished fluid intake all combine with the effects of the inappropriate immune response to induce shock and eventual multiorgan failure.

Patients who do survive often have prolonged recoveries with hair loss and desquamation of areas initially affected by rash. Weakness, myalgias, and arthralgias were also common among survivors of the Kikwit outbreak in which convalescent survivors were compared to controls. In human cases, lesions are common throughout the tissues with endothelial cells and macrophages in many organs having demonstrable virus antigens as well as histological changes and virus antigens in the parenchyma [69–71].

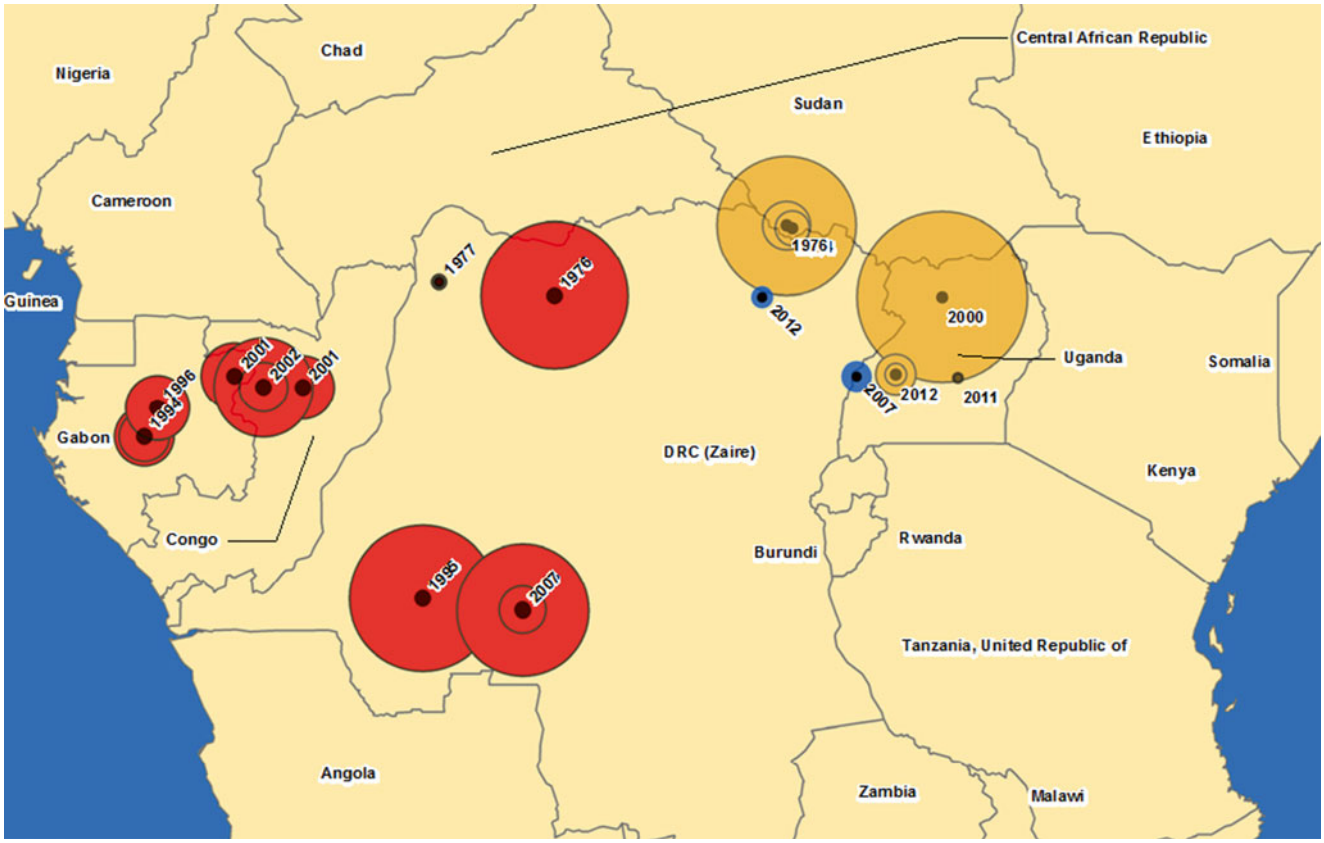

Fig. 14.2 Map of African Ebola virus outbreaks and the year in which each occurred. The circles are proportional to the number of cases in the outbreak (see Table 14.2 for the number of cases). The color of circles indicates which Ebola virus caused the outbreak: Ebola Zaire (*red*), Ebola Sudan (*burnt orange*), and Ebola Bundibugyo (*blue*). In order to show sufficient detail of the area in which the majority of outbreaks have occurred, the 1994 single case of Ebola Ivory Coast is not shown, and the 1996 South African imported case of Ebola Zaire is not shown. The 1976, 1979, and 2004 Ebola Sudan outbreaks overlap each other and the dates are obscured in the figure

5 Laboratory Diagnosis

As for all virus diseases, detection of the virus (or its antigens or nucleic acids) or detection of the resulting antibody response in patients is the primary means of confirming a specific diagnosis. Virus antigens are also found in the skin, a feature which has been exploited to allow for diagnosis using skin biopsies fixed in formalin, which may aid in surveillance for the filoviruses. Until the early 1990s, virus isolation and identification by electron microscopy or reference immune reagents remained the definitive means of definitive diagnosis. This was difficult as high-containment laboratories, of which there were very few in the world, were required to carry out this work safely. Detection of antibodies was also somewhat fraught with issues as the indirect fluorescent antibody test was prone to yield false positives in the sera of individuals who had no previous exposure to filoviruses and the antibody response requires some time to become apparent, and in many instances patients died without yet having detectable antibodies. Following the outbreak of a new Ebola virus in Reston in 1989, a number of newer technologies were applied to the diagnosis of Ebola virus infections. An antigen detection assay, utilized extensively in the Reston outbreak and its investigation, allowed for a rapid and specific identification of Ebola virus in the blood of acutely ill individuals even in remote areas [72]. Continued difficulties with the nonspecific detection of antibodies by the indirect fluorescent antibody test in primates during the Reston investigation led to the adaption of enzyme-linked immunosorbent assays to the detection of both IgM antibody in acute Ebola infections and IgG antibody in individuals surviving infection [73] and the application of these assays to human patients in the Kikwit outbreak [74]. These assays also had the advantage that they could be adapted for use in the field during outbreaks or outbreak investigations by utilizing appropriate personal protective equipment and inactivating the clinical specimens using heat and detergent. This allowed the diagnosis of patients quickly in either onsite or at a local laboratory, depending on the logistical support and demands of the particular outbreak. At about the same time that the antigen detection and serological assays were being developed, the

use of reverse transcriptase-polymerase chain reactions for the detection and diagnosis of RNA viruses advanced where it too began to play a role in Ebola diagnosis [75] which eventually led to it being deployed to the field setting along with the ELISA tests [29, 76]. Another means of diagnosis, particularly useful in remote areas, was described and shown to be of utility during the Kikwit outbreak and employs the use of formalin-fixed skin snips as a means of avoiding invasive postmortem exams and the safety and convenience of using formalin for transport of the biopsy to a suitable laboratory for diagnostic testing [72].

6 Epidemiology and Ecology

6.1 Epidemiology

The epidemiology of the filoviruses is largely comprised of the person-to-person transmission that occurs once the virus has been introduced into the human population. The distribution of the virus and, for the most part, the disease is dictated by the underlying ecology of these zoonotic viruses that reside in reservoir or maintenance hosts.

The high mortality associated with filovirus infection has created an aura of fear associated with the outbreaks. At a local level, particularly in the developing world where the virus appears to be resident, this is understandable. The history of outbreaks is marked by caregivers as early victims of the disease. However, intense investigations during more recent outbreaks have provided valuable information on how transmission occurs and the level of protection that is necessary to protect caregivers and aid in stopping the chain of transmission that continues to fuel the epidemics. This was afforded by an earlier response than had occurred in the initial Ebola outbreaks of the 1970s and was particularly taken advantage of during the Kikwit outbreak in 1995.

The principle lessons are that transmission of the filoviruses is by direct contact with infectious material and that moderate infection control practices, rigorously applied and controlled, can almost immediately stop transmission [77]. In most of the endemic areas, this is a matter of providing resources and training to health-care facilities where they may be available on only a limited basis and are not routinely used in the everyday practice of health care. In the developed world, standards of care and the employment of standard precautions for patient contact have addressed the majority of the risks associated with caring for patients with these infections as has been demonstrated in a number of instances of imported cases where no, or, at worst, very limited, transmission to care givers has occurred [31, 40].

Risks to individuals in the community who do not have direct contact with infected patients are practically nonexistent. Assessment of risks during the outbreak in Kikwit gave ample testament to the association of infection to exposure to seriously ill patients or the cadavers of individuals who had died of Ebola infection [78, 79]. Nevertheless, some care and education to avoid transmission via blood-borne routes by traditional medicine practices or reuse of needles in local pharmacies remain a concern.

Even though the filoviruses can be readily transmitted by the experimental creation of small particle aerosols, the role of aerosol transmission in outbreaks is, at most, minimal as attested by the lack of transmission in the community, other than by direct contact, and by studies of family members sharing small enclosed spaces with infected patients [78].

6.2 Ecology

The reservoirs of the filoviruses are just beginning to be understood, and only recently has the primary host for Marburg virus, rousette fruit bats (*Rousettus aegyptiacus*), been established with a degree of certainty [80–82]. The reservoir host(s) for the Ebola viruses is less certain, but indications are that, like Marburg virus, bats are the leading candidates [83, 84]. Anecdotal accounts of outbreaks going back to the Sudan outbreaks of the 1970s, in which the initial cases in both 1976 and 1979 worked in a cotton factory in which there were resident bats [85], have suggested to many in the field that bats were leading candidates, but hard scientific data was lacking until more recent circumstances provided stronger circumstantial and scientific support for this contention.

The big break for Marburg virus came when an outbreak in Durba in the Democratic Republic of Congo (formerly Zaire) which began in 1999 provided a combination of scientific and circumstantial evidence that led to further concentration on bats as the reservoir. The outbreak in this instance was not a typical filovirus outbreak with a single introduction of the virus and a serial passage of that virus through a chain of human cases; rather, in this instance, it was a series of small outbreaks. This could be discerned because the viruses that caused each of the mini-outbreaks could be genetically differentiated and the epidemiology of each small chain leads back to a single source of the virus. This source was a former commercial mine now being exploited by free-lance miners who were at the beginning of the transmission chain for each of the small mini-outbreaks [27]. Investigation of the mine found that bats were the principal fauna and genetic evidence of Marburg virus could be found in animals collected during the investigation [86]. These findings were further advanced by investigations of cases occurring or originating in Uganda that were associated with large concentrations of bats first at the Kamwenge mines [28, 30], where miners were infected, and then at a cave in Queen Elizabeth Park, where two tourists became infected [31, 32].

Subsequent investigations of the bat populations have allowed the repeated isolation of the virus from the rousette bats that are the principal fauna of these two locations and the demonstration of the persistence of the virus in the bat populations [80, 81].

7 Prevention

Prevention of filovirus outbreaks is possible. In the first instance, now that the reservoirs of the viruses are becoming apparent, education of local populations should enable them to avoid the source of the virus. However, this may be culturally difficult in hunter-gatherer populations that have strong traditional values that may be difficult to change, particularly in economically developing areas where hunting remains an important source of nutrition.

7.1 Infection Control

Outbreaks of disease are more often the product of exposure of individuals to the primary case who is infected by interaction with the reservoir. The lesson of the last 20 years is that infection control in health-care facilities, often the source of amplification of outbreaks, can stop the transmission of the viruses from patients in these settings. Unfortunately, this does require that these facilities have the basic supplies that are part of the daily routine of medical care in the developed world, what have come to be known as standard precautions. Without the ability to use these simple measures, including routine hand washing and single use of needles and syringes, outbreaks are likely to continue to be amplified by health-care facilities in the endemic areas.

7.2 Vaccines

Vaccines are being developed and have reached a stage where nonhuman primates can be protected from infection by Ebola and Marburg viruses [87–91]. Some of these vaccines have even demonstrated their potential use as therapeutics for treatment of individuals following exposure to a filovirus if the vaccine is administered relatively soon after infection [92]. While almost all funding for the development of filovirus vaccines is driven by biodefense concerns, the vaccines are of practical use in providing protection to individuals who have occupational exposure because they work with the agents in the laboratory and to individuals who respond to outbreaks that have similar risks of exposure from caring for patients in the field. There, in reality, is probably little use for filovirus vaccines as a means of protecting populations in endemic areas because the outbreaks are so sporadic and focal that the cost of immunization would not be economically viable; at best they might be used in the midst of an outbreak to provide protection to at-risk individuals, particularly if the protection is rapid, as the postexposure treatment use of certain of these vaccines is effective as pointed out above. One added note about the use of vaccines, there is at best limited cross-protection among the Ebola virus vaccines, and no cross-protection between Marburg virus and Ebola viruses; multivalent vaccines will have to be developed or the vaccines targeted at outbreaks with known filovirus etiology.

8 Treatment

Aside from vaccines used postexposure, there have been some advances in therapeutics for filoviruses.

8.1 Monoclonal Antibodies

Monoclonal antibodies have recently been shown to protect nonhuman primates against Ebola Zaire infection [92–95]. Efforts are underway to improve the utility of some of these preparations by making them more compatible for use as human therapeutics. As was the case for the vaccines, the monoclonal preparations are directed against specific filoviruses and would be most effective when used in situations where the specific virus causing disease was known. Monoclonal antibody cocktails would be needed to broaden protection against multiple viruses.

8.2 iRNAs

Specific iRNAs have recently been shown to be effective in the treatment of Ebola virus-infected animals [96, 97]. However, like vaccines, these are targeted and exhibit specificity of action which means that a cocktail of iRNAs would be required for situations in which the virus was not yet identified or if a new filovirus were to appear. Closely related to the use of iRNAs has been the use of antisense phosphorodiamidate morpholino oligomers (PMOs) which are also targeted at specific RNA sequences but increase the stability, affinity, and access into cells of the antisense nucleotides. In a recent study, they have been used and demonstrated to successfully treat up to 75 % of rhesus macaques from Ebola Zaire infection when used either pre- or post-virus challenge [98].

8.3 Small Molecule Inhibitors

Other small molecule inhibitors of the viruses have been described from in vitro screening exercises or testing of

compounds with antiviral activity with other non-filovirus viruses. However, there has only been limited screening of these compounds in in vivo screening in filovirus small animal models, which have not been great predictors of positive performance of vaccines and antiviral drugs in primates [99].

9 Unresolved Problems

Even though the reservoir of Marburg virus is now reasonably established as the cave-dwelling fruit bat, *Rousettus aegyptiacus*, the definitive reservoir of the Ebola viruses has remained elusive. As discussed in previous sections, although a number of species of fruit bats have been identified by RT-PCR as containing Ebola virus RNA, virus isolation and studies demonstrating persistence of the virus in reservoir populations remain lacking.

There remain a number of issues with the filoviruses. The foremost is probably the lack of approved vaccines or therapeutics for use in the rare individuals who are exposed in endemic areas during epidemics or individuals exposed during the course of lab work with the agents. Much progress has been made in finding candidate vaccines that have been effective in experimental use in primates, but the regulatory pathway for licensure of these products is dependent on the application of the so-called animal rule. This mechanism went into effect as a means of approving medical countermeasures that are unable to undergo traditional efficacy trials for ethical or practical reasons as 21 CFRParts 314 and 601 in July, 2002. However, the interpretation and implementation of the animal rule have remained a somewhat controversial pathway for product approval with much interpretation of various facets of the rule remaining illusive and resulting in only a few products being approved (and those being for medical countermeasures previously licensed for other purposes) [100, 101]. These issues will have to be resolved to provide a predictable pathway for approval of medical countermeasures for the filoviruses.

Field and laboratory research on the filoviruses have never been simple. High-containment laboratories capable of doing research have always been scarce. The post-9/11 environment has created other obstacles that are associated with these viruses being considered as "weapons of mass destruction" that have tangibly increased the practical burdens and expense of working with the agents. The public health importance of the filoviruses in endemic areas is emphasized by the number of outbreaks that have occurred recently (see Tables 14.1 and 14.2). The practical and administrative aspects of moving diagnostic specimens from suspected outbreaks, making virus isolates, curating virus strains, and clearing and training scientific personnel have all increased with the frequent advent of additional layers of security regulations that are levied on labs and personnel that work with these viruses.

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Flaviviruses: Dengue

15

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1 Introduction

Dengue is the world's most important human arboviral disease with indigenous and endemic transmission in more than 100 tropical and subtropical countries. There are numerous other locales that experience non-sustained epidemic transmission with cases in returning travelers or military personnel [1, 2]. More than half the population of the world is at risk of being infected with a dengue virus (DENV). Despite its importance dengue is under-recognized and underreported with current literature estimating 400 million infections each year with 100 million being clinically apparent [3]. The human, community, country, and regional cost of dengue in terms of mortality, morbidity, and health care resource utilization is significant and growing in scope [4–9]. There are numerous factors that are believed to contribute to the increase in dengue burden, which include (1) rising number of susceptible hosts (population growth), (2) expanding *Aedes* mosquito vector populations (ineffective vector control, increasing breeding sites, changing ecology), (3) increasing DENV distribution (travel), and (4) the convergence of the these three: urbanization, poverty, and decaying infrastructure [10–12].

DENV infection produces a spectrum of clinical presentations from asymptomatic infection to a nonspecific febrile illness termed dengue fever (DF) to severe dengue known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [13]. The original World Health Organization (WHO) clinical classification scheme has recently been under review and an alternative classification has been proposed to account for instances where biochemical and clinical markers of severe disease are present in cases of otherwise uncomplicated dengue fever. Reconciling the value of the new scheme for both research and patient care purposes has been controversial [14–18]. Why some human-vector-virus interactions result in infection and others do not and why some infections result in no disease and others severe multisystem organ failure and death are incompletely understood. The pathophysiology of dengue is multifactorial with host (age, gender, clinical comorbidities, immunologic background), vector (salivary proteins), and virus (type and genotype virulence, escape mutants) factors and the interplay between them all playing a role [19–23]. There is no licensed anti-dengue antiviral or vaccine to treat or prevent dengue disease. Vector control, except in rare circumstances, has been insufficient to significantly curtail dengue expansion. Fortunately, there are numerous efforts underway to explore methods to strategically and effectively reduce vector populations or alter their ability to become infected and/or transmit dengue [24, 25]. Anti-dengue therapeutic development efforts have focused primarily on reducing viral burden (as measured in the peripheral circulation) or reducing the magnitude of the host pro-inflammatory response. New drug development and seeking new indications for existing drugs are both being actively explored [26–28]. The dengue vaccine pipeline is abundant with candidates at various stages of preclinical and clinical development [29–32].

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2 Historical Background

Dengue's nonspecific clinical features make the interpretation of historical records for evidence of past epidemics difficult. David Bylon is sometimes credited with the first clinical description of dengue [33]. In 1779, he observed an epidemic of febrile disease in Batavia, a disease he termed "knokkel-koorts," knuckle or joint fever [34]. At the same time (1779), August Hirsch described a similar epidemic in Cairo [35]. Dr. Benjamin Rush provided one of the first in-depth clinical descriptions of dengue after observing numerous patients during a 1780 epidemic in Philadelphia, Pennsylvania [36]. Rush wrote, "…its more general name among all classes of people was, the Break-bone fever." The term "dengue" is derived from the Swahili phrase "Ka dinga pepo," where Ka means "a kind of"; dinga, "sudden cramp-like seizure"; and pepo, "plague." The phrase is believed to have crossed from Africa to the Caribbean in 1827 [37, 38]. Cubans identified this phrase with the Spanish word "dengue," which represented the painful and stiff gait of people afflicted with the disease. Spanish archives indicate the use of "quebranta huesos" (breakbone) by a physician in Puerto Rico describing a febrile illness in 1771 and the use of "dengue" by the Queen of Spain in 1801 to describe an acute febrile illness with bone and joint pains, hemorrhage, and jaundice [39].

Early research into the etiology of dengue implicated bacteriological, protozoan, and spirochetal causes [40, 41]. Ashburn and Craig provided evidence for the viral etiology of dengue making it the second human viral pathogen identified after the yellow fever virus [40, 42, 43]. Siler and colleagues built upon the work of Graham in exploring the dengue vector and transmission dynamics [41, 44]. Large outbreaks during World War II and human infection experiments resulted in the isolation of dengue virus types 1 and 2, identified the presence and possible protective role of anti-dengue virus neutralizing antibodies, and described the protective and disease attenuating capacity of homotypic and heterotypic immunity, respectively [43, 45, 46]. In 1956, a dengue epidemic occurred in Manila resulting in the identification and naming of dengue virus type 3 and 4 viruses [47].

Dengue has a consistent presence in Asia and a sporadic pattern in the Americas. In 1897, North Queensland reported a dengue outbreak clinical manifested as epistaxis, hematemesis, and gingival bleeding [48]. Severe dengue was implicated in major disease outbreaks in Greece in 1928 and in Formosa in 1931 [49–51]. After World War II, intensified and sequential transmission of multiple dengue virus types began in Southeast Asia, leading to hemorrhagic fever outbreaks. In the 1950s, the dengue viruses would be associated with Philippine hemorrhagic fever and Thai hemorrhagic fever, both later identified as dengue hemorrhagic fever (DHF) [52, 53]. Outbreaks in the Americas have been postulated since the 1600s, associated with dengue virus introduction into port cities by international commerce. Vector eradication efforts initiated in the 1940s were initially successful, but the program ultimately ended and reinfestation and the return of dengue occurred in the region by the early 1980s [54]. Dengue has been increasingly reported from the Middle East and Africa reaffirming the global spread of dengue [55, 56].

3 The Dengue Viruses

3.1 Evolution of the Dengue Viruses

The evolution of the flaviviruses has been covered in another chapter (Chap. 16). This section covers specifically the evolution of the dengue viruses (DENV) and why understanding its evolution is important in how these viruses have evolved into a global health problem and challenging dengue vaccine development. The dengue viruses evolved in fundamentally different ways from their flavivirus brothers though retaining the properties responsible for the characteristics of flaviviral infection in humans such as fever, myalgias, headache, hepatitis, encephalitis, and in particular hemorrhagic fever. The continent of origin of the dengue viruses is not known, though circumstantial evidence suggests an African origin based on the many flaviviruses that circulate there now, its principal mosquito vector *Aedes aegypti* is thought to have originated in Africa, and DENV circulates in an African sylvatic cycle with nonhuman primates [57]. An argument can be made that the DENVs originated in Asia or perhaps evolved into four separate serotypes in Asia based on the hyperendemicity of DENV in this geographic region and the phylogenetic relationship of the Asian sylvatic strains to current circulating DENV strains [58, 59]. It is estimated that DENV evolved into four antigenically distinct serotypes approximately 1,000 years ago, and each of these four serotypes emerged independently into a non-sylvatic endemic cycle of transmission between humans and *Aedes aegypti* approximately 125–320 years ago [59, 60]. The Asian relationship of the DENV is historically important as the first cases of the more severe form of DENV infection, dengue hemorrhagic fever (DHF), made its appearance in the 1950s first in the Philippines and then in Thailand [61]. The current Asian strains of each serotype are considered more severe than the American strains [62]. The evidence suggests that Asia plays a pivotal role historically and currently in creating viruses that produce severe illness and due to its population growth and urbanization, contributing to the increase in genetic diversity which has increased by a factor between 14 and 20 in the last 30 years [63]. In prospective cohort studies in Northern Thailand and hospitalized children in Bangkok, rates of genetic diversity have been high among all serotypes in any given year,

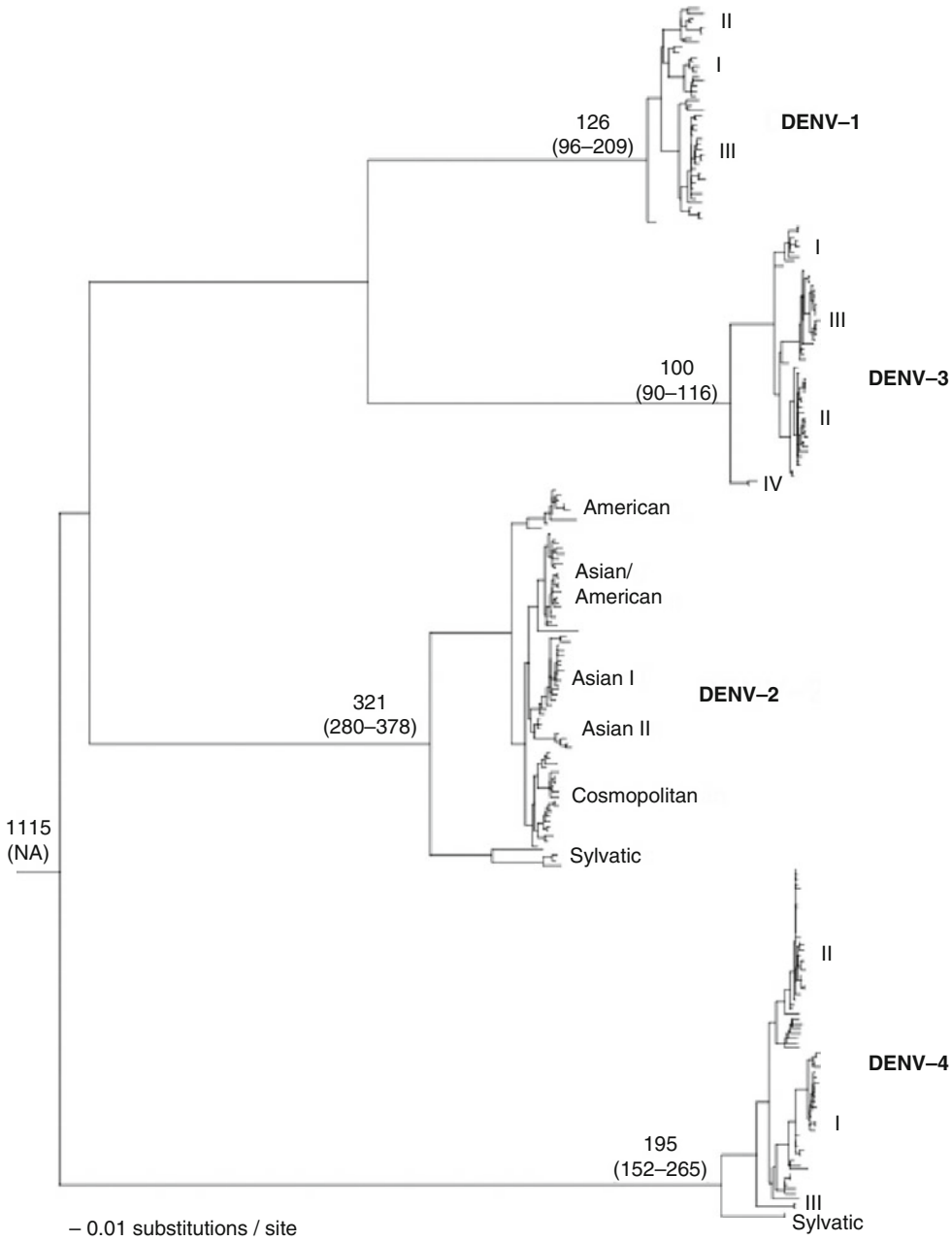


Fig. 15.1 The evolutionary history of the DENV as determined by phylogenetic analysis [68]

restricted flight of the vector has accounted for spatial genetic conservation, and major genetic clade replacements have occurred over time [64–67]. The change of DENV from a sylvatic cycle to a primarily human-vector cycle, especially in Asia, changed the viruses in a fundamental way. Each serotype adapted to infecting and replicating in a human population with high levels of flavivirus antibody from previous DENV infections or in Asia from DENV and Japanese encephalitis virus (JEV) infections. As a result, DENV have become adept at escaping heterologous neutralizing antibody and using it as a means to attain high viral load levels and more severe disease, i.e., antibody enhancement. Today the DENVs continue to evolve at a rapid rate, Asian strains are geographically distributed throughout the world, and DHF has become a global health problem.

3.1.1 DENV Phylogeny

The phylogeny of DENV demonstrates the genetic diversity of these viruses and its evolution as displayed in Fig. 15.1 [68]. The four serotypes diverge from each other by 30 % across their polyproteins, conferring their serotypic distinction. Variation among genes among the viruses range from highly variable for the C and E proteins to relatively conserved for NS3 and NS4B. Within a serotype there is a high degree of genetic variation with the formation of genotypes. There are three genotypes (I, II, and III) for DENV-1, four

genotypes (I, II, III, and IV) for DENV-3, six genotypes (American, Asian/American, Asian I, Asian II, Cosmopolitan, and Sylvatic) for DENV-2, and four genotypes (I, II, III, and sylvatic) for DENV-4. Understanding the genotypes is important in comprehending the epidemiology of the DENVs because genotypes arise in specific geographic regions and can be tracked as they spread to other areas of the world, as occurred with the Asian strains of DENV-2 introduced into the Americas. The other important reason for understanding the genotypes is that the occurrence of severe dengue illness is associated with specific genotypes, the Asian strain of DENV-2, for example. Finally there may be a valid concern for understanding the genotypes for vaccine development as certain circulating genotypes may not be covered by potential dengue vaccines that are being developed currently.

3.2 Flavivirus Genome and Structure

The DENV genome is typical of all the flaviviruses and consists of a single-stranded, positive sense RNA of 11 kilobases in length [69]. The DENV genome has an open reading frame (ORF) of over 10,000 bases that encodes polyproteins of 3,386–3,434 amino acids with the order of proteins 5′ –C-prM(M)-E-NS I -NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′ [69]. The structural proteins consist of the proteins C (capsid) and M (membrane or its precursor prM) and the E (envelope) protein. The nonstructural proteins consist of the NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 proteins. The reader is referred to an excellent review of the DENV genomic structure and the protein structures from each gene and their function by Perera and Kuhn [70]. Figure 15.2 displays the organizational structure of the DENV genome and the functions of each protein and its structure from this publication.

3.3 The E Protein

The E protein of DENV is the receptor that mediates virus-host cell binding and entry into cells by membrane fusion, and it is the target of serotype-specific neutralizing antibody during the human immune response following infection. The E protein is therefore the major target for vaccine developers in developing a protective tetravalent vaccine. As the DENVs evolved to infect the host in the presence of preexisting antibody, the E protein developed properties with which to evade the host response by utilizing non-neutralizing or sub-neutralizing concentrations of antibodies to mediate the phenomenon of antibody-dependent enhancement (ADE) of infection. This enhancement phenomenon is thought to result in severe hemorrhagic disease. The E protein is a glycoprotein that has dimers arranged in a herringbone pattern consisting of three domains termed the structurally central N-terminal domain I, domain II which contains the hydrophobic "fusion" peptide that allows virus-cell fusion, and the C-terminal domain III [71, 72]. Domain III is important in virus binding and as a neutralizing site for human antibody and important in protection following infection [73, 74]. Figure 15.3 demonstrates the crystal structure of the E protein and its conformation under varying conditions [70]. In panel (a) is the structure of the immature virion at a neutral pH where it exists as a heterodimer with 60 trimeric spikes that extend from the surface and the structure that exists as it develops in the endoplasmic reticulum (ER) of the host cell. Panel (b) displays the immature virion at a low pH as it encounters the trans-Golgi network (TGN). In this form the heterodimers form dimers giving a smooth surface to its structure. In panel (c) the prM protein is shown cleaved into the M protein by the host endoprotease, furin. In (d) the mature virion is seen secreted in the extracellular space. This figure is an elegant schematic of the processing of the E protein and the effects of pH on its conformational structure as it develops into a mature virion.

The E protein's structure was crystallized and its conformational structure revealed by cryoelectron microscopy by Kuhn and colleagues and demonstrated in Fig. 15.4 [75]. Domain I is red, domain II in yellow, and the neutralizing domain III in red. The fusion peptide is shown in green.

3.4 The Nonstructural Proteins

The nonstructural proteins of dengue virus play an important role in viral replication in the host cell and in immune evasion allowing viral replication. As shown in Fig. 15.2, there are five nonstructural dengue virus proteins, NS1 to NS5. NS1 is involved in RNA replication of the virus and thought to act with NS4A as an RNA replicase; it is secreted in the extracellular space by infected cells and can be measured in the serum of patients infected with DENV. NS2A binds to NS3 and NS5 and is involved in the recruitment of RNA templates to the membrane-bound replicase. NS2B is a membrane-associated protein and a cofactor for the serine protease function of NS3. NS3 is a cytoplasmic protein involved in the polyprotein processing of the virus and RNA replication. NS4A and NS4B are hydrophobic proteins that are membrane associated and function in RNA replication. NS5 is the largest of the nonstructural proteins and serves as the viral polymerase and methyltransferase. Host immune evasion is performed by the inhibition of interferon α and β production in human dendritic cells in which the nonstructural proteins play a major role [76, 77]. The nonstructural proteins (NS2A, NS4A, NS4B, and NS5) in particular inhibit the mammalian interferon signaling pathway [78].

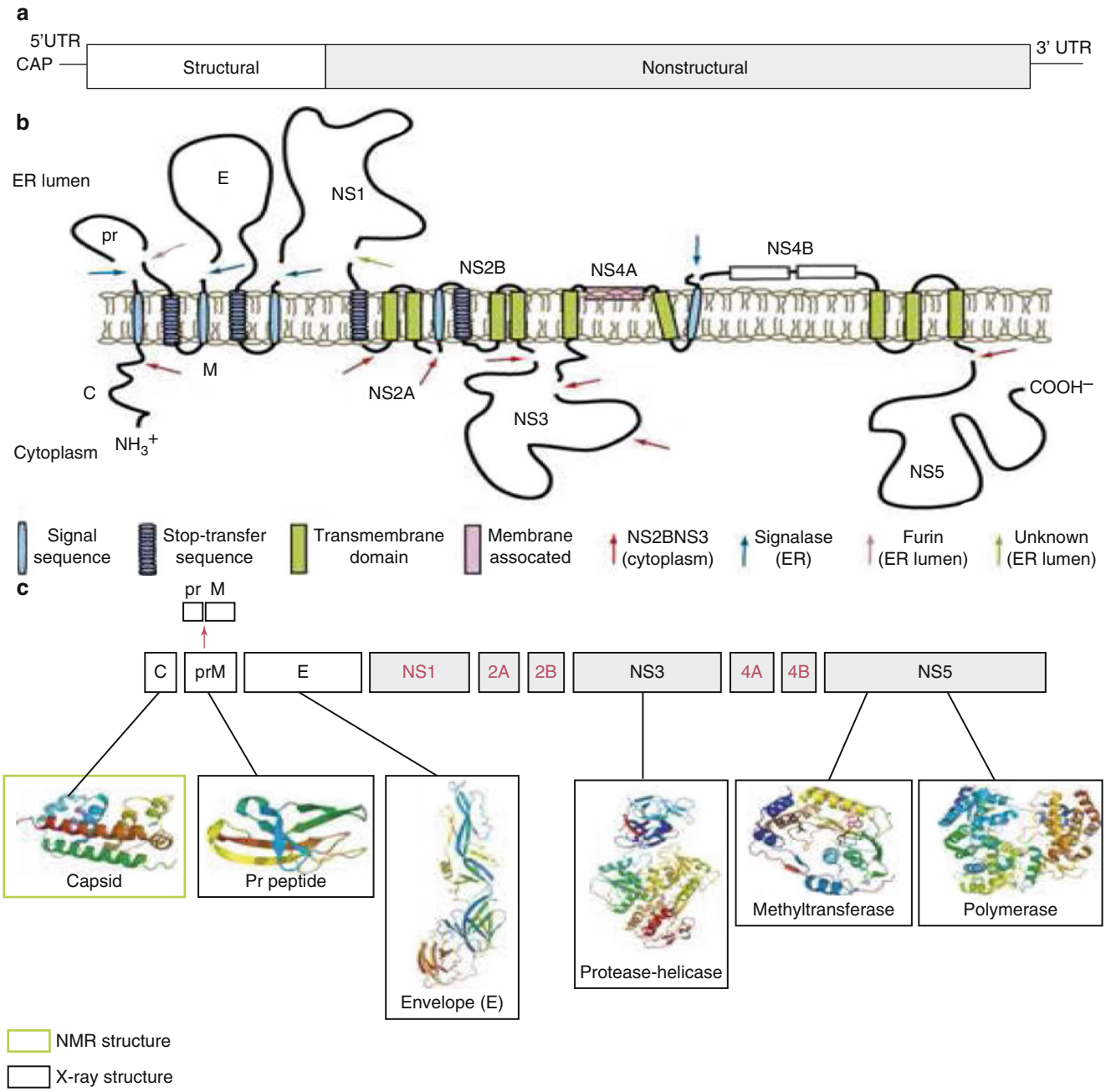


Fig. 15.2 Organizational structure of the DENV genome and its structural and nonstructural proteins [70]

NS5, for example, binds to STAT2 and reduces its expression essential for host cell interferon signaling [78]. The multiple ways DENV evades the host immune response is illustrated by findings that suggest DENV may use preexisting enhancing antibodies not only to promote its entry into Fc receptor-bearing cells but also to form DENV-antibody complexes inside human monocytic cells [79]. These complexes downregulate type 1 interferon production and upregulate cytokine IL-10, which in turn downregulates secondary antiviral responses, such as nitric oxide radical production [79].

3.5 Virus-Host Interactions

This section discusses the complex interplay of viral evolution and E protein assembly and its structural components that influence the host immune response and the role of the nonstructural proteins in virion replication and assembly and are equally important in evading the host innate immune response. The readers are referred to several excellent reviews referenced in this section and in addition an excellent summary of these complexities illustrated in Fig. 15.5 by Rothman [80]. This figure demonstrates the human antibody response

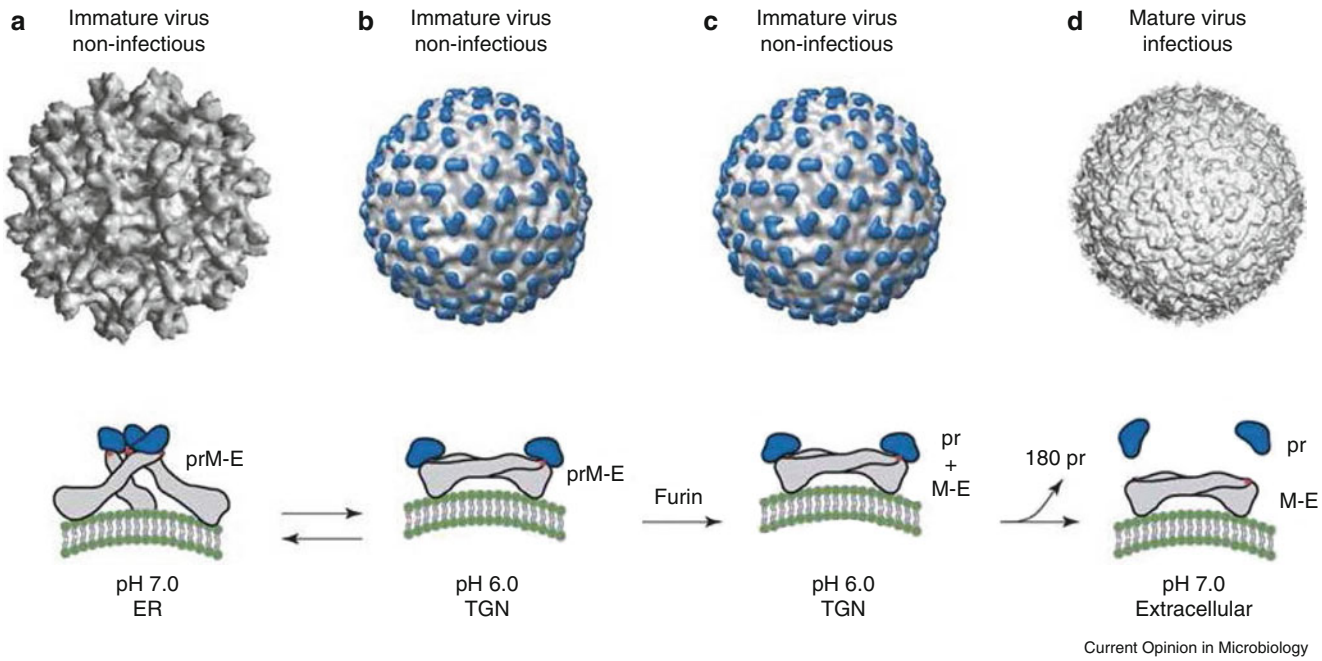


Fig. 15.3 Structures of the E protein and its conformations under varying conditions. (**a**) Structure of the E protein as an immature non-infectious virus in the endoplasmic reticulum (*ER*). (**b**) Structure of the E protein as an immature non-infectious virus in the trans golgi network (*TGN*). (**c**) Structure of the E protein as an immature non-infectious virus in the TGN after furin digestion. (**d**) E protein structure in a mature infectious virus (*Current Opinion in Microbiology*) [70])

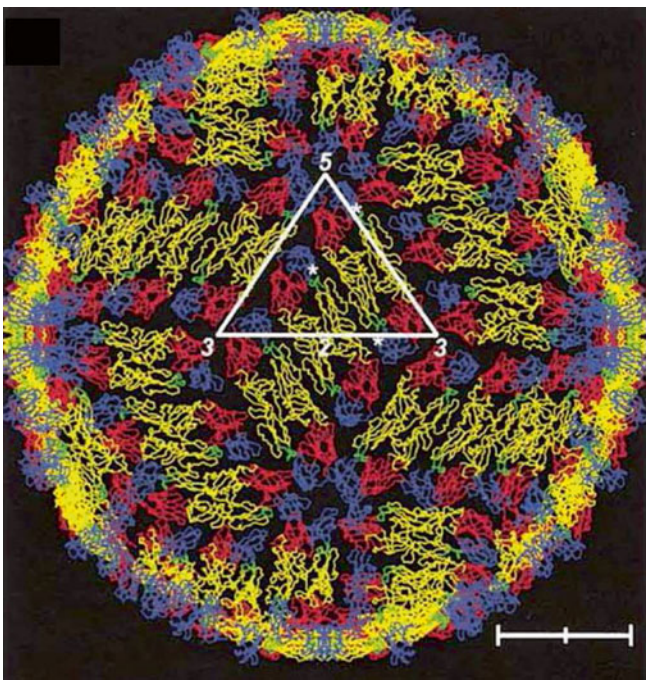
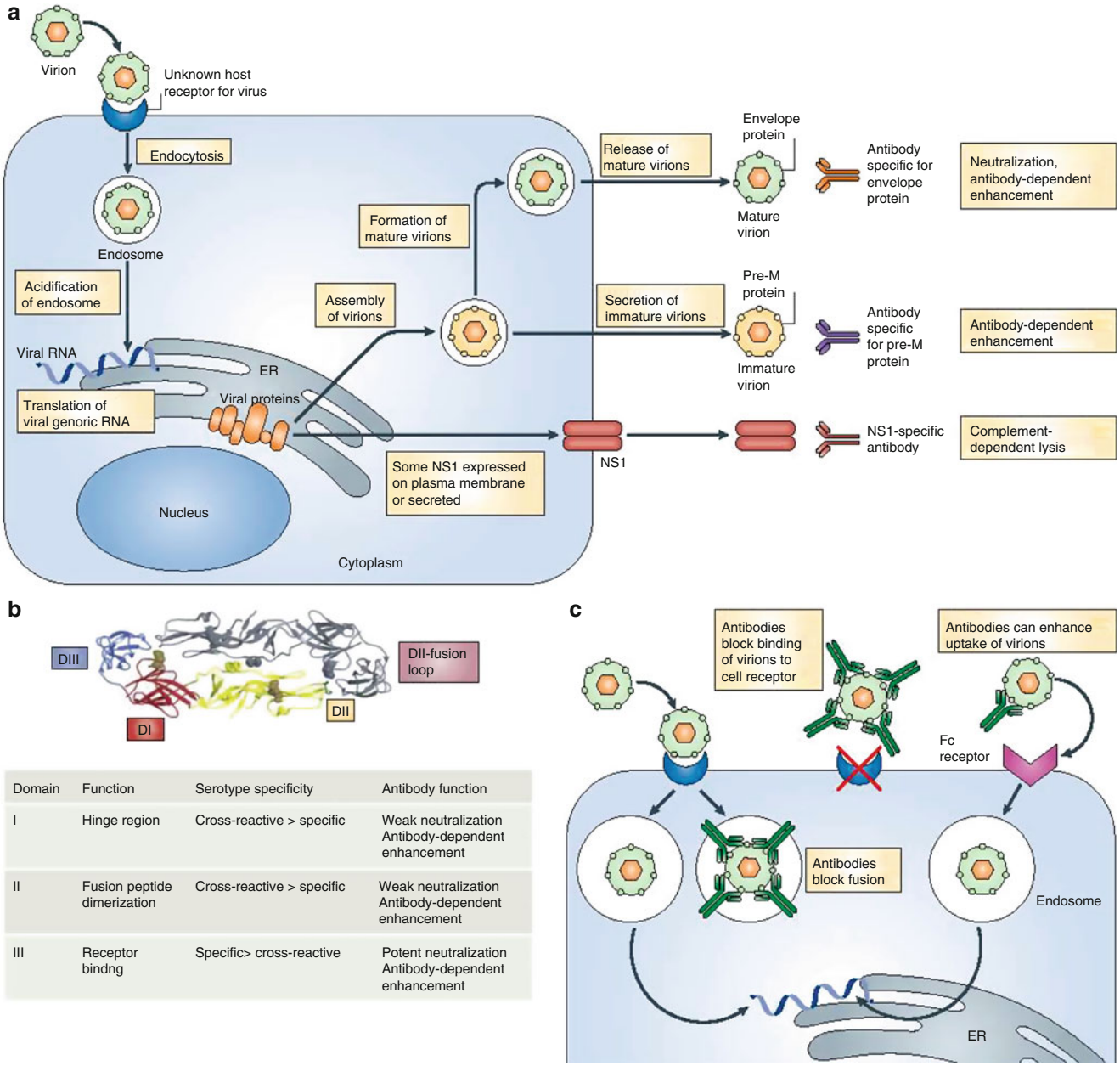


Fig. 15.4 Structure of the DENV E protein demonstrating domains I, II, and III and the fusion peptide [75]. Domains I, II, and III are in *red, yellow*, and *blue*, respectively. The fusion peptide is shown in *green*. The C-terminal residue 395 is shown as a *white asterisk* for monomers within the defined icosahedral asymmetric unit. Scale bar represents 100 Å

to dengue proteins as the virion enters the host cell and replicates. In (a), the mature virion binds to the cell surface receptor via the E protein in its conformational structure as shown in Fig. 15.3. The virion is internalized through endocytosis, and in the resultant lysosomes, acidification drops the pH, leading to a conformational change of the E protein as shown, and the virions are internalized through endocytosis. The acidification of the endocytic vesicle leads to the rearrangement of the surface E protein as shown in Fig. 15.2, fusion of the viral and vesicle membranes, uncoating of the virus, and release of viral RNA into the cytoplasm. Viral genomic RNA is then translated to produce viral proteins in the endoplasmic reticulum, and the viral proteins and newly synthesized viral RNA assemble into immature virions within the lumen. The cleavage of the viral precursor membrane occurs by the host cell enzyme furin, forming mature virions that are then secreted from the cell. In this figure also is shown how NS1 forms and secreted from the plasma membrane of the cell. As demonstrated, host antibody forms against both mature and immature virions that can neutralize virus or result in ADE. Anti-NS1 antibodies can cause complement-dependent lysis of virus-infected cells. In (b), the structure of the dengue virus E glycoprotein ectodomain and its three domains are shown with antibody specific to each. In (c), the mechanisms of neutralization and enhancement by dengue virus-specific antibodies are shown. Neutralization can occur when high levels of epitope-specific antibody block the binding of virions to the cellular receptor or can block fusion at the post-binding stage. At lower concentrations, antibodies can enhance the uptake of virions into cells by interacting with immunoglobulin (Fc) receptors and enhancing binding and cellular uptake of virus.



Domain	Function	Serotype specificity	Antibody function
I	Hinge region	Cross-reactive > specific	Weak neutralization Antibody-dependent enhancement
II	Fusion peptide dimerization	Cross-reactive > specific	Weak neutralization Antibody-dependent enhancement
III	Receptor bindng	Specific> cross-reactive	Potent neutralization Antibody-dependent enhancement

Fig. 15.5 Human antibody responses to dengue virus protein targets and antibody functions. (**a**) DENV binds to host cell and internalized with viral protein production and virus assembly. (**b**) Structure of the E protein and their domains. (**c**) Mechanism of antibody mediated neutralization and enhancement by DENV specific antibodies [80]

4 Methodology Involved in Epidemiologic Analysis of Dengue

4.1 Sources of Data

A variety of sources of data are used to estimate the occurrence of dengue in countries where it is endemic. These include hospital admissions, clinic visits, and prospective studies. The majority of countries rely on syndromic surveillance diagnosing patients by clinical symptoms consistent with dengue fever or dengue hemorrhagic fever using WHO criteria [81]. The reliance on syndromic surveillance is in large part due to the lack of affordable DENV diagnostics available and the lack of infrastructure in which to perform them. There are several problems with using syndromic surveillance, and these include an inability to detect other causes of fever in susceptible populations from other diseases with similar presentation such as leptospirosis and an underestimation of the true burden of dengue infection as the majority of cases are subclinical or of less severity, not requiring hospitalization or presentation to a health clinic. Incidence of dengue burden can be measured more reliably in prospective cohort studies, but these studies are expensive and require

technical expertise often lacking in endemic countries [82]. To compensate for this underestimation of dengue burden by syndromic surveillance, much has been written about using expansion factors (also called multiplication factors in some manuscripts) to estimate the true burden of infection. Expansion factors use a combination of incidence data from prospective studies to correct for the underestimation of disease burden by syndromic surveillance. For example, incidence data from cohort studies performed in children in Thailand and Cambodia were used as expansion factors to calculate the national burden of infection [83]. From these cohort studies the annual incidence of laboratory-confirmed dengue was 23/1,000–25/1,000 in Thailand and 41/1,000 in Cambodia. Age-specific rates of confirmed dengue were compared to the same national data and an expansion factor based on the degree of underestimation calculated and used to readjust the national rate. In Thailand, the median readjusted provincial number of dengue cases was 229,886 (range 210,612–331,236) annually during 2003–2007, and in Cambodia, the median was 111,178 (range 80,452–357,135). The degree of underestimation of the burden of dengue nationally was 8.7-fold in Thailand and 9.1-fold in Cambodia. Similar approaches have been used to estimate the true burden of infection in Southeast Asia and South America [84–87]. While using expansion factors to calculate national burden is well meaning, it is still inherently inaccurate due to the variable nature of DENV transmission spatially and temporally. Prospective studies have demonstrated a high degree of spatial and temporal variation on the incidence and burden of dengue among neighborhoods and districts and from year to year [88]. To capture the true burden of dengue infection in endemic countries requires an investment in training, infrastructure development, and accurate yet inexpensive dengue diagnostics combined with ongoing prospective studies to reflect national incidence.

4.2 Serologic Surveys

Serologic surveys to estimate serotype-specific DENV incidence have played an important role in understanding DENV transmission and burden of infection. The methods of serologic surveys and their importance have been discussed more extensively in Chaps. 3, 4, and 16, to which the reader is referred for more information. Serologic surveys involve sampling a defined population and measuring the amount of specific antibody to the targeted DENV protein which will indicate past or current infection. This is performed by a number of assays, but all currently employ a variation of the enzyme-linked immunosorbent assay (ELISA) or plaque reduction neutralization titer tests (PRNT). The results give a point prevalence of past or current DENV infection in different geographic areas or subpopulations or those at particular risk for infection. Several early prospective serologic surveys conducted in Thailand estimated risk factors for DHF and the incidence of dengue burden in defined populations. They established the important contribution of serologic surveys to the understanding of the pathogenesis of DENV infection [89, 90]. In one of the first prospective studies on DENV infection, Burke and colleagues prospectively studied 1, 757 children in a Bangkok school in June of 1980 [91]. At the start of the study, 50 % of children had DENV antibodies to at least 1 DENV serotype by the age of 7 years. Sequential serologic surveys of this population demonstrated that 87 % who became infected during the study period were asymptomatic; of those remaining who were symptomatic, 53 % were clinically recognized as cases of DHF requiring hospitalization. Incidence rates ranged from 5.5 % in children with preexisting DENV antibody to 6.3 % in those who were serologically naïve. None of the children with primary dengue infection required hospitalization as compared to 12 % of secondary infections. The authors concluded that preexistent dengue immunity was a significant risk factor for the development of DHF.

4.3 Dengue Diagnostics

4.3.1 Introduction

A variety of laboratory methods and diagnostic tests are available for diagnosing past or current DENV infection. They can be divided into two broad categories, those that detect the virus (viral isolation, RNA detection) and those that detect the host response via antibody production specific to DENV. Understanding the viral-host interaction and pathogenesis during a DENV infection is essential in understanding the predictive value of each assay by clinical illness day.

4.3.2 Virus Isolation and RNA Detection

Since the first isolation of a virus, tobacco mosaic virus in 1935 by Stanley, viral isolation has been the mainstay by which to detect viral infection [92]. DENV (DENV-1) was first isolated during an outbreak in the Nagasaki-Sasebo area of Japan brought in from returning seamen in 1942 by Kumura and Hotta and subsequently by Sabin and Schlesinger the following year [43, 45]. DENV isolation was performed by taking serum from ill patients within 48 h of their first fever and inoculating mice intracerebrally. Serial mouse brain passage isolated the virus, which was then identified through neutralization by convalescent sera of patients clinically ill with DENV infection. Though cell lines had been used to propagate viruses such as vaccinia virus and yellow fever virus since the early 1900s, the development of tissue culture lines for viral isolation by Weller and colleagues, first used for poliovirus culture, transformed the ability to isolate

and study viruses in the laboratory [93, 94]. Today a variety of cell lines are used to isolate and study DENV and include but are not limited to the mammalian monkey kidney cell lines, Vero cells and LLC-MK2 cells, as well as the mosquito cell lines from *Aedes albopictus* (C636) and *Aedes pseudoscutellaris* (AP61) [95, 96]. All have similar sensitivity and specificity for isolation, and their usefulness is limited to the viremic period of patients discussed below.

Amplification of the DENV ribonucleic acid (RNA) genome by polymerase chain reaction (PCR) provides a highly sensitive and specific assay in which to isolate the viral genetic material [97–99]. Specific primers and probes and nested PCR permits DENV serotyping with a reaction time of 4 h and represents a rapid means of detecting DENV both for diagnosis and for epidemiologic characterization of serotype-specific transmission. Today DENV PCR has become the mainstay technique to diagnose and serotype dengue-infected patients. Real-time quantitative PCR allows an estimation of the amount of virus (i.e., viral load) during clinical illness, thereby providing information on the pathogenesis of severe dengue illness [100].

4.3.3 Serologic Tests

Serologic testing for past or current DENV infection relies on the host response to produce specific antibody to the targeted DENV proteins that indicates host recognition of infection. Current serologic testing involves detection of either IgM or IgG antibody, the former indicating current active infection and the latter either current or past infection. Two important concepts are involved in understanding serologic testing for DENV infection. The first is that IgG antibody from current or past infection displays a high degree of cross-reactivity to other DENV serotypes or other flaviviruses. IgG antibody to a specific infecting DENV serotype is termed "homotypic" and antibody to another noninfecting serotype "heterotypic" or cross-reactive. This is particularly important as DENV occurs as four distinct serotypes. Infection from one serotype confers long-term immunity to that serotype but not to the others. Thus, the human host can be infected from multiple DENV serotypes during a lifetime. The second concept is that the host response to the first DENV infection differs from the response to subsequent DENV infections. The first infection is termed primary dengue and the second or additional DENV infection termed secondary dengue. The second or subsequent DENV infection is characterized by a blunted IgM response, a rapid and heightened IgG response, and cross-reactive T-cell responses inherently involved in the pathogenesis of DHF discussed in more detail below. This heightened host response during a secondary infection is termed "antigenic sin" and was first described by Francis and defined as the ability of the host immune system to utilize immunologic memory based on a previous similar infection [101]. This has been applied to DENV infection where antigenic sin forms the basis for severe dengue illness [102].

It is not within the scope of this section to discuss every available assay for detecting DENV infection. Most are experimental and performed in research laboratories and only a few assays have been licensed and are commercially available. The traditional DENV serologic diagnostics will be discussed in detail as most assays are variations of these and are essential in understanding the complexities of serology during and after infection.

Plaque Reduction Neutralization Test (PRNT)

The plaque reduction neutralization test utilizes the ability of a DENV to infect a single cell in a cell culture under agar, forming areas of cell death and clearing (plaques), and the ability of neutralizing IgG antibody to form complexes with the virus, preventing cell entry and thus plaque formation. The PRNT was first adapted to DENV by Russell and colleagues in 1967 [103]. The endpoint for the PRNT was calculated as the titer of neutralizing antibody that inhibits 50 % of the plaque formation known as the PRNT50. Originally used with LLC-MK2 cells, a variety of PRNT assays exist now utilizing the same principles but with differing cell lines such as Vero cells and assay formats including the use of microwell plates and a micro-focus assay [104]. The PRNT is an important assay for determining previous and current DENV infection. Two critical issues limit the interpretability of the results. The first is antibody cross-reactivity, making serotype-specific interpretation difficult, and the second is intra and inter-assay variability. The latter issue has been discussed extensively by Thomas and colleagues, demonstrating with one sera set a high degree of variability of the PRNT depending on the cell lines used, the type of prototype DENV, and the use of complement in the assay [105]. This is an important issue as the PRNT has been used by DENV vaccine developers in determining protective immunity from vaccines.

Hemagglutination Inhibition (HAI)

The hemagglutination inhibition assay (HAI) is a technique widely used to measure antibody to a variety of viruses including DENV and first described by Hirst in 1942 to measure influenza virus antibody [106]. As described in Chap. 3, the assay utilizes the ability of viruses to adhere to red blood cells (RBC) and form clusters thereby agglutinating them from solution. The HAI assay was adapted to DENV using goose RBCs and originally a powerful assay to measure DENV antibody though not much in use today except in specialized DENV research laboratory The HAI is distinct from the PRNT as it measures any DENV antibody that adheres to the serotype-specific DENV, not just neutralizing antibody. The advantages of the DENV HAI are that it is a high-throughput assay that can accommodate several hundred

samples in a short period of time, the reagents are relatively low cost, and intra- and inter-assay variabilities are well controlled. The disadvantage of the HAI test is similar to that of the PRNT in that cross-reactive antibody makes it difficult to determine serotype-specific infection. Unlike the PRNT, the HAI can be used to determine acute primary or secondary DENV infection by WHO criteria: a fourfold rise in HAI antibody titer between acute and convalescent sera is diagnostic for an acute DENV infection; the sera pair that is ≥7 days and the convalescent titer that is ≤ to 1:1,280 for an acute primary DENV infection; the convalescent titer that is ≥ than 1:2,560 for an acute secondary infection; and no fourfold rise in HAI titer between the acute and convalescent titer and the convalescent that titer is > 1:2,560 for a recent secondary flavivirus infection [81].

Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is an easily reproducible high-throughput assay in which to measure both IgM and IgG DENV antibody. In general, the indirect ELISA is the most widely performed assay for measuring DENV antibody. Like the HAI test, all DENV IgG antibodies are quantified and not just neutralizing antibody. Innis and colleagues were the first to develop an ELISA specifically to measure DENV IgG and also IgM, the latter known as an IgM antibody capture ELISA or MAC-ELISA [107]. This assay was unique as it capitalized on the observation that in secondary DENV infection the IgM to IgG ratio was lower than in primary DENV infection. In sera of children infected with DENV in Thailand, this assay demonstrated a sensitivity of 78 % in admission sera and 97 % in paired sera. Dengue infections were classified as either primary or a secondary DENV infection by determining the ratio of units of dengue IgM to IgG antibody where an IgM to IgG ratio of ≥1.78 was indicative of primary dengue and <1.78 secondary infection. The dengue IgM/IgG ELISA assay is commercially available and offers the advantage of being reproducible, technically within the scope of clinical laboratories; high throughput; and relatively inexpensive to perform. A characteristic feature of DENV infection is that the virus in infected cells secretes free NS1 protein into the extracellular space. NS1 can be measured in the serum of patients and correlates directly with viral replication and viral load. The NS1 ELISA assay is commercially available and uses an antibody capture of NS1 protein and a secondary enzyme-linked detection antibody to produce a colorimetric reaction that measures the amount of NS1 in the sample. The NS1 ELISA assay was 82 % sensitive in diagnosing patients with acute dengue [108]. The advantages of the ELISA assay for NS1 are its high-throughput capacity and ease of performance in most clinical laboratories. The disadvantage is that NS1 correlates to DENV viremia, and thus, detection is limited to the first 7–10 days of clinical illness.

Lateral Flow Immunochromatographic Assay

This assay use porous paper to allow the wicking and conjugation of the test sample; it is in essence an ELISA assay on paper. A number of DENV lateral flow assays have been developed, and some (e.g., the NS1 rapid lateral flow assay) are commercially available in endemic countries. Studies have demonstrated that this assay is less sensitive (72–73 %) than its ELISA format counterpart [108, 109]. The lateral flow assay has been developed to detect dengue IgM and IgG, potentially broadening the time window for clinical diagnosis of infection to several weeks while IgM is Detectable [110]. The lateral flow tests, also known as rapid diagnostic tests (RDTs), have a number of distinct advantages. The first is that they are potentially point-of-care diagnostic devices that can be done rapidly at the bedside or in villages where dengue infection is suspected. The second and third are that a cold chain is not needed to store material and interpreting the result requires nothing beyond the ability to determine the color lines that indicate a positive or negative test. The disadvantage is that in general they are less sensitive and specific than an ELISA format and PRNT-, or PCR-based assays. Table 15.1 summarizes the advantages and limitations of available DENV diagnostic tests [111].

4.3.4 Dengue Diagnostics During Clinical Illness

Figure 15.6a, b summarizes the importance of knowing the day of clinical illness, whether the suspected dengue infection is primary or secondary and the appropriateness of the diagnostic assay for the time of collection. In general, assays that detect the virus, whether by isolation or RNA detection, require viremia in the patient and are limited to the first week of infection. Assays that rely on host antibody detection, whether IgG or IgM, differ in primary and secondary detection with IgM higher and longer lasting in the first infection as compared to secondary infection. Figure 15.6 also illustrates the importance of obtaining an acute and a convalescent sample in both primary and secondary infections to determine a fourfold rise in IgG antibody as a criterion for diagnosis.

5 Descriptive Epidemiology

5.1 Incidence and Geographic Distribution

DENV is considered the most common arboviral infection in tropical and subtropical regions of the world. As mentioned in a previous section, the frequency of dengue infection is underestimated due to the emphasis on reporting of hospitalized cases by syndromic surveillance by most countries endemic for dengue. A recent analysis with mathematical modeling of the geographic distribution and global incidence of dengue in 2010 demonstrated that DENV is endemic

Table 15.1 Summary of DENV diagnostic assay[a]

Diagnostic tests	Advantages	Limitations
Viral isolation and identification	Confirms infection	Requires acute sample during the viremic period (first 7 days of illness)
	Is serotype specific	Needs specialized expertise and laboratory
	Allows additional scientific investigation into the virus	Requires several weeks to isolate virus and is not high throughput
		Does not differentiate between primary and secondary infection
		Can be expensive
RNA detection (PCR format)	Confirms infection	Has potential false-positives due to contamination
	Sensitive and specific	Requires acute sample during the viremic period (first 7 days of illness)
	Identifies serotype and genotype	Needs specialized expertise and laboratory
	Has rapid results in 24–48 h	Does not differentiate between primary and secondary infection
Antigen detection		
ELISA format for NS1 for example	Confirms infection and sensitive and specific	Is not as sensitive as virus isolation or RNA detection
	Is easy to perform and high throughput	Requires basic clinical laboratory and training
	Is less expensive than virus isolation or RNA detection	Requires acute sample during the viremic period (first 7 days of illness)
		Does not differentiate between primary and secondary infection
Lateral flow assay for NS1 for example	Confirms infection	Is not as sensitive as virus isolation, RNA detection, or ELISA format
	Is easy to perform and high throughput	Requires acute sample during the viremic period (first 7 days of illness)
	Is less expensive than virus isolation or RNA detection	Does not differentiate between primary and secondary infection
	Point of care and does not require specialized lab equipment or cold chain	
Serologic tests		
Plaque reduction neutralization test (PRNT)	Confirms infection	Is expensive and time-consuming to perform
	Is serotype specific in primary infection	Requires specialized training and laboratory
	Is considered the "gold standard"	Does not differentiate between primary and secondary infection
	Is considered a correlate for protection	Has high degree of antibody cross-reactivity
Hemagglutination inhibition (HAI)	Confirms infection	Requires specialized training and laboratory
	Confirms primary and secondary infection	Reagents such as goose red blood cells may be difficult to obtain
	Is relatively inexpensive	Confirmation may require two or more serum samples
	Is high throughput	
IgM/IgG ELISA	Is easy to perform	IgM levels can be low in secondary infections
	Confirms infection and sensitive and specific	Requires specialized training and laboratory
	Is high throughput	
	Confirms primary and secondary dengue	
Lateral flow assay for IgM/IgG	Confirms infection	Is not as sensitive as ELISA
	Is easy to perform and high throughput	Requires acute sample
	Is less expensive than ELISA	Does not differentiate between primary and secondary infection
	Point of care and does not require specialized lab equipment or cold chain	

[a]Adapted from Peeling et al. [111]

throughout the tropics with spatial variations and influenced by climate such as rainfall and temperature as well as urbanization [3]. The authors estimated that the number of DENV infections per year is 390 million (95 % confidence interval 284–528) of which 96 million (67–136) are symptomatic [3]. This estimate is greater than three times the dengue burden as determined by the WHO and further emphasizes importance of this infection for global health [18].

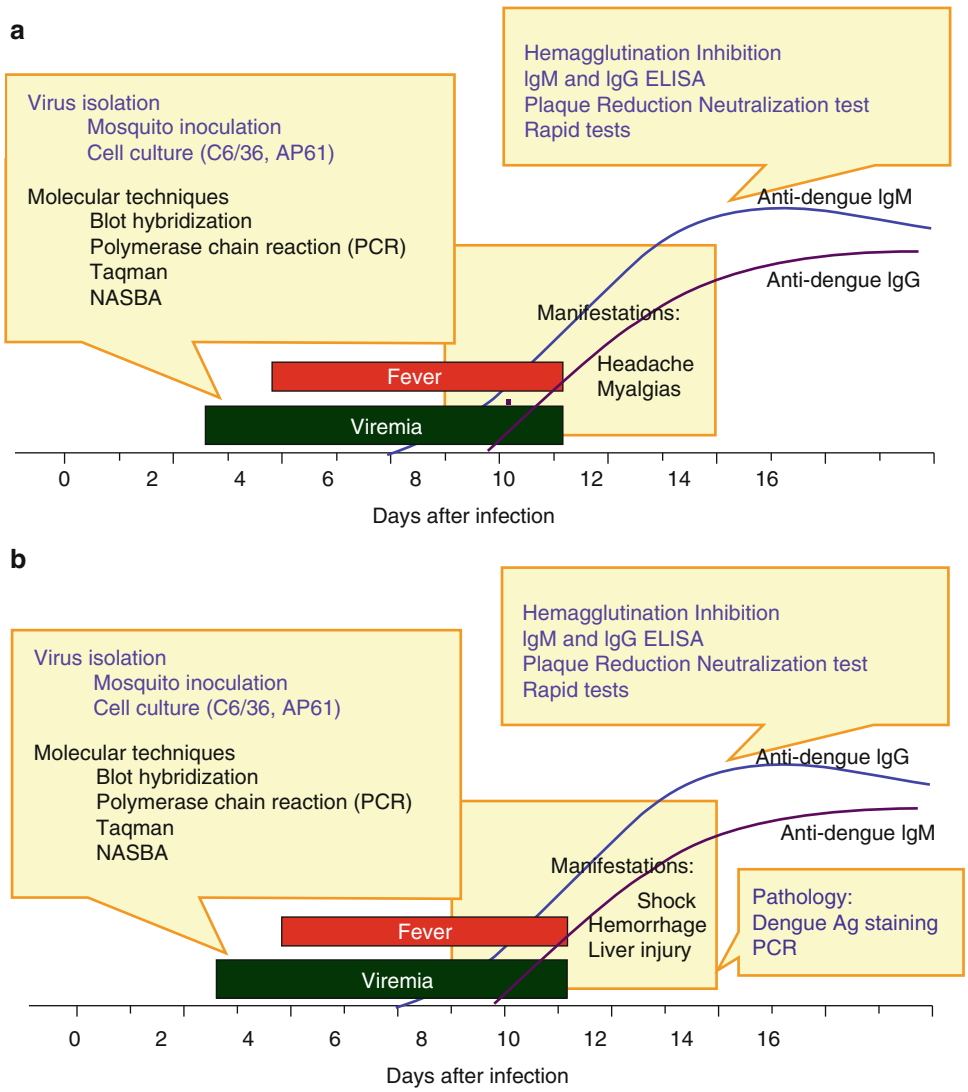


Fig. 15.6 (**a**) Types of dengue diagnostics appropriate by day of infection in primary dengue. (**b**) Types of dengue diagnostics appropriate by day of infection in secondary dengue

5.2 Epidemic Behavior and Contagiousness

5.2.1 Studies of DENV in Thailand

During the 1950s, collaboration between the United States and Southeast Asian countries through the Southeast Asian Treaty Organization (SEATO) created a number of host-country laboratories to respond to and study endemic diseases. In Thailand the SEATO General Medical Research Project originally was located at Mahidol University in Bangkok and later became a joint Thai and US military project named the Armed Forces Research Institute of Medical Sciences (AFRIMS) located on the Thai military medical complex. Bangkok and Thailand became the epicenter for severe dengue illness and allowed Thai and US investigators to study all aspects of DENV, an effort that has continued for more than half of a century and produced seminal advances in DENV epidemiology, vector transmission, and pathogenesis. Early studies of hospitalized children in Bangkok characterized the clinical severity of DHF resulting in thrombocytopenia, leukopenia, coagulopathy and plasma leakage [112]. Research on severe DENV resulted in the observation that both secondary DENV infection and primary infection of infants with declining maternal antibody were risk factors for DHF, and the theory of ADE as an important force in the pathogenesis of DHF was born [113]. Prospective studies of DENV in children living in Bangkok established the role of enhancing antibody in the peripheral blood mononuclear cells of children in producing severe dengue illness and DHF [114]. Additional prospective studies demonstrated the importance of viral load and T-cell responses in disease severity and the circulation of all DENV serotypes and their spatial and temporal diversity [88, 115–117].

5.3 Spatial and Temporal Distribution

DENV transmission, being vector-borne, is dependent on both the propagation of the *Aedes aegypti* vector mosquito

and the availability of viremic individuals from whom the mosquito can become infected. Thus, DENV transmission is seasonal and has both spatial and temporal distributions. The experience in Thailand in many ways illustrates how DENV can emerge in a population and through the combination of increasing urbanization, a high birth rate providing a continuous pool of susceptible individuals, and climate changes, evolved from a seasonal disease with periodic epidemics to a hyperendemic one involving transmission of all four serotypes. A classic paper by Nisalak and colleagues illustrates this concept nicely [118]. Dengue virus serotype was determined by viral isolation and PCR in hospitalized children with suspected dengue at the Queen Sirikit National Institute of Child Health in Bangkok, Thailand, from 1973 to 1999. During that period, acute dengue was diagnosed in 15,569 children with 4,846 viral isolations. All four DENV serotypes circulated with DENV-3 the most frequent serotype in primary dengue and DENV-2 in secondary infection and DHF. However, the change in predominant serotype from year to year was striking, with DENV-1 the major cause of the outbreak from 1990 to 1992, DENV-2 from 1973 to 1986 and 1988–1989, DENV-3 in 1987 and 1995–1999, and DENV-4 from 1993 to 1994. In Thailand there has been a progressive increase in the number of dengue cases from several thousand per year in the 1960s to 200–300,000 cases per year in the last decade. In the Americas the more recent pattern resembles the experience in Thailand during the 1970s and 1980s. DENV is introduced in a country producing localized epidemics and through the movement of infected individuals and transportation of the mosquito vector, the disease spreads and becomes endemic in a region. Continued urbanization and an increase in birth rate provide high vector burden and a pool of susceptible individuals for all four serotypes, and the country becomes hyperendemic for DENV. Like in Thailand, one serotype emerges as the predominant serotype causing an outbreak in any given year. As the population becomes infected and develops herd immunity to that serotype, another serotype becomes predominant and thus the cycle is repeated every year. Long-term prospective cohort studies of children in Northern Thailand from 1998 to the present illustrate the fine-scale spatial and temporal distribution of DENV transmission in a fixed geographic area. Using a school-absence surveillance system, absent students were evaluated for fever or a history of fever. If present, acute and convalescent blood samples were tested and DENV serotype determined by isolation and PCR [88, 119, 120]. DENV infection incidence and serotype circulation varied geographically and from year to year with one school having a DENV-3 outbreak and several miles away another school having a DENV-2 outbreak. The following year the same schools had outbreaks with different DENV serotypes. These studies demonstrate the complexity and remarkable diversity in DENV serotype transmission. A recent study of DENV transmission in Bangkok using the location of dengue patients' homes and the infecting serotype found evidence of localized transmission at distances of under 1 km [121]. In summary, there is a diverse spatial and temporal distribution of DENV transmission illustrating the complexities of transmission between the host and the mosquito vector. *Aedes aegypti,* as an urban mosquito, has a limited range of approximately 200–500 m from its breeding site as determined by capture-release studies [122]. Infected viremic individuals move from one *A. aegypti* area to another and introduce the virus to feeding vectors that then become infected. After an extrinsic incubation period of approximately 14 days, mosquitoes can then feed and infect the susceptible human host, typically within a 500 M radius, thereby propagating a restricted spatial outbreak. A useful metaphor is to imagine a large-scale DENV outbreak as throwing a series of small pebbles in a pond. Each pebble represents the location of one infected individual that infects an *A. aegypti* mosquito. The waves from the one pebble represent individuals who are infected from that mosquito producing a spatially confined outbreak. As more and more pebbles are thrown in the pond, the numerous resulting waves create more and more spatially confined outbreaks producing a large outbreak across a country or region. This wave concept was illustrated by a mathematical model of how DENV travels across Thailand during the epidemic season in each of several years [123]. The authors demonstrated the existence of a spatial-temporal traveling wave in the incidence of DHF with the wave emanating from Bangkok moving radially at a speed of 148 km per month.

5.4 Age and Sex Distribution

In countries with endemic DENV where the force of infection is high, dengue illness becomes primarily a pediatric disease. Maternal DENV antibody is transplacentally transferred to the infant conferring a degree of protection that lasts for approximately 12 months [124]. The first infection with DENV typically occurs before the age of five as a primary infection and goes largely unrecognized as just another febrile illness that infants experience. The second infection often occurs between the age of 5 and the teenage years and as a secondary infection produces severe DENV illness. The age for severe infection ranges between 5 and 15 years and both sexes are equally affected [115]. In a recent study of children in Bangkok with severe infection, ages ranged from 18 months to 15 years with a mean age of 8.6 years. The mean age of children diagnosed with DF was 8.5 years and 8.7 years for children with DHF [125]. Similar age and sex distributions are seen in the regions of the Americas where DENV infections are endemic [126].

5.4.1 Phenomenon of the Increasing Median Age in SE Asia

First in Thailand and then in other Southeast Asian countries, it was observed that the median age of severe dengue illness was increasing over time. To determine the reasons for this observation, an analysis was performed from 72 provinces of Thailand to examine the force of infection and demographic and climactic variables [127]. The force of infection was found to have declined by 2 % each year, with the strongest predictor for this change being the median age of the population. The increase in median age was explained by a reduced birth rate and a shift in the population age structure to include older individuals. Lower birth and death rates are thought to decrease the number of susceptible individuals in the population as a result of increased longevity of immune individuals. If true, other countries with shifts in the age of the population to older individuals may also see an increase in the age of severe dengue illness.

5.5 Occurrence in Different Settings

5.5.1 Prospective Studies in Families and Schools

A number of prospective studies conducted in families and schools have played an important role in increasing our understanding of DENV transmission and the pathogenesis of subclinical and severe infection. The readers are referred to a review that discusses these studies in more detail [82]. Table 15.2 summarizes these studies and their salient study findings. Burke and colleagues performed a 2-year (1980–1981) school-based study involving 1,757 children, ages 4–16 years, in Bangkok, Thailand [91]. DENV-antibody testing demonstrated a high antibody prevalence with 50 % of the children having evidence of preexisting dengue antibody, indicating infection prior to the age of 7 years. The majority (87 %) of the students became infected during the study period but remained asymptomatic. Of those who developed symptomatic illness, 53 % were classified as having DHF. Overall incidence was 6.3 % and a symptomatic-to-asymptomatic ratio of 1:8. The risk for DHF in secondary infection was ≥6.5. Table 15.2 summarizes the incidence of infection and the symptomatic-to-asymptomatic ratio in the various cohorts. An important result of these prospective studies has been a better understanding of the full burden of subclinical infection, particularly in those patients who contribute to the pool of potential hosts for mosquito infections but go unreported in most surveillance programs. Another value of the prospective studies is to determine the full economic burden of dengue disease. In the Thai studies, incidence and economic impact were used to calculate the disability-adjusted life years (DALYs) lost to DENV infections [117]. The mean DALYs per million population was 465 per year which accounted for 15 % of DALYs lost to all febrile illnesses. Nonhospitalized patients were an underappreciated economic burden that represented a substantial proportion of the overall disease burden; 44–73 % of the total DALYs were lost to dengue each year. During high incidence years, the number of DALYs lost to dengue was greater than that calculated for important tropical diseases such as meningitis and hepatitis B and was three times greater than reported by WHO [128].

5.5.2 Military

The US military considers developing a DENV vaccine a high priority and has sustained an effort to develop an effective vaccine for over 50 years [129]. The reason for this effort is the impact that DENV infection has had on US military operations since the Spanish-American War [2, 130]. Before WWII soldiers stationed in Panama and the Philippines experienced major outbreaks of DENV infection. During WWII several hundred soldiers stationed in Queensland, Australia, were hospitalized with dengue, and outbreaks occurred in soldiers throughout the South Pacific. Severe outbreaks occurred in US soldiers landing in Saipan in July 1944; the initial incidence of 300 cases per 1,000 rose to

Table 15.2 Prospective studies in families and schools[a]

Study site	Population size	Age range (years)	Study period	Incidence (average)			Symptomatic: asymptomatic ratio
				Dengue infection (%)	Symptomatic dengue (%)	Hospitalized dengue (%)	
Bangkok, Thailand [91]	1,757	4–16	1980–1981	11.8	0.7	0.4	1:8
Rayong, Thailand [90]	1,056	4–14	1980–1981	9.4		0.1	
Yangon, Myanmar [167]	12,489	1–9	1984–1988	5.1		0.3	
Yogyakarta, Indonesia [341]	1,837	4–9	1995–1996	29.2	0.6	0.4	
Kamphaeng Phet I, Thailand [115]	2,119	7–11	1998–2002	7.3	3.9	1.0	1:0.9
Managua, Nicaragua [342]	1,186	4–16	2001–2002	9.0	0.85		1:13–1:6
Kamphaeng Phet II, Thailand [119]	2,095	4–16	2004–2006	6.1	2.0	0.5	1:3.0
West Java, Indonesia [343]	2,536	18–66	2000–2002	7.4	1.8	0.1	1:3

Adapted from Endy et al. [82]

[a]Number in cohort tested for dengue antibody (incidence denominator)

3,500 per 1,000 [2]. After WWII dengue was a significant cause of illness and noncombat injuries in soldiers stationed in Vietnam and most recently during operations in both Haiti and Somalia. Soldiers deployed to Haiti in particular suffered from acute dengue illness, which was responsible for 30 % of all febrile illness [131]. DENV infections continue to be documented in deploying military personnel.

5.5.3 Risk to Travelers

Dengue poses a significant risk to travelers vacationing or working in dengue-endemic countries and is now considered the most common cause of febrile illness in returning travelers [132]. Information from the US Centers for Disease Control and Prevention's laboratory-based Passive Dengue Surveillance System (PDSS) described trends in travel-associated dengue from January 1, 1996 to December 31, 2005. Of 1,196 suspected dengue infections in returning travelers, 334 (28 %) were confirmed laboratory positive. Regions where travelers were at particularly high risk for infection were the Caribbean, Mexico, Central America, and Asia [132]. Dengue in returning travelers was examined by the GeoSentinel Surveillance Network [133]. Dengue cases demonstrated regional and seasonal peaks—June to September for Southeast Asia, October for South Central Asia, March for South America, and August to October for the Caribbean. In Southeast Asia, for example, the annual incidence of dengue in travelers increased from 50 cases per 1,000 in non-epidemic years to 159 cases per 1,000 during epidemic years [133].

5.5.4 Adult Symptomatic Dengue Infection

As noted, DENV infection in endemic countries due to the high force of infection is primarily a pediatric disease. However, DENV infection occurs at all ages. In countries that experience sporadic DENV outbreaks because of recent introductions, such as islands, all ages are infected and severe illness will occur in adults. Current knowledge of the pathogenesis of adult DHF is limited to a few studies. A prospective study performed in Thai patients in Phetchabun Provincial Hospital demonstrated significant clinical differences in adults as compared to children [134]. More common in adults than in children were manifestations such as petechiae, melena, headache, retro-orbital pain, joint pain, myalgia, nausea, and emesis. A retrospective study of Taiwanese adults and children during a DENV-2 outbreak in 2002 demonstrated that adult patients presented more frequently with arthralgias, myalgias, headache, and abdominal pain as compared to children [135]. Adults had more severe thrombocytopenia, elevated alanine aminotransferase (ALT) levels, and greater incidence of upper gastrointestinal bleeding and DHF. Clearly different in adults than in children are the higher rates of comorbidities such as diabetes mellitus, hypertension, chronic obstructive pulmonary disease, and previous stroke that contributed to the severity of dengue illness. In a recent study of 309 adults with DHF, causes of fatality were massive gastrointestinal (GI) bleeding, dengue shock syndrome (DSS), subarachnoid hemorrhage, and bacteremia from *Klebsiella pneumoniae* [136]. The influence of comorbidities and the pathogenesis of severe dengue illness in adults should be a high priority for future research.

6 Pathogenesis and Immunity

Most human infections with DENV originate from a transmission cycle between infected humans and mosquitoes of the genus *Aedes*, of which *A. aegypti* constitutes the most important vector for epidemic dengue worldwide [137]. Sylvatic transmission of DENV between nonhuman primates and other *Aedes* species has been documented in forest areas of Africa and Asia [138, 139]. Some evidence suggests that this cycle served as the original source of the viruses now circulating in the human population, but the latter viruses have since evolved as separate lineages with little evidence of ongoing interaction between these transmission cycles. Mosquitoes acquire DENV through feeding on the blood of viremic hosts. Viral infection of the mosquito midgut epithelium leads to dissemination and later infection of salivary gland tissue [140]. At that point, the mosquito is capable of transmitting the virus to a vertebrate host during subsequent blood feedings and remains infectious for the remainder of its lifetime. The time required for the mosquito to become infectious, or extrinsic incubation period, is modulated by ambient temperature, with higher temperatures resulting in a shorter extrinsic incubation period and therefore a greater potential for transmission [141].

DENV injected into the dermis during mosquito feeding most likely initially infects cells locally. Many different cell types can be infected with DENV in vitro, including human fibroblasts, but Langerhans cells or tissue macrophages are considered more likely initial targets of infection [142]. Additional components of the mosquito saliva may enhance infectivity, based on experiments in animal models, through mechanisms that are poorly defined [143]. The intrinsic (in humans) incubation period typically ranges from 4 to 9 days but can extend up to 14 days [141]. In experimental models, DENV is detected in local and regional lymph nodes and likely undergoes some replication there [144, 145]. Following the local/regional replication of DENV, systemic dissemination occurs. Viremia is detectable in nearly all symptomatic infections, often reaching very high titers within the first few days of symptoms [146, 147]. Dendritic cells, monocytes, and macrophages are considered the principal cellular targets; there is also substantial evidence of infection of B lymphocytes and hepatocytes [148–150]. Virus is detected principally in the liver and tissues of the reticuloendothelial

system including the bone marrow [151]. There is limited evidence for infection of other cells types in vivo, including mast cells, vascular endothelial cells, neurons, myocytes, and platelets; however, the frequency and significance of these phenomena remain controversial.

Innate immune responses, especially the production of type I interferons (IFN), are induced in both infected cells and in natural IFN-producing cells such as plasmacytoid dendritic cells [152, 153], and these responses likely account for many of the systemic symptoms and signs of infection. Plasma levels of IFN follow kinetics similar to plasma viral titers and are often at or near peak levels at the onset of fever and systemic symptoms [154]. Although DENV has evolved several immune evasion mechanisms, inhibiting both IFN production and response [155], studies in laboratory mice suggest that these innate responses are critical to the control of DENV infection [156].

Activation of adaptive immune responses can be detected within the first few days, with an increase in the circulating levels of activated T lymphocytes, plasmablasts, NK cells, DENV-specific T lymphocytes and antibodies, soluble markers of immune activation, and a wide array of both pro-inflammatory and anti-inflammatory cytokines [157–160]. This immune activation coincides with a rapid clearance of virus from both plasma and peripheral blood monocytes and lymphocytes, as well as the most serious symptoms and signs of dengue, including maximal thrombocytopenia, bleeding, elevation of hepatic transaminases, and plasma leakage.

Leukopenia, affecting both neutrophils and lymphocytes, is present throughout much of the symptomatic phase, and its severity does not correlate with other measures of disease severity [161]. Other common laboratory abnormalities include thombocytopenia and elevations in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT); these abnormalities reach their extremes late in illness, coincident with plasma leakage, and correlate with the overall severity of illness. In fatal cases, the principal findings are serous effusions in pleural and peritoneal cavities, widely distributed microhemorrhages, and hepatocellular injury that can range from mild to severe [162]. The paucity of histologic findings in the vasculature has suggested that the enhanced vascular permeability represents physiologic disturbance to a greater extent than structural damage to blood vessel walls. Edema of the lung parenchyma is uncommon, even in patients with severe plasma leakage, and may reflect overly aggressive fluid administration rather than a consequence of DENV infection per se. Inflammation of the brain, heart, and other tissues has been described in isolated cases or small case series but is atypical.

DENV infection of bone marrow cells may in part explain the cytopenias [163], but platelet consumption is also increased, particularly in severe cases [164, 165]. Bleeding does not correlate well with the severity of thrombocytopenia, however. Other contributing factors may include coagulopathy due to clotting factor consumption and inhibitory antibodies recognizing cross-reactive determinants on clotting or hemostatic proteins [166]. The pathophysiologic basis of dengue-associated plasma leakage has been and remains a source of controversy. The competing hypotheses of a viral mechanism versus an immunologic mechanism for this syndrome have largely been decided in favor of a principally immunologic mechanism on the basis of strong epidemiologic data showing an association with prior DENV exposure [91, 167]. Nevertheless, viral factors are obviously also involved, as some DENV genotypes appear to be incapable of triggering plasma leakage [168]. Elevated production of factors promoting vascular permeability—TNF-α and vascular endothelial growth factor (VEGF) among others—is clearly correlated with the extent of plasma leakage [158]. The sources of these factors and reasons for their increased production remain unclear, however. High level viremia appears to be necessary to initiate this cascade of events, but is not sufficient to cause plasma leakage. Furthermore, plasma leakage occurs several days after the peak of viremia and instead closer in time to the period of rapid clearance of viremia; these observations support the immune-mediated model of pathogenesis [147]. Both DENV-infected cells, such as monocytes, macrophages, dendritic cells, and mast cells, and responding effector cells, such as NK and T cells, produce factors that are capable of contributing to vascular permeability under experimental conditions [152, 169–171]. Given this variety of potential mechanisms, it is possible that no one single pathway explains plasma leakage in all cases.

Patients who have recovered from acute dengue illness demonstrate antibodies and T lymphocytes that recognize both serotype-specific and serotype-cross-reactive determinants. The principal targets of DENV-specific antibodies are the E, pre-M, and NS1 proteins [172–174]. Antibodies to the E protein mediate neutralization in vitro and in vivo to various degrees determined by both the antibody affinity and the epitope targeted; antibodies directed at domain III of the protein tend to display greater serotype specificity and neutralization activity [172]. Antibodies with lower affinity and those with high affinity when present at concentrations below the threshold for neutralization form immune complexes with virions and facilitate uptake into and productive infection of immunoglobulin receptor-bearing cells in vitro and in vivo, i.e., ADE [175]. In addition to increasing the mass of infected cells, infection via ADE may also modify infection by (a) increasing the number of progeny virions produced by each infected cell and (b) inducing IL-10 and other cytokines via signaling from the immunoglobulin receptor(s) [176]. Antibodies to pre-M bind only to immature or partially

mature virions. Although a pre-M-specific monoclonal antibody was reported to protect mice from a lethal DENV challenge [177], other studies have found these antibodies to have poor neutralizing activity but to be capable of enhancing the infectivity of immature virions [178]. Antibodies to NS1, in contrast, are incapable of binding to virions; these antibodies bind both to soluble NS1, which is released from infected cells and is found in the circulation, and to NS1 on the surface of infected cells [179, 180]. Binding of anti-NS1 antibodies to cells can trigger complement-mediated and/or cell-mediated cytotoxicity [181, 182]. Under some conditions, soluble NS1 can attach to the surface of uninfected cells, thereby causing uninfected cells to be targeted by the same processes [183]. Some antibodies to NS1 also bind in a cross-reactive fashion to determinants on components of the coagulation pathway or platelet/endothelial cell surface proteins [166].

Epitopes recognized by CD4 and CD8 T lymphocytes have been identified in all of the DENV proteins [184–186]. Several studies have found a concentration of epitopes in the NS3 protein, however, raising the possibility that this protein has features associated with higher levels of MHC class I and II presentation or has sequence constraints that limit its ability to mutate toward reduced immunogenicity. Upon contact with DENV-infected cells, DENV-specific T cells secrete predominantly type 1 cytokines (IFN-γ, TNF-α, and IL-2) and lyse antigen-presenting cells [187, 188]. An intriguing feature of the serotype-cross-reactive T-cell response is that the sequence differences between serotypes (usually 1–3 residues of the 9 amino acids in the epitope) result in both quantitative and qualitative changes in the effector functions expressed by T cells, sometime referred to as heterologous immunity [170, 171, 189, 190]. In particular, the production of the antiviral cytokine IFN-γ is reportedly diminished, while the production of pro-inflammatory cytokines such as TNF-α and MIP-1β is unchanged.

The immune response differs substantially between the first (primary) DENV infection and subsequent (secondary, tertiary, and quarternary) DENV infections. The differences are attributed to two factors. First, at the time of the secondary and subsequent infections, memory T and B lymphocytes are already present, having been induced by the earlier primary infection. Second, because reinfection with the same serotype is not known to occur, secondary and subsequent infections involve antigenic differences from the earlier infection. Anti-DENV IgG antibodies rise earlier and reach dramatically higher peak titers, while IgM antibody responses are blunted, and the antibodies produced are predominantly serotype cross-reactive. Most of the serotype-cross-reactive antibodies display lower affinity for epitopes on the newly infecting DENV than for the previously encountered DENV, however [174, 191, 192]. The frequencies of circulating DENV-specific T lymphocytes and their expression of activation markers are similarly high in primary and subsequent infections [190]. However, a greater proportion of DENV-specific T lymphocytes target more conserved regions of the DENV proteome and show serotype cross-reactivity in their responses.

The immune responses induced by DENV infection described above can have either protective or pathological effects during the second or subsequent DENV infection. Protective immunity is mainly serotype specific. Protective immunity can be ascribed to high-avidity E protein-specific neutralizing antibodies based on experimental challenge studies in animal models [193]. However, in vitro assays of neutralizing antibody are a poor measure of protective immunity, especially in individuals who have had several prior DENV infections, in whom antibody responses are highly serotype cross-reactive [116]. Antibodies to the pre-M and NS1 proteins have also shown protective efficacy in animal models, but the applicability of these findings to human infection is unclear. Studies of protection mediated by DENV-specific T lymphocytes are more limited. However, one prospective cohort study conducted in Thailand found an association between higher baseline DENV-specific T-cell IFN-γ responses and subsequent subclinical DENV infection [194], and a survey of healthy adults from Sri Lanka found higher DENV-specific T-cell IFN-γ responses in subjects with HLA alleles that were associated with a lower risk of DHF [186].

Except for a short (several months) period of resistance to challenge with other serotypes, individuals who have had one (primary) DENV infection are not only at risk for infection with any of the other three serotypes, but they are actually at increased risk for development of dengue-related plasma leakage compared to individuals who have never had prior exposure to DENV [20, 82, 91]. This finding is the principal basis for proposing the existence of pathologic immune responses to DENV. Both DENV-specific antibodies and T cells have pathologic potential, through mechanisms discussed above. The observation by Halstead of enhanced DENV infection due to ADE (see above) provided a model for antibody-mediated immunopathology [195] and has also been invoked to explain dengue-associated plasma leakage among infants in endemic countries, who acquire DENV-specific antibodies transplacentally from their DENV-immune mothers [114]. At odds with this model are the observations that (a) plasma leakage occurs several days after the peak viremia, (b) anti-DENV antibodies are neither necessary nor sufficient for the occurrence of plasma leakage, and (c) preinfection serum ADE activity has not been correlated with disease severity [196, 197]. Immune activation and cytokine production thus appear to be a critical link in the pathogenetic cascade. One study has suggested an association with T-cell production of TNF-α [198], but the in vivo evidence for a specific T-cell mechanism remains extremely limited.

7 Patterns of Host Response

7.1 Common Clinical Features

Many DENV infections resolve without a recognized illness, particularly in children [115]. Among patients with laboratory-confirmed dengue illness, constitutional symptoms are most often reported, including fever, headache, eye pain, body pain, and joint pain (60–90 % of patients) [199]. Rash, typically macular or maculopapular, is reported in approximately half of the subjects. Gastrointestinal symptoms—nausea, vomiting, or diarrhea—occur in 30–50 % of patients, and respiratory symptoms, including cough, sore throat, and nasal congestion, are somewhat less common (~1/3 of patients). The frequency of symptoms is influenced by the patient's age, sex, and previous history of DENV exposure (primary vs. secondary DENV infections) [199]. All symptoms are less frequent in children. Joint pain, body pain, and rashes are more commonly reported in females. Rashes have been more frequently reported in primary DENV infections, whereas constitutional and gastrointestinal symptoms have been more common in patients experiencing a secondary DENV infection.

Fever accompanied by prominent headache, retro-orbital pain, and muscle and joint pains forms the syndrome of classical dengue fever (DF), which had historically evoked more descriptive terminology such as "breakbone fever" [39]. However, these classic symptoms have been reported in a minority of cases in studies of travelers or military personnel [131, 200–202]. The duration of symptoms in DF typically ranges 5–7 days [202], with some patients showing a biphasic ("saddleback") fever curve. Periods of marked fatigue lasting days to weeks after the acute illness are occasionally reported, especially in adults.

Hemorrhagic manifestations occur often in patients with DF, with spontaneous bleeding in as many as two thirds of cases [158]. The skin (petechiae) and nose are the most common bleeding sites; gastrointestinal or genitourinary bleeding is less common [202]. In rare cases, bleeding is severe enough to be life threatening, but this clinical presentation needs to be differentiated from dengue hemorrhagic fever (DHF), described below.

The physical examination in patients with DF is generally nonspecific, with fever and rash being the most common findings. Typical laboratory findings include leukopenia, thrombocytopenia, and elevated hepatic transaminases [131, 161, 203]. Thrombocytopenia can be profound, with platelet counts <100,000 cells/mm^3 observed in one quarter to one half of patients. Serum aspartate transaminase (AST) levels are characteristically more elevated than alanine aminotransferase (ALT) levels; elevations are usually modest (2–5 × upper limit of normal), but marked elevations (5–15 times the upper limit of normal) are occasionally noted [202, 203].

7.2 Dengue Hemorrhagic Fever

Plasma leakage is the most worrisome manifestation of DENV infection, although other less common syndromes can be life threatening. For historical reasons, this syndrome is referred to as dengue hemorrhagic fever (DHF) and has been defined based on the presence of four cardinal features [3, 5, 18]: fever lasting 2–7 days; increased vascular permeability as evidenced by hemoconcentration (20 % or greater rise in hematocrit above baseline value), pleural effusion, or ascites [15]; marked thrombocytopenia (platelet count 100,000 cells/mm^3 or lower); and a hemorrhagic tendency as demonstrated by a positive tourniquet test or spontaneous bleeding. The term dengue shock syndrome (DSS) is used when shock is present along with these four criteria [18].

The increase in vascular permeability leading to plasma leakage in DHF develops rapidly, over a period of 24–48 h, and characteristically occurs between 3 and 7 days after the onset of illness [204]. This time point coincides with the disappearance of fever, the nadir of platelet count, and the peak of serum aminotransferase elevations [205]. Abdominal pain is reported shortly before the onset of plasma leakage in ~60 % of patients with DHF [206–208]. Intense abdominal pain, persistent vomiting, change from fever to hypothermia, and marked restlessness or lethargy, especially occurring during this window of time, have been proposed as "alarm signs" for clinicians of possible impending shock [209].

The hemorrhagic manifestations are variable in severity among patients with DHF. Spontaneous petechiae or ecchymoses have been reported in approximately one half of cases with DHF [206, 208]. The tourniquet test, performed by inflating a blood pressure cuff on the arm to a level halfway between systolic and diastolic pressure for 5 min, elicits petechiae in the remaining cases. Less common sites of spontaneous bleeding include the nose, the gastrointestinal tract (hematemesis more often than melena), and genitourinary tract (more often in women).

Leukopenia and thrombocytopenia are common laboratory findings in DHF, the latter being a component of the WHO case definition. Serum transaminase levels are significantly different between DF and DHF, and they have been proposed as useful markers of illness severity prior to clinically evident plasma leakage [203, 210].

There continues to be debate as to whether DF and DHF are different diseases, implying distinct pathogenesis mechanisms, or whether DHF represents an extreme along a continuous spectrum of disease severity encompassing both patient populations. Some investigators have highlighted cases that have been difficult to classify, usually where plasma leakage is apparent, but the other three criteria of DHF were not all met [211]. Although such cases occur, they clearly represent a minority of patients, as most patients with plasma leakage display the full picture of DHF [158].

7.3 Atypical Dengue Syndromes

In rare cases, liver failure or neurologic dysfunction is the predominant clinical feature in a patient with DENV infection [212–215]. Liver failure may have been a consequence of prolonged shock in some cases, rather than a direct viral effect. A wide range of neurological manifestations have been described in patients with laboratory-confirmed DENV infection, including encephalopathy, seizures, pure motor weakness, mononeuropathies, polyneuropathies, Guillain-Barré syndrome, and transverse myelitis [212, 213, 216, 217]; some patients have none of the characteristic features of DF or DHF. A causal association has been supported by the occasional detection of DENV in cerebrospinal fluid. Myocarditis, cholecystitis, and retinal vasculitis [218], have been reported in isolated patients with serologic evidence of acute dengue, but a causal association with DENV is not definitively established since subclinical DENV infections are common in endemic areas.

7.4 Differential Diagnosis

The clinical features of DF are largely nonspecific. The differential diagnosis for this syndrome includes influenza, enteroviral infection, measles, and rubella. In residents of or travelers to endemic areas, malaria, leptospirosis, and typhoid fever are also relevant for consideration [219]. Over 90 % of patients in dengue-endemic areas who meet all of the criteria in the case definition for DHF have serologic evidence of DENV infection [158]. Bacterial sepsis, leptospirosis, malaria, and other viral hemorrhagic fevers are worthy of consideration, however.

8 Control and Prevention

8.1 Treatment

There is no licensed therapeutic agent or vaccine to prevent dengue disease. The recommended treatment for patients with suspected acute DENV infection includes supportive care, acetaminophen for relief of fever and aches, and monitoring to detect plasma leakage and other severe manifestations at the earliest stage possible. Aspirin and nonsteroidal anti-inflammatory drugs are best avoided to minimize the risk for bleeding and Reye syndrome. There are no antiviral drugs available at the present time with demonstrated efficacy in dengue. Chloroquine, which shows some antiviral activity in vitro, was ineffective in a high-quality clinical trial [220].

There has been substantial interest in the potential value of immunomodulation as a strategy to reduce the severity of dengue illness, based on the model of immune-mediated pathogenesis described above. In controlled clinical trials, corticosteroids have not proven effective either in patients with established shock [221] or when given earlier in illness [222]. Other adjunctive therapies, for example, pentoxifylline or intravenous immunoglobulins, have only been tested in uncontrolled case series or small clinical trials.

8.2 Vector Control

Efforts to control endemic and epidemic dengue transmission and reduce disease burden have been limited largely to vector control and personal protective measures to reduce human-vector interaction [223]. The World Health Organization (WHO) promotes a concept of Integrated Vector Management (IVM) with the aim to improve efficacy, cost-effectiveness, ecological soundness, and sustainability of disease-vector control. Program pillars include environmental management (i.e., reduce potential breeding containers, modify human behavior), chemical control (i.e., larvicides/adulticides and residual and space spraying), and biologic control (i.e., fish, predatory copepods) [224]. There are historic examples of successful vector control campaigns. In the late 1940s, Dr. Fred Soper led an *A. aegypti* eradication effort in the Americas; between 1948 and 1962, the species was eliminated from 21 countries. Unfortunately, success was short lived due to reduced funding, lack of program leadership, lack of political will, and a deficit of highly trained people to design and execute the programs [223]. In 1973, Singapore completed the implementation of a vector control program to reduce the premise index (i.e., the percentage of houses infested with *A. aegypti* larvae and/or pupae), bringing the index to ~2 % with an associated 15-year period of low dengue disease incidence. However, in the 1990s, despite maintaining the premise index ~2 %, dengue surged [225]. Singapore's experience highlighted the complexity of managing vector control programs and the need for a more evidenced-based and data-driven approach to designing and implementing control programs. In addition, a more strategic application of tools capable of defining and modeling dengue disease and vector population dynamics in space and time was needed.

Interesting advances have been made in vector-based interventions to include biocontrol and genetic modification of vector populations [226]. One example is the proposed use of *Wolbachia*, an endosymbiotic bacterium estimated to be present in up to 66 % of insect species [227]. The bacterium infects the gonads, where it ensures transmission to the next host generation (from mother to egg) and manipulates reproductive capabilities [226]. The manipulations directly or indirectly benefit the infected females assisting with its spread through host populations [228]. Other changes

associated with *Wolbachia* infections include a shortened lifespan, altered locomotor activity, and poor blood feeding in mosquitoes [229–231]. *Wolbachia* use to control insect populations has been explored since 1967 [232]. Three *Wolbachia* strains have been successfully introduced into the *A. aegypti* populations, setting the stage for release studies [230, 233, 234]. Mosquito population reduction and suppression, decreased pathogen transmission, and/or decreased pathogen replication in mosquito species are desirable outcomes [226]. Recent, small-scale releases demonstrated the ability for genetically modified males to persist in nature and reduce wild-type populations [235, 236]. Regulatory issues, proof-of-concept trial design, and sustainability are a few of the issues requiring extensive thought prior to large-scale use [237].

Vector control through any means will require community buy-in and, in some cases, active participation. Political will and sustained financial resources will be required for any effort to be effective in reducing disease burden. Providing scientists and operational personnel state-of-the-art education and access to advanced epidemiologic tools (global positioning systems, modeling software, etc.) will increase the likelihood of program success.

8.3 Vaccines

The dengue vaccine pipeline in 2013 is robust; the following is a review of the approaches currently being tested in human trials. Initial development efforts began in 1929 with unsuccessful attempts to produce an inactivated virus vaccine using phenol, formalin, or bile [46]. During World War II, live attenuated virus (LAV) vaccines were explored by serially passaging DENV-1 and DENV-2 strains in suckling mouse brain [45, 238, 239]. Mouse brain-derived candidates were eventually abandoned for cell culture-based vaccines. Currently, six different vaccine approaches have been tested in human clinical trials, and a single candidate is in phase 3 clinical trials.

Flaviviral live attenuated virus (LAV) vaccines (yellow fever (YF), Japanese encephalitis) have proved to be safe and durably efficacious [240–243]. LAV vaccines replicate in the recipient, presenting all viral antigens in the vaccine construct, and eliciting both antibody and T-cell responses, resembling those seen after natural infection. A tetravalent vaccine approach (i.e., DENV-1 + DENV-2 + DENV-3 + DENV-4) is designed to induce primary-type immune responses to all four dengue viruses simultaneously. The first major effort at live attenuated dengue vaccine development was made at Mahidol University in Bangkok; investigators used the classical method of serial passage of virus in dog (PDK) and nonhuman primate (PGMK) cell lines [244–250]. The effort experienced early success, but tetravalent formulation and balancing immunogenicity (i.e., neutralizing antibody responses to each dengue virus type) with safety proved difficult [251–254].

The Walter Reed Army Institute of Research (WRAIR) also developed LAV candidates by serial PDK passage of each dengue virus type with final passages in fetal rhesus lung cells (FRhL). Early development efforts identified dengue virus strains and promising formulations combining variations of attenuation and dengue virus antigen concentrations [255–263]. A single formulation was tested in Thai schoolchildren and toddlers. Vaccination was well tolerated and, although variable neutralizing antibody responses were observed across cohorts, was sufficiently immunogenic to pursue continued development of the candidate [264, 265]. Newly derived vaccine lots were tested in adults in the United States and in Thailand and Puerto Rico across a broad age range (12 months to 50 years). There were no overt safety signals in over 300 vaccine recipients, and rates of seroconversion were moderate to high [264, 266]. Although the candidate demonstrated promise, the WRAIR and its corporate partner indefinitely suspended development in pursuit of a superior target product profile (i.e., shorter dosing schedule).

The US National Institutes of Health (NIH) constructed viable cDNA clones of DENV-4 and induced a 30-nucleotide deletion in the 3′ untranslated region (i.e., directed mutagenesis). The result was lower viremia levels than the parental strain and retained immunogenicity. Numerous monovalent vaccine candidates were tested in phase 1 clinical trials to identify optimal candidates for tetravalent formulation [267–275]. Tetravalent candidates (admixtures, TetraVax-DV) were prepared and are being tested in phase 1/2 clinical trials [276].

The insertion of dengue *preM* and *E* genes into the cDNA backbone of YF 17D as a vaccine development platform (i.e., chimera) was pioneered at the St. Louis University Health Sciences Center, further developed by Acambis, Inc., and licensed for manufacture to Sanofi Pasteur [277–281]. Phase 1 clinical trials of monovalent vaccine preparations in YF-naïve and YF-primed volunteers demonstrated acceptable safety and immunogenicity profiles (i.e., neutralizing antibody responses) [282, 283]. Expanded trials with tetravalent preparations in volunteers of various ages, genetic backgrounds, and flavivirus priming status (i.e., preexisting immunity to YF, dengue, Japanese encephalitis) demonstrated a consistently excellent safety profile and robust, balanced neutralizing antibody profiles [284–288]. Sanofi's ChimeriVax (a construct containing yellow fever and dengue components) was the first dengue vaccine candidate tested in a clinical endpoint efficacy study. The phase 2b trial was an observer-blinded, randomized, controlled, single-center trial in healthy Thai schoolchildren ($N = 4{,}002$) aged 4–11 years who were randomly assigned (2:1) to receive three injections

of dengue vaccine or control (rabies vaccine or placebo) at 0, 6, and 12 months. All subjects were followed for dengue illness. The primary objective was to assess protective efficacy against virologically confirmed, symptomatic dengue, irrespective of severity or serotype, occurring 1 month or longer after the third injection. Although the vaccine was safe and neutralizing antibody responses were moderate to high, overall efficacy was 30.2 % (95 % CI −13.4 to 56.6) and differed by serotype [289]. The results of this trial were disappointing and highlighted the numerous, still unanswered, questions which exist regarding immuno-protective profiles (see below). Phase 3 trials in Latin America and Asia are ongoing.

The US Centers for Disease Control and Prevention (CDC) developed a tetravalent chimeric dengue vaccine by introducing DENV-1, DENV-3, and DENV-4 *prM* and *E* genes into cDNA derived from an attenuated LAV DENV-2 component [290–294]. Dengue-dengue chimeras were formulated as a DENV-1/DENV-2, DENV-2, DENV-3/DENV-2, and DENV-4/DENV-2 tetravalent vaccine candidates (DENVax) and licensed to Inviragen, Inc. [295, 296]. Phase 1 clinical trials in dengue-endemic and dengue-non-endemic areas are underway [297]. Another approach to producing viral vaccines is the use of inactivated whole virus or viral subunits. Such vaccines have potential advantages to include the inability to revert to a more pathogenic phenotype, they are unlikely to produce immune interference when combined into a tetravalent formulation, and, theoretically, there could be fewer safety issues. Inactivated flaviviral vaccines have been licensed and are in wide use to prevent Japanese encephalitis and tick-borne encephalitis [298–300].

These vaccines have certain potential disadvantages. Killed or subunit vaccines raise antibodies to only a portion of the structural proteins and normal virion-based structural conformation. High concentrations of antigens and multiple doses may also be required to induce a potent and protective immune response. Another concern is the potential for an adverse response resembling the occurrence of atypical measles and respiratory syncytial virus (RSV) infection following vaccination and subsequent wild-type exposure [301, 302]. Merck & Co. is developing a dengue vaccine candidate produced in a Drosophila S2 cell expression system [303–305]. A single DENV-1 monovalent trial has been completed and a phase 1 tetravalent trial is under way [297].

The WRAIR developed a purified inactivated virus (PIV) dengue vaccine candidate by inactivating with formalin each of the dengue virus types [305–308]. A phase 1 trial of a monovalent DENV-1 PIV adjuvanted with alum was completed in a small number of US flavivirus-naïve volunteers. The candidate had an acceptable safety profile with low to moderate immunogenicity. GlaxoSmithKline Vaccines (GSK) and the WRAIR are currently testing a tetravalent PIV formulation adjuvanted with GSK's proprietary adjuvant systems. Phase 1 trials in the United States and Puerto Rico are underway.

DNA vaccines consist of one or more plasmids containing DENV genes reproduced to high copy number in bacteria such as *E. coli*. The plasmid contains a eukaryotic promoter and termination sequence to drive transcription and presentation to the immune system. DNA vaccine advantages include ease of production, stability and ability to be transported at room temperature, ability to accept new genes, ability to immunize against multiple pathogens with a single construct, and reduced reactogenicity [309]. A DENV-1 monovalent DNA vaccine trial enrolled 22 flavivirus-naïve US volunteers. None of the low-dosage recipients and only five of 11 high-dosage recipients developed neutralizing antibodies. The safety profile was acceptable and supported exploration of an adjuvanted DNA dengue vaccine [310].

In addition to the dengue vaccine candidates listed above in clinical development, there are also numerous in preclinical development (i.e., mice and nonhuman primate studies). Without an animal model of disease, a human challenge model, or a correlate of protection, it will be a slow and arduous process to advance candidates through efficacy trials. Despite this, the field is hopeful a dengue vaccine can be licensed in an endemic country within 5 years.

9 Unresolved Problems

Unraveling the virologic and immunologic complexities of the dengue viruses, virus-vector-host interactions, and dengue disease has been challenging. The existence and co-circulation of four antigenically distinct viruses, the absence of an animal model of disease, the absence of a dengue human infection model, and gaps in our comprehensive understanding of what constitutes immunopathologic versus immuno-protective profiles have contributed to difficulties in prognosticating, in a clinically relevant way, dengue disease severity and the development of anti-dengue virus therapeutics and protective vaccine candidates. The disconnect between vaccine immunogenicity, as measured using a standard plaque reduction neutralizing antibody test, and clinical outcome following wild-type virus exposure further complicates the field. The following are a few of the knowledge gaps and areas of current scientific interest and exploration.

There is no well-characterized and comprehensive animal model of dengue disease. However, significant resources have been allocated and development progress has been made [311, 312, 313, 314]. Two models currently being explored include the hu-HSC mouse model created by human hematopoietic stem cell (HSC) engraftment and the

BLT mouse model prepared by co-transplantation of the human fetal liver, thymus, and HSC [315]. Humanized mouse models with a transplanted human immune system have the ability to generate T cells, B cells, macrophages, dendritic cells, and natural killer (NK) cells. Theoretically, these mice possess the immunologic "building blocks" required to generate the pro-inflammatory cascade of immunologic events required to induce clinical outcomes such as plasma leakage and hemorrhage. Until further development and optimization is achieved, mouse models will be absent from the "critical path" components of advanced drug or vaccine development programs.

Following dengue virus exposure, nonhuman primates develop infection as evidenced by measurable replicating peripheral viremia and neutralizing antibody and cellular immune responses. Nonhuman primates do not consistently develop objective and measurable clinical or biochemical markers of disease (i.e., elevated temperature, failure to feed, lethargy, rash, depressed white blood cell and platelet count, evidence of plasma leakage, or hemorrhage) [316–318]. A single study reported a hemorrhagic phenotype following intravenous administration of a high-titer DENV-2 strain, but these findings have not been reproduced [319]. Despite this, the nonhuman primate dengue virus infection and viremia model has been a required and necessary component of dengue vaccine development programs and the transition between preclinical and clinical development phases. How informative the nonhuman primate model is as it relates to predicting human responses to dengue virus exposure or vaccination with candidate dengue vaccines remains unclear. The observation of low clinical efficacy against DENV-2 infection following vaccination despite protective efficacy in nonhuman primates underscores the need to rethink the utility of the model and the need to further optimize or explore other options [249, 279].

Identifying which cytokines, factors, and/or other circulating immune mediators contribute to clinical disease or protection from the same is one challenge, and how to best measure these is another. Neutralizing antibodies have been associated with protection from other flaviviral diseases such as yellow fever and Japanese encephalitis [320]. It is therefore reasonable to assume that neutralizing antibodies play a similarly protective role in dengue. Measurement of neutralizing antibodies has classically been accomplished using a plaque reduction neutralization test (PRNT) [321]. Although very informative, properly performing and interpreting PRNT results requires advanced skill and experience. Variability among laboratories and technicians has been observed. Furthermore, the assay lacks robustness with minimal modifications in assay reagents or testing conditions producing significant variability in results [105]. It is for these reasons direct comparison of immunogenicity readouts between vaccine candidates and developers has been difficult [322]. Newer methods of measuring neutralizing antibodies have been proposed to increase throughput, reduce human error, reduce assay-to-assay variation, and increase the biologic and physiologic relevancy of the measurement [323–325].

Variation also permeates the measurement of cell-mediated immune responses. It is generally accepted that components of the cellular immune system contribute to both the disease severity and the immune profile which protects against disease following homotypic reinfection [186, 326–333]. What is less clear is the sequence of immunologic events which leads to one outcome (antiviral activity, asymptomatic, or highly attenuated illness) versus another (pro-inflammatory state, disease). How components of the humoral and cellular immune systems interact qualitatively, quantitatively, and kinetically during in vivo infection is incompletely understood. Furthermore, the immunologic events that may determine the resulting clinical phenotype likely occur prior to the onset of symptoms. As a result, when and how to collect the appropriate biologic samples following infection remains unknown, and important immunologic events are not measured.

Key to the study of dengue immunopathogenesis is being able to link biologic samples (i.e., whole blood, peripheral blood mononuclear cells, etc.) collected before, during, and after dengue virus exposure and to associate these with various clinical phenotypes observed following infection (i.e., asymptomatic infection, dengue fever, severe dengue). Establishing a correlate and/or surrogate of protection is probably not possible without this approach. The complexity of this task is not trivial as it involves establishing prospective cohorts in dengue-endemic areas with sustained and relatively high annual clinical attack rates (1.5–2.0 %). To complete comprehensive immunologic evaluations also requires a sample with a significant volume of biologic material, a challenge in places where dengue disease is preferentially experienced by toddlers and children. Prospective studies are performed over multiple years and are very costly. Despite these challenges, numerous cohorts have been established in Asia and Latin America and are making important contributions to our understanding of dengue [82, 115, 197, 334–339].

Additional challenges to the dengue field include understanding (1) how viral diversity over time and space may or may not impact disease severity or what diversity means for developing a protective vaccine, (2) immune response maturation following infection over time and the impact of waning immunity on both durable protection from disease and vaccination policies once a vaccine is available, and (3) the role of protective and non-protective heterotypic immunity [29, 340]. It is possible that the world will have a safe, efficacious, and effective dengue vaccine available before many of the above questions have been answered.

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Flaviviruses: Yellow Fever, Japanese B, West Nile, and Others

16

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1 Introduction

Historically and currently the flaviviruses are important human pathogens both as endemic viruses restricted to specific geographic areas and as emerging pathogens. Yellow fever, West Nile virus, and the dengue viruses (not discussed in this chapter) represent previous, current, and future important emerging pathogens that have produced large epidemics and human deaths. Why are these viruses such a threat to human populations as emerging pathogens? Several important factors interplay to create these pathogens as agents of emerging diseases. As mostly arthropod-borne viruses (arboviruses), they have complex life cycles involving both arthropods and vertebrate hosts with a life cycle involving all life stages of the arthropods (mosquitoes, ticks, and midges) and their reservoirs of vertebrates (birds or rodents) and ultimately higher vertebrates (humans) through the bite of the infected arthropod vector. The flaviviruses all contain ribonucleic acid (RNA) as their genetic core and as such have a high rate of mutations and thus adapt quickly to changes in vector competence and the environment. Climate change, urbanization, and increasing ease of travel have created opportunities for the vector to spread and expand into human populations. The combination of these factors produces a family of viruses that can change and emerge quickly as important human pathogens.

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Yellow fever virus (discussed in detail in this chapter) was the first arbovirus identified in the 1800s as responsible for large epidemics of hemorrhagic fever in Africa and North, Central, and South America. By 1960 scientists recognized serologically two distinct arboviruses: the group A arboviruses now known as the family *Togaviridae* and the group B arboviruses, renamed the family *Flaviviridae* [1]. Serologic comparisons among the flaviviruses revealed cross-reactivity within groups distinguishing the flaviviruses as mosquito borne, tick borne, or nonvectored [2]. According to the International Committee on Taxonomy of Viruses, a subgroup of the Division of Virology of the International Union of Microbiology Societies, the Family *Flaviviridae* is comprised of three genera: *Flavivirus*, *Hepacivirus*, and *Pestivirus* (http://ictvonline.org/index.asp). Information on their isolation, morphology, sensitivity to inactivation by chemicals, arthropod vectors, vertebrate hosts, laboratory propagation, serologic reactions, geographic distribution, clinical manifestations, and epidemiology is found in the *International Catalogue of Arthropod-Borne Viruses*, compiled by the American Committee on Arthropod-Borne Viruses (ACAV) [3]. This exhaustive reference source has been used freely in preparing the text that follows. *Hepacivirus* and *Pestivirus* are discussed elsewhere in this textbook, and the focus of this chapter will be on the pathogenic viruses within the genera *Flavivirus*. The dengue viruses are discussed in a separate chapter. Within the genus *Flavivirus* there are 53 species of viruses and displayed in Table 16.1.

Despite their species variation, the flaviviruses have a remarkable genetic conservation throughout its genus [4]. The genome is a single positive-stranded RNA, 11 kilobases in length, encoding the viral proteins from an open reading frame (ORF) of over 10,000 bases of approximately 3,434 amino acids [4]. The proteins encoded from the ORF is 5′–C-prM (M)-E-NS I-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′. The structural proteins C (capsid), M (membrane), and E (envelope) comprise the outer coat of the flaviviruses and the epitopes responsible for attachment to host cells and cell entry. The nonstructural proteins include the highly

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Table 16.1 Taxonomy of viruses of the family *Flaviviridae*, genera *Flavivirus*

Group (by vector)	Abbreviation	Geographic distribution	Principal vector species	Human disease
Tick-borne viruses				
Mammalian tick-borne virus complex				
Alkhurma hemorrhagic fever virus	AHFV	Egypt, Sudan	Camel tick	Hemorrhagic fever
Gadgets Gully virus	GGYV	Australia	*Ixodes uriae*	Unknown
Kadam virus	KADV	Uganda, Saudi Arabia	*Rhipicephalus pravus*	Unknown
Kyasanur Forest disease virus	KFDV	India, China	*Haemaphysalis spinigera*	Hemorrhagic fever
Langat virus	LGTV	Malaysia, Thailand, Siberia	*Ixodes granulatus*	Fever
Omsk hemorrhagic fever virus	OHFV	Russia and Central Asia	*Dermacentor pictus*	Hemorrhagic Fever
Powassan virus	POWV	Canada and Northern United States	*Ixodes* spp.	Encephalitis
Royal Farm virus	RFV	Afghanistan	*Argas* spp.	Unknown
Karshi virus	KSIV	Central Asia	*Ornithodoros papillipes*	Encephalitis
Tick-borne encephalitis virus	TBEV	Europe, Asia	*Ixodes* spp.	Encephalitis
European subtype		Europe, Asia	*Ixodes* spp.	Encephalitis
Far Eastern subtype				
Siberian subtype				
Louping ill virus	LIV	United Kingdom	*Ixodes* spp.	Encephalitis
Irish subtype		Ireland	*Ixodes* spp.	Encephalitis
British subtype		United Kingdom	*Ixodes* spp.	Encephalitis
Spanish subtype		Spain	*Ixodes* spp.	Encephalitis
Turkish subtype		Turkey	*Ixodes* spp.	Encephalitis
Mosquito-borne viruses				
Aroa virus complex				
Aroa virus	AROAV	Venezuela	Unknown	Unknown
Bussuquara virus	BSQV	Brazil, Colombia, Panama	*Culex* spp.	Fever
Iguape virus	IGUV	Brazil	Unknown	Unknown
Naranjal virus	NJLV	Ecuador	*Culex* spp.	Unknown
Dengue virus complex				
Dengue virus				
Dengue virus type 1	DENV-1	Tropical and subtropical regions of the world for all	*Aedes aegypti* for all	Fever, Hemorrhagic fever, encephalitis
Dengue virus type 2	DENV-2			
Dengue virus type 3	DENV-3			
Dengue virus type 4	DENV-4			
Kedougou virus	KEDV	Senegal, Central Africa	*Aedes* spp.	Unknown
Japanese encephalitis virus complex				
Cacipacore virus	CPCV	Brazil	Unknown	Unknown
Koutango virus	KOUV	Senegal, Central Africa	*Aedes* spp.	Unknown
Japanese encephalitis virus	JEV	Asia	*Culex* spp.	Encephalitis
Murray Valley encephalitis virus	MVEV	Australia	*Culex annulirostris*	Encephalitis
Alfuy virus	ALFV	Australia	Unknown	Unknown
St. Louis encephalitis virus	SLEV	North, Central, and South America	*Culex* spp.	Encephalitis
Usutu virus	USUV	Africa	Mosquitoes	Fever, rash
West Nile virus	WNV	Worldwide	*Culex* spp.	Encephalitis
Kunjin virus	KUNV	Australia, Asia	*Culex* spp.	Fever, rash
Yaounde virus	YAOV	Cameroon	*Culex* spp.	Unknown
Kokobera virus complex				
Kokobera virus	KOKV	Australia	*Culex annulirostris*	Unknown
Stratford virus	STRV	Australia	*Aedes vigilax*	Unknown
Ntaya virus complex				
Bagaza virus	BAGV	Africa	*Culex* spp.	Fever
Ilheus virus	ILHV	Central and South America	Mosquitoes	Fever
Rocio virus	ROCV	Brazil	Mosquitoes	Encephalitis
Israel turkey meningoencephalitis virus	ITV	Israel	Unknown	Unknown
Ntaya virus	NTAV	Africa	Mosquitoes	Fever

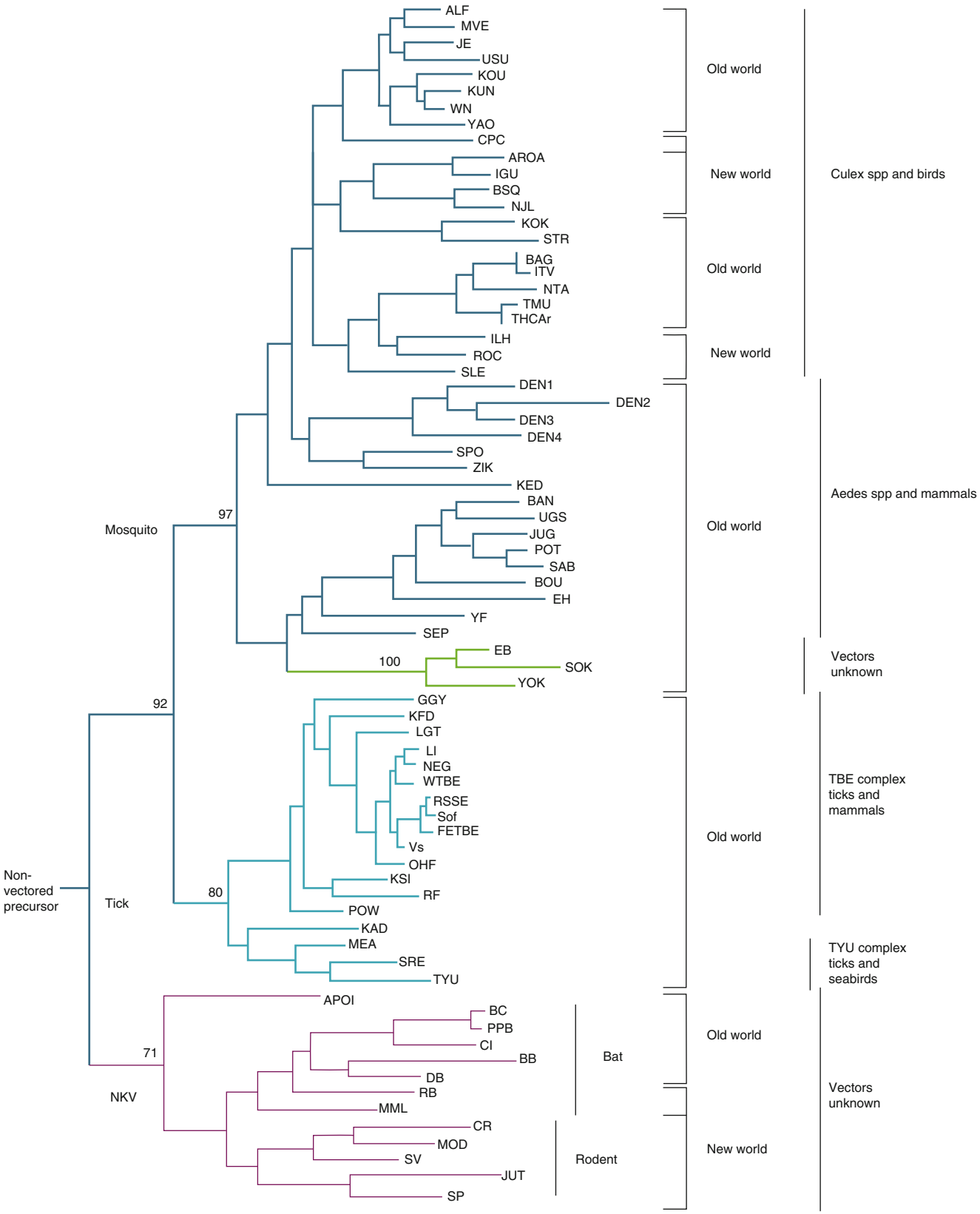
Table 16.1 (continued)

Group (by vector)	Abbreviation	Geographic distribution	Principal vector species	Human disease
Tembusu virus	TMUV	Malaysia, Thailand	*Culex* spp.	Unknown
Spondweni virus complex				
Zika virus	ZIKV	Africa, Asia	*Aedes* spp.	Fever, rash
Spondweni virus	SPOV	Africa	*Aedes circumluteolus*	Unknown
Yellow fever virus complex				
Banzi virus	BANV	Africa	*Culex* spp.	Fever
Bouboui virus	BOUV	Africa	Unknown	Unknown
Edge Hill virus	EHV	Australia	Mosquitoes	Unknown
Jugra virus	JUGV	Malaysia	Mosquitoes	Unknown
Saboya virus	SABV	Senegal	*Tatera kempi*	Unknown
Potiskum virus	POTV	Nigeria	Unknown	Unknown
Sepik virus	SEPV	New Guinea	Mosquitoes	Fever
Uganda S virus	UGSV	Africa	Mosquitoes	Unknown
Wesselsbron virus	WESSV	Africa, Asia	*Aedes* spp.	Fever
Yellow fever virus	YFV	Africa, South America	*Aedes aegypti*	Hemorrhagic fever
Viruses with no known arthropod vector				
Entebbe bat virus complex				
Entebbe bat virus	ENTV	Uganda	Unknown	Unknown
Sokoluk virus	SOKV	Kyrgyzstan	Unknown	Unknown
Yokose virus	YOKV	Japan	Unknown	Unknown
Modoc virus complex				
Apoi virus	APOIV	Japan	Unknown	Unknown
Cowbone Ridge virus	CRV	USA	Unknown	Unknown
Jutiapa virus	JUTV	Guatemala	Unknown	Unknown
Modoc virus	MODV	USA	Unknown	Unknown
Sal Vieja virus	SVV	USA	Unknown	Unknown
San Perlita virus	SPV	USA	Unknown	Unknown
Rio Bravo virus complex				
Bukalasa bat virus	BBV	Uganda	Unknown	Unknown
Carey Island virus	CIV	Malaysia	Unknown	Unknown
Dakar bat virus	DBV	Africa	Unknown	Fever
Montana myotis leukoencephalitis virus	MMLV	USA	Unknown	Unknown
Phnom Penh bat virus	PPBV	Cambodia	Unknown	Unknown
Batu Cave virus	BCV	Malaysia	Unknown	Unknown
Rio Bravo virus	RBV	USA, Mexico	Unknown	Fever

Adapted from Calisher et al. [1]

conserved proteins NS1, NS3, and NS5 and the four small hydrophobic proteins NS2A, NS2B, NS4A, and NS4B [4].

Molecular and phylogenetic analysis of the genus *Flavivirus* reveals a family of viruses that has evolved rapidly from a progenitor virus arising possibly in Africa several thousands of years ago. With evolution the flaviviruses have shown substantial ecological diversification with different lineages adapting to different vectors and modes of transmission. They have also evolved unique strategies to evade the host's innate and adaptive immunity [5]. Figure 16.1 displays the phylogenetic tree of the genus *Flavivirus* showing the association of the groups of related viruses with their invertebrate vectors, vertebrate hosts, and geographic distribution. All human pathogenic flaviviruses are transmitted by insect vectors, and all but the dengue viruses have an animal host (i.e., they are zoonotic). The ecological speciations of these viruses are diverse. For example, the Japanese encephalitis virus (JEV) group (e.g., Japanese encephalitis virus, West Nile virus (WNV), St. Louis encephalitis (SLE)) is maintained in a cycle of transmission between birds and *Culex* mosquitoes. Mammalian hosts, humans, and horses are infected but are dead-end hosts as the viremia achieved is too low for subsequent transmission to an insect vector. The tick-borne encephalitis group is transmitted among rodents by a tick vector; as with the JEV group, humans are a dead-end host. Yellow fever virus (YFV) is primarily maintained in a sylvatic cycle involving nonhuman primates and *Aedes* mosquitoes; but it has shown the capacity to spread in urban areas using *Aedes aegypti* mosquitoes and humans as its primary reservoir, resulting in extensive urban epidemics.


ALF
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PPB
CI
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RB
MML
CR
MOD
SV
JUT
SP
Old world
New world
Old world
New world
Old world
Old world
Old world
New world
Culex spp and birds
Aedes spp and mammals
Vectors unknown
TBE complex ticks and mammals
TYU complex ticks and seabirds
Vectors unknown
Bat
Rodent
Mosquito
Tick
NKV
Non-vectored precursor
97
92
100
80
71

Analysis of the genetic changes of the flaviviruses over time reveals a group of viruses that have evolved rapidly. Methods using coalescent theory and a maximum likelihood (ML) demographic model reveal that the flaviviruses are growing at an exponential rate, with specific viruses such as the dengue viruses increasing rapidly in the recent past and Japanese encephalitis virus changing from constant population size to exponential growth within the last century [6]. For St. Louis encephalitis virus (SLEV), a Bayesian coalescent approach was used on the envelope gene sequence substitution rates and dates of divergence in the Americas [7]. The mean substitution rate estimated for all SLEV was 4.1×10^{-4} substitutions/site/year. The direction of the gene flow was South to North between 158N and 308N latitude consistent with migratory bird patterns from their northern breeding grounds after having acquired infection while wintering in the region of the Gulf of Mexico. This is an elegant example of the role of the vector and the vertebrate host especially migratory birds in influencing the gene flow and evolution of the flaviviruses. The dengue viruses (DENVs) are also an example of an emerged flavivirus and global health problem that have changed dramatically over the past century. DENV has evolved to a molecular clock that is serotype and genotype specific [8]. Phylogenetic analysis and time analysis suggest that dengue viruses serotypes separated within the last 1,000 years, and the change of DENV from a sylvatic cycle to sustained human transmission may have occurred between 125 and 320 years ago [9].

In this chapter we will review the characteristics of the flaviviruses, their modes of transmission, and their role in causing severe human clinical infection and emerging diseases of potential epidemiologic significance.

2 Historical Background

Yellow fever and its emergence as a deadly epidemic disease is a model on how diseases emerge to become a large recurrent epidemic disease in urban populations. Originally an African virus unknown to the western world prior to the 1600s, was introduced to the Western Hemisphere by the transportation of slaves from Africa with the first reported outbreak of yellow fever in the Yucatan in 1648 [10]. The transportation of the mosquito vector and infected passengers by ship and introduction of the virus into naive susceptible populations resulted over the next 200 years in the transmission of the virus and illness to many large urban populations with outbreaks occurring in the tropical Americas, costal North America, and Europe. Yellow fever became known in the Hispanic world as *La Vomito* for the black vomit that accompanies the hemorrhagic phase and yellow jack among the Europeans for the international signal flag for quarantine and its characteristic yellow and black squares known by the same name. During the summer months, epidemics occurred in New York in 1668, Boston in 1691, and Charleston in 1699, as well as later in the cities of the Gulf of Mexico and the Mississippi River. In 1793 a major yellow fever epidemic occurred in Philadelphia. Philadelphia at that time was the United States' largest and most cosmopolitan city. French refugees from a slave rebellion in Haiti arrived on the banks of the Delaware River which bounds the east side of the city bringing yellow fever with them [11]. In July of that year, cases developed among the working class living along the Delaware River who suffered high fevers, yellowing of the skin and eyes, hemorrhage, and death. By August 19 the epidemic potential was recognized by Dr. Benjamin Rush, professor at the University of Pennsylvania, founder of the College of Physicians of Philadelphia, and signer of the Declaration of Independence. Dr. Rush later gave an accounting of his clinical experience during this epidemic in a publication entitled, "An Account of the Bilious Remitting Yellow Fever, as It Appeared in the City of Philadelphia, in the Year 1793." The epidemic peaked in October of that year with total deaths estimated to be 4,041–5,000 in a population of 45,000 (crude mortality of 9–11 %). Subsequently Philadelphia experienced outbreaks of yellow fever in 1797, 1798, 1799, 1802, 1803, and 1805.

Fig. 16.1 Phylogenetic tree showing the association of the groups of related viruses with their invertebrate vectors, vertebrate hosts, and geographic distribution. *ALF* Alfuy, *MVE* Murray Valley encephalitis, *JE* Japanese encephalitis, *USU* Usutu, *KOU* Koutango, *KUN* Kunjin, *WN* West Nile, *YAO* Yaounde, *CPC* Cacipacore, *ARO* Aroa, *IGU* Iguape, *NJL* Naranjal, *KOK* Kokobera, *STR* Stratford, *BAG* Bagaza, *IT* Israel Turkey meningoencephalomyelitis virus, *TMU* Tembusu, *THCAr* strain of Tembusu, *ILH* Ilheus, *ROC* Rocio, *SLE* St. Louis encephalitis, *DEN* dengue, *SPO* Spondweni, *ZIK* Zika forest, *KED* Kedougou, *UGS* Uganda S, *JUG* Jugra, *POT* Potiskum, *SAB* Saboya, *BOU* Bouboui, *EH* Edge Hill, *YF* yellow fever, *SEP* Sepik, *EB* Entebbe bat, *SOK* Sokoluk, *YOK* Yokose, *GGY* Gadgets Gully, *KFD* Kyasanur Forest disease, *LGT* Langat, *LI* Louping ill, *NEG* Negishi, *Sof* Sofjin, *FETBE* far eastern TBE, *Vs* Vasilchenko, *OHF* Omsk haemorrhage fever, *KSI* Karshi, *RF* Royal Farm, *POW* Powassan, *KAD* Kadam, *MEA* Meaban, *SRE* Saumarez Reef, *TYLI* Tyuleniy, *APOI* Apoi, *BC* Batu Cave, *PPB* Phnom Penh bat, *CI* Carey Island, *BB* Bukalasa bat, *DB* Dakar bat, *RB* Rio Bravo, *MML* Montana myotis leucoencephalitis, *CR* Cowbone Ridge, *MOD* Modoc, *SV* Sal Vieja, *JUT* Jutiapa, *SP* San Perlita, *TBE* tick-borne encephalitis, *WTBE* Western European TBE, *RSSE* Russian spring and summer encephalitis, *NKV* refers to viruses with no known vector (Adapted from Ref. [2] with permission of the publisher) Reprinted with permission from [296]

Yellow fever took its toll on deployed US soldiers in the tropical Americas, especially in Cuba. Army Surgeon-General George Miller Sternberg created the US Army Yellow Fever Commission in 1893 and directed Major Walter Reed to conduct studies to establish its etiology. Together with his colleagues and Carlos Finlay, a series of human clinical trials were performed in Havana, Cuba, during 1900–1901 to discover the cause of yellow fever. Walter Reed, Jas Carroll, and Aristides Agramonte published in the JAMA in 1901 *The Etiology of Yellow Fever: An Additional Note* where they discussed the methods of their studies, results, analysis, and concluded the following: (1) the mosquito serves as the intermediate host for the parasite of yellow fever; (2) yellow fever is transmitted to the nonimmune individual by means of the bite of the mosquito that has previously fed on the blood of those sick with this disease; (3) an interval of about 12 days or more after contamination appears to be necessary before the mosquito is capable of conveying the infection; (4) yellow fever is not conveyed by fomites, and hence disinfection of articles of clothing, bedding, or merchandise, supposedly contaminated by contact with the sick with this disease, is unnecessary; and (5) the spread of yellow fever can be most effectually controlled by measures directed to the destruction of mosquitoes and the protection of the sick against the bites of those Insects [12].

With this information, General William C. Gorgas engineered the elimination of the mosquito initially from Havana and then later from the environs of the Panama Canal construction site and subsequently yellow fever cases disappeared [13–16]. In 1932, Soper and colleagues showed that there was a jungle cycle of yellow fever in the absence of *A. aegypti*. This significant observation meant that yellow fever could not be stopped just by controlling the mosquitoes in the cities. Theiler and Smith succeeded in cultivating the Asibi strain of yellow fever virus, first in monkeys and then in embryonated eggs, and attenuated it by passage [17]. In 1937, they announced their discovery of an attenuated vaccine—the 17D strain [18]. This vaccine is used throughout the world today to prevent yellow fever.

The discovery of the role of *Aedes aegypti* in the transmission and spread of yellow fever introduced the concept of mosquito control as an effective measure to disrupt yellow fever transmission. The International Health Board and the Rockefeller Foundation instituted mosquito control strategies including the use of a larvicidal, Paris green, throughout the United States and Central and South America [19]. These techniques were applied to malaria control during the years from 1924 to 1925 [19]. World War II created the Rockefeller Foundation Health Commission in 1942 to support national defense and malaria control for US forces. The need for lousicides to combat typhus introduced a new insecticide developed by the Swiss firm, Geigy, called dichlorodiphenyltrichloroethane (DDT) [19]. Led by Fred Soper, the Rockefeller team demonstrated the effectiveness of DDT as a lousicides and in disrupting typhus epidemics. DDT was soon used in aerial and ground spraying for Allied Forces during a malaria outbreak in Italy and was found to be highly effective. DDT became a key component of the World Health Organization's global malaria eradication campaign in 1955 [19]. This campaign resulted in the elimination of both the malaria mosquito vector and *Aedes aegypti* throughout South America and the virtual elimination of malaria, yellow fever, and dengue throughout the Americas. The growing concerns of the environmental effects of DDT led to the end of the use of DDT as an effective mosquito control larvicide in 1969 [20]. The cessation of the DDT-based mosquito control programs in the Americas and the social disruption that resulted from World War II allowed the emergence of dengue in Asia and its reintroduction and resurgence in the Americas.

By the 1950s, scientists of the Rockefeller Foundation, the US Army and Navy, the US Public Health Service, several US universities, and many foreign governments recognized that arboviruses could be recovered in nature from apparently healthy arthropods and wild vertebrate animals. This search in the natural cycles of arboviruses resulted in the discovery of over 500 different arboviruses with about 100 of them causing human disease. Unfortunately, the control of arbovirus infections has not kept pace with the discovery and spread of disease. Antiviral drugs for arboviruses are not commercially available. Except for yellow fever, tick-borne encephalitis, and Japanese encephalitis, there are no widely used vaccines available for humans. Source reduction and control measures against the mosquito vector such as pesticides and biological larvicides have neither been sustainable nor effective in vector control.

3 Methodology Involved in Epidemiologic Analysis

3.1 Sources of Mortality and Morbidity Data

Mortality data are collected systematically but passively by national governments for many arboviruses. Data are published in the Morbidity and Mortality Weekly Report of the US Centers for Disease Control and Prevention, in the Weekly Epidemiological Report of the Pan American Health Organization, and in the Weekly Epidemiological Report of the World Health Organization. The mortality data are grossly underreported but may serve as a comparative data base, since underreporting may be uniform throughout much of the world. The reporting of yellow fever mortality in Africa, for instance, was found to be about 10 % of the true figure [14, 21].

The information flow to the World Health Organization is sometimes facilitated by informal networks of scientists and interested citizens. Nevertheless, the organization is constrained from action until official reports are received. This constraint often means a delay in control of a disease of regional or world importance.

The same sources supply morbidity data as supply mortality data. In the United States, of the arbovirus diseases, those

producing neuroinvasive and non-neuroinvasive illnesses are reported and include the California serogroup virus, Eastern and Western equine encephalitis virus, and the flaviviruses Powassan virus, St. Louis encephalitis virus, and West Nile virus [22]. In addition, yellow fever is still reportable in the United States. Monath documented the time elapsed between onset of an epidemic of St. Louis encephalitis and its recognition [23]. That time varied between 2 and 8 weeks. In the Corpus Christi, Texas, epidemic of 1966, nearly 707 of the cases had occurred before recognition.

3.2 Serologic Surveys

Serologic surveys entail the sampling of a defined population and measuring the amount of specific antibody to the targeted protein which will indicate past or current infection. Because of the large sample size that is tested, high-throughput assays such as enzyme-linked immunosorbent assay (ELISA), complement fixation (CF), or hemagglutination inhibition (HAI or HI) are performed with confirmation by more time-intensive assays such as the plaque reduction neutralization titers (PRNT). The results give a point prevalence of past or current infection in different geographic areas and subpopulations or those at particular risk for infection. Distribution of the antibody in a population can give clues to the ecological conditions necessary for maintenance of the virus as well as the human behavior that might place persons at risk for infection. Surveys of different age groups will show if the virus is more prevalent as the population ages, typical of an endemically transmitted agent. Another prevalence pattern in which antibody is present only in persons born before a certain year may indicate an epidemic in that year. Alternatively, a constant percentage of antibodies in each age group may indicate a recent introduction of the virus in a naïve population. Broadly based serologic surveys of large populations can provide extensive information about virus distribution in different geographic areas, rural versus urban populations, different age and sex groups, and different occupational types. Arbovirus serosurveys have limitations. Cross-reactions occur among viruses of the same serogroup. This is especially true of the HI test and ELISA with flaviviruses. The neutralization test is more specific and should be used where feasible. Surveys with HI or ELISA must be interpreted with caution unless one is certain that only one flavivirus circulates in the region, or unless the results have been confirmed by neutralization test with a portion of the negative and positive sera.

The serosurvey usually will not indicate when the infection responsible for the antibody occurred. If the antibody is suspected to be of recent origin, the IgM antibody-capture ELISA is useful for detection of infections originating within the prior 3–6 months. A serosurvey is not a reliable indicator of natural infection in populations vaccinated for tick-borne encephalitis, yellow fever, or Japanese encephalitis. On the other hand, the survey is an excellent tool for determination of vaccination coverage and protection.

One very important use of serosurveys is in the calculation of the basic reproductive ratio, R naught (R_0) [24], which is defined as the number of new individuals in a susceptible population who are infected by a single individual during his/her infectious period (see also Chaps. 1 and 5). The higher the number, e.g., R_0 >1, the more transmissible the infection. For vector-borne diseases, in particular, R_0 is important and complicated as it takes into account the susceptible and infected vectors as well as the susceptible and infected vertebrate hosts; R_0 is thereby influenced by both the intrinsic and extrinsic incubation periods. Serosurveys can calculate the R_0 as a measurement of the epidemic impact of a pathogen on a population but also as an estimate on the impact of interventions such as vector control on reducing disease in a population. Furthermore, a concept integrally related to R_0 is the "critical vaccination threshold," which is the number of persons in a population needed to vaccinate in order to drive R_0 to <1. Thus, determining R_0 by serosurveys is important not only in gauging the epidemic potential of a pathogen but also in determining the interventions necessary to control an epidemic. Age-stratified seroprevalence studies with cohorts including ages surrounding the age of peak incidence allows estimation of the force of infection over previous years based on different exposure rates across age groups and a calculation of R_0 [25].

3.3 Laboratory Methods

The laboratory is an all-important resource and key cornerstone in the study of the epidemiology of arbovirus diseases as well as understanding the pathogenesis of severe illness. Diagnosis can rarely be made with certainty by clinical examination and understanding the clinical course of symptoms, viremia, and antibody rise essential in utilizing the appropriate laboratory assays. Isolation of virus from arthropods, wild and domestic animals, and people is essential to determine the natural history of infection with these agents. Figure 16.2 demonstrates the typical flavivirus infection after inoculation from a vector assuming an incubation period of 6 days. Viremia occurs prior to the first clinical symptoms with early symptoms, commonly fever, headache, myalgias, and laboratory findings of leukopenia and thrombocytopenia. Antibody rises late in the clinical illness with first IgM followed by IgG. IgM can persist for several months and disappear, while IgG can persist for years after the infection. From a laboratory perspective the utility of the assay is dependent on the stage of infection. PCR-based assays and viral isolation are useful during the days of viremia in the early part of the clinical illness, whereas antibody-based assays are useful during the latter part of the illness and in convalescent sera.

Nonstructural protein 1 or NS1 is a highly conserved 48-kDa glycoprotein that is a requirement for flavivirus

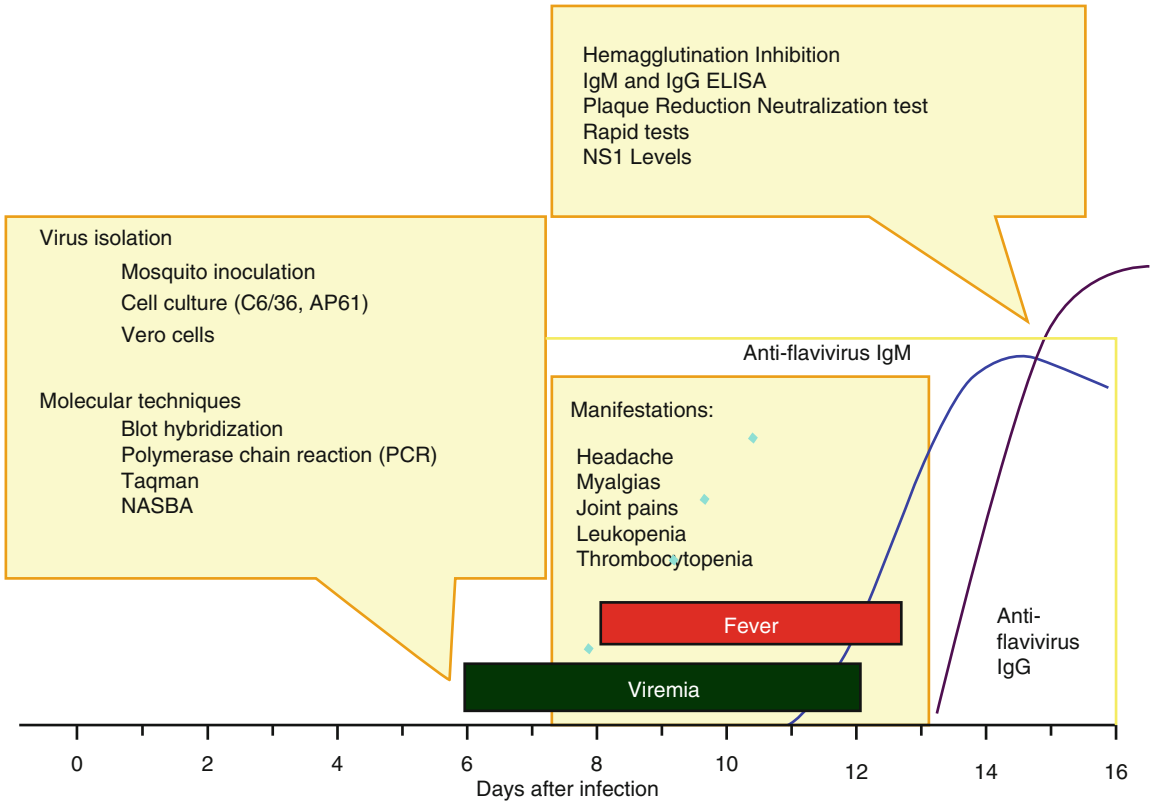

Fig. 16.2 Typical flavivirus infection and the human host response by clinical course and appropriate diagnostic assay

RNA replication [26]. Interestingly NS1 exists in the cell as a homodimer associated with organelle membranes and is actively transported to the mammalian cell surface where it is secreted as a soluble hexamer. Thus, NS1 can be detected in serum during flavivirus infections and has a half-life longer than detectable RNA in serum [27]. NS1 ELISA-based assays have become available for dengue virus infections and have the potential to be used for other flaviviruses [27]. The advantage of these assays is that they can be used throughout the early, middle, and late clinical course of infection with a sensitivity and specificity equivalent to PCR.

Virus isolation is key in understanding the virus and its effects on the vector and human population, but isolation is also the most challenging for laboratories. It requires serum from the early part of clinical illness, −80 °C or colder freezers, and special laboratory techniques. Viral isolation requires inoculation of a small amount of serum into laboratory animals, arthropods like mosquitoes, or cell culture. Cell culture systems (vertebrate or insect cell cultures) are used for both virus isolation and neutralization titers.

In the study of material derived from patients, it is ideal to have acute and convalescent sera available. As demonstrated in Fig. 16.2, antibody titer rises rapidly from the acute to the convalescent serum, and a fourfold rise between the two samples is indicative of an acute infection. Demonstration of IgM in ELISA permits a presumptive diagnosis with a single serum.

Details of the techniques of CF, HI, and virus neutralization relating specifically to arboviruses are available in current manuals. Fluorescent antibody (FA) techniques, antigen-based ELISA, often coupled with monoclonal antibody, and PCR are widely used for antibody, antigen, and RNA detection, respectively. Such advances are greatly extending the scope of field and laboratory investigations.

3.4 Genetic Sequencing

Molecular methods such as polymerase chain reaction (PCR) and the ability to detect and amplify small amounts of RNA or DNA to identify specific pathogens have revolutionized our diagnostic capabilities in identifying patients who are acutely ill from flavivirus infections [28]. Reverse transcriptase PCR (RT-PCR) methods are highly sensitive and specific in detecting the infecting arbovirus during viremia [29, 30]. Real-time assays that utilize exonuclease fluorogenic assays such as TaqMan® (Applied Biosystems, CA, USA) and SYBR® Green (Molecular Probes, Inc., OR, USA) have significantly shortened the time of detection of RNA and DNA in a specimen but also added the ability to quantify the amount of virus and correlate it to disease severity [31]. Interest and need for point of care devices that can be utilized at a clinic with little expertise has prompted the development of new molecular platforms such as "isother-

mal" methodologies including nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), and loop-mediated isothermal amplification (LAMP) technologies [32]. This allows an isothermal reaction and amplification to be performed at room temperature with potential reduction in time to detection to a matter of minutes.

Sequencing of the arboviruses has transformed our ability to understand the genetics and epidemiology of these viruses as well as the relatedness of each virus as demonstrated in Fig. 16.1. Genetic sequencing has rapidly evolved from a time-intensive process to technology for rapid full-length automated sequencing. Partial and full-length sequencing of the arboviruses has allowed phylogenetic analysis and revealed a series of clades defined by their epidemiology and disease associations [33]. Phylogenetic analysis has identified mosquito-borne, tick-borne, and no-known-vector (NKV) virus clades, which have been further divided into clades associated to their principal vertebrate host. Sequencing coupled with phylogenetic analysis is a powerful tool to understand the correlation between epidemiology, disease, and geography. It furthers our understanding of the complex evolutionary relationships between the virus, vector, and its vertebrate host. Metagenomics takes this concept further. It is the coordinated study of multiple genomes—the population of genetic material and, as applied to viruses, the entire community of viruses within the host environment [34]. This approach utilizes direct sequencing and bioinformatics to reconstruct the viral population and as such reveals the genetic diversity and evolution of the virus and its vector hosts. Especially powerful is the use of metagenomics when standard techniques fail to identify a viral infection, such as in emerging diseases. For example, metagenomics was used to identify specific viruses from samples collected from an enterovirus surveillance program in the Netherlands [34]. Samples were identified using conventional techniques including PCR that demonstrated cytopathic effects in cell culture without a virus. Metagenomics identified new viruses in all the samples.

3.5 Mathematical Modeling

Mathematical modeling is a powerful technique used to understand the interactions of the arbovirus, its vector, and host infection that produce endemic transmission or explosive epidemics in populations [35]. Mathematical models have the potential to predict epidemics and, equally importantly, to identify interventions by which to prevent or diminish the spread of the arbovirus through the use of vector reduction, personal protection methods, antivirals, or vaccines. Models have progressed from the basic susceptible, infected, and recovered SIR model to more advance modeling techniques using stochastic models in which the number of individuals in any class is an integer with events occurring randomly with a given probability based on the associated deterministic model. The value of stochastic models is the generation of different epidemics capturing the variability in the epidemic profile [35]. For example, a stochastic metapopulation model with spatiotemporal transmissibility scenarios was used to model the spread and transmission of yellow fever [36]. This model was useful to understand and assess the risk of yellow fever in producing urban outbreaks and to identify locations at risk for yellow fever introduction and subsequent transmission.

4 Biological Characteristics of the Virus That Affect the Epidemiologic Pattern

If you were to look at the world from the eyes of a flavivirus, you would find a bewildering array of environments you were forced to adapt and propagate in. The vector invertebrate environment is quite different from the vertebrate environment in requiring the virus to propagate at a lower body temperature and to utilize different cellular receptors for attachment and cellular entry. The virus upon infection of its vector host from a blood meal will replicate, escape from the midgut, evade vector host defenses, and replicate in high concentration in the salivary glands. Environmental factors including temperature and humidity affect vector longevity and viral replication and overwintering until the next transmission season. Upon infecting their vertebrate host after the bite of the infected vector, the flavivirus faces a new host environment with a different ambient temperature, new receptors for host cell entry and replication, and new host innate and adaptive immunity. Flaviviruses are remarkably adept and successful at adapting to an array of environments to replicate and produce infection and ultimately epidemics in human populations. Ultimately the biological characteristics of the flavivirus that affect human epidemiology rely on its ability to be successful in the host vector. The capacity of the virus to replicate in the vector is influenced by many factors including the environment, ecology, vector behavior, and viral molecular factors. The interaction of these factors is called the vectorial capacity. The extension of vectorial capacity to infect the host is known as vector competence—the intrinsic ability of a vector to become infected and to subsequently transmit a pathogen to a susceptible host [37].

The viral molecular factors involved in vectorial capacity and the ability of the virus to be highly specific for its vector host are determined by how arboviruses exist as a collection of variable genomes within a host known as a mutant spectrum, mutant swarm, or quasispecies [37]. During a replicative cycle, RNA viruses and its high mutation rate will develop as a distribution of genetic variants which is influenced by a balance between mutation and selection. Selection pressure will determine the most fit virus for the given environment with the least fit viruses going into

extinction. This Darwinian survival of the fittest is known as viral adaptability. Genetic bottlenecks, defined as survival of a minority of one generation to become the majority of the next generation, can occur in flaviviruses and have been well described for dengue virus [38]. During the course of an infection in a vector, host genetic bottlenecks can occur, for example, when the virus leaves the midgut or replicates in the salivary glands allowing the selection of one mutant to become the dominant genotype. Thus, bottlenecks diminish viral diversity by selecting a subpopulation of viruses particularly adapted to that environment; as a result, frequent bottlenecks enhance the evolution of phenotypic robustness of the virus [37].

Viral fitness is inherently tied to vector fitness. A particular virus strain that is selected for rapid killing of its vector host will have little success at survival to its next host, and an attenuated virus that does not replicate well in its vector may not propagate to the vertebrate host. Viral replication may disrupt the physiology of the vector host through disruption of digestion, salivation, or changes in the vector midgut [39, 40]. Other consequences to vector fitness from viral infection include energy costs associated with immune activation, behavioral changes resulting in decreased feeding, and alterations in mating and propagation. A meta-analysis of studies across a range of mosquito–virus systems showed that arboviruses do reduce the survival of their mosquito vectors, but the overall impact on vector fitness varies by the virus taxonomic group and mode of transmission [41]. Alphaviruses, for example, were associated with the highest virulence levels in mosquitoes and the least for the bunyaviruses. The conclusion was that virus and vector fitness are interwoven and dependent on multiple selection factors that are evolving. The complexities of this relationship between virus and vector fitness coupled with the viral fitness required to propagate in a vertebrate host highlight the exquisite balance needed to maintain the life cycle and the disease process. This "trade-off" hypothesis suggests that the replication in arthropod then vertebrate hosts shapes virus evolution. It is when this trade-off results in an increase in both vectorial capacity and vertebrate infection that human epidemics, global expansion, and emerging diseases may occur. An example of this is the emergence of chikungunya virus (CHIKV) infection in 2004 from the Indian Ocean to India, attributed to a single amino acid mutation that enhanced vector capacity in a secondary vector *Aedes albopictus* [42]. Another example is the emergence of a new lineage of West Nile virus (WNV) in North America that was adapted for more efficient transmission in *Culex* mosquitoes [43]. When vectorial capacity for an arbovirus is diminished or vector resistance to virus infection is induced, virus transmission may potentially be completely interrupted. The occurrence of this phenomenon may been realized recently with the observation that infection of *Aedes aegypti* by Wolbachia, an intracellular bacterium, confers resistance of the mosquito to DENV infection [44]. These findings are now being used to infect natural *A. aegypti* mosquitoes to diminish DENV transmission [45].

5 Epidemiology

The epidemiology of flavivirus infections in humans as noted in previous sections is a complex interplay between viral evolution, selection pressures, vectorial capacity, and risk of human infection from the vector and its spread in human populations. In practical terms, many factors account for the dramatic changes in the epidemiology of the flaviviruses: increasing travel and the speed of travel where an individual can go from rural areas of Africa or Asia to major metropolitan areas in less than 24 h, expanding trade and commerce, population growth, increasing urbanization and population detritus leading to increasing vector breeding areas, deforestation, and climate change [46]. Examples of this changing epidemiology are the spread of the dengue viruses throughout Asia, the Americas, and most recently Florida; resurgence of yellow fever in South America and Africa; spread of West Nile virus through North and Central America; and the spread of Japanese encephalitis in Southwest Asia [46].

5.1 Incidence and Prevalence

All infections with pathogenic flaviviruses discussed in this chapter are acute and can be diagnosed either through molecular means during the time of infection or by serology consistent with acute infection, i.e., detection of IgM antibody or a fourfold rise in antibody titer between acute and convalescent sera. Measurement of incidence requires documenting acute infection through the application of clinical criteria consistent with the flavivirus infection. All acute flavivirus infections progress from a classic presentation of fever, chills, myalgias, and headache to a more flavivirus-specific syndrome of an acute febrile illness, hemorrhagic fever, or encephalitis. These specific syndromes fall into a spectrum of overlapping clinical presentations, making it difficult to distinguish one flavivirus from another or from other pathogens based on clinical symptoms and signs alone.

All infections display a biological gradation ("iceberg") of clinical responses and disease severity both at a cellular and host response level (see Chap. 1). In the case of flaviviruses, although unobserved (below the "waterline" of the iceberg), subclinical infections are viremic and do contribute to the transmission and infection of the vector and thus represent a very important component of the epidemic. Further along the gradient of infection are mild ambulatory illness, moderate illness requiring medical care, more severe illness requiring hospitalization, and fulminant illness requiring intensive care with potentially fatal outcome. The shape of the gradient or "iceberg" varies among the flaviviruses and depends on the preexisting immunity in the population, vectorial capacity, and environmental factors. The subclinical infections are not well studied due to the requirement for long-term prospective studies of cohorts with the ability to diagnose asymptomatic infections. The best documentation of subclinical infection

has come from the prospective cohort studies conducted in Northern Thailand where at least 50 % of all DENV infections were subclinical [47, 48]. In general, a large majority of flavivirus infections occur below the waterline of detection and subclinical. The more severe forms of infection resulting in death occur in less than 5 % of all infections. Understanding the iceberg concept of infection is critical in thinking on the epidemiology and incidence of flavivirus infections. Surveillance confined to hospitalized cases will detect only 10–20 % of all cases. Extended to the ambulatory clinics, surveillance can detect an additional 30 % of all infections. It is apparent that even with detection of 50 % of acute cases, the incidence of infection is being grossly underestimated.

In contrast, seroprevalence studies (see Chap. 2) of flavivirus infection can define populations at risk, give a historical account of the transmission, and provide an estimate of the burden of infection in a population. A classic arbovirus seroprevalence study was the mapping of the worldwide distribution of yellow fever by Sawyer et al. in 1937 [49]. The virus neutralization test was performed using adult white mice as test animals. Yellow fever virus was found to be more widely distributed in South America and Africa than had been earlier suspected and was absent from Europe, Asia, and Australia. Neutralization tests measure durable antibody that persists for years. High rates of antibody prevalence could result from continued, widespread virus activity and/or from a large outbreak with a high attack rate. For example, yellow fever neutralization tests performed on sera collected from residents of Trinidad, West Indies, in 1953 revealed no immunes under the age of 15 years and therefore no apparent virus activity later than 1938. This situation changed dramatically with the reappearance of yellow fever on the island in epidemic form in 1954 [50].

Incidence and prevalence studies provide two views on flavivirus infection. The former can uncover newly occurring infections in the population and evidence of an outbreak; the latter offer a historical perspective on previous outbreaks and subpopulations that may be at risk. In most countries appropriate diagnostics are not available to diagnose a specific flavivirus infection, and clinical criteria are mostly used; as a result their population incidence is grossly underreported.

5.2 Epidemic Behavior and Geographic Distribution

Arbovirus infections are worldwide in distribution and may occur whenever the appropriate mosquito or other arthropod vectors abound in proximity to humans and a suitable amplifying host. Table 16.1 displays both the human pathogenic flavivirus and those where illness hasn't been documented, vector if known and human illness if known. The flaviviruses occur globally as both regional endemic diseases and epidemic diseases that can spread worldwide. The dengue viruses are now the most common mosquito-borne arboviral infection worldwide (http://www.who.int/csr/disease/dengue/en/). Other significant flaviviruses that have vast global distributions include West Nile virus, tick-borne encephalitis, and yellow fever. Regional flavivirus threats that produce epidemic diseases include Japanese encephalitis in Asia and West Nile virus and St. Louis encephalitis in North and South America. All are endemic diseases that produce transmission and disease every year with occasional epidemics.

5.3 Temporal Distribution

Temporal distribution is a consequence of overwintering and seasonal breeding and is region specific. If birds are the primary vertebrate host, temporal distribution is dependent on their migratory patterns, nesting, and hatching of young immunologically naïve chicks. In general, seasonal breeding of the vector occurs in spring, summer, and fall, although this is affected by the climate with warmer years having transmission during the winter months. Climate change and global warming will affect the temporal distribution of the flavivirus by changing the abundance of the vector. Global warming will increase the distribution of the vector as well as cause more rainfall and thus create more potential areas for breeding in the case of mosquitoes. The natural weather pattern that occurs approximately every 5 years, La Niña/El Niño-Southern Oscillation, or ENSO, can have dramatic effects on rainfall, flooding, and vector-borne diseases including the flaviviruses [51, 52]. This periodic climate change occurs across the tropical Pacific Ocean with variations in surface temperature (warming known as El Niño and cooling as La Niña). In a recent study, Murray Valley encephalitis virus (MVEV) in northern Australia and Papua New Guinea was monitored and correlated to the occurrence of rainfall [53]. Using multi-satellite precipitation analysis, the authors found that increases in monthly rainfall and monthly number of days above average rainfall increased the risk of MVEV activity at a time lag of 2–3 months. Clearly climate change and weather have an effect on the temporal distribution of vector-borne infections with a particular impact on the flaviviruses.

5.4 Age, Sex, and Other Demographic Factors

Infections with arboviruses can occur at any age. The age distribution depends on the degree of exposure to the particular transmitting arthropod relating to age, sex, occupation, and recreational habits of the individual or group of individuals. In highly endemic areas where transmission occurs every year, such as with Japanese encephalitis and dengue viruses, flavivirus infection is seen primarily in children, with adults

protected by the antibody from previous infections [54, 55]. In areas where transmission is sporadic and the entire population is at risk, extremes of age becomes a risk for severe infection such as seen in WNV infection in adults over the age of 50 years [56].

5.5 Other Factors

Genetic factors are known to influence disease severity from flavivirus infection and thus directly the epidemiology of arbovirus infections. Nutrition may have an effect on increasing disease severity in specific flavivirus infections such as in dengue hemorrhagic fever though it is unknown if there is an effect with other infections [57]. Certainly malnutrition can suppress the immune response and affect disease severity and thus the epidemiology. Genetic factors are very likely associated with disease severity. For example, in case–control gene association studies performed on cohorts of DENV-infected patients in Asia and the Caribbean, specific genes have shown associations with disease severity. These genes include the HLA molecules, the cell receptors for IgG (FcGII), vitamin D, and ICAM3 (DCSIGN or CD209) [58]. Also pathogen recognition molecules such as mannose-binding lectin (MBL) have associated with disease severity as well as the blood cell-related antigens including ABO and human platelet antigens (HPA1 and HPA2) [58]. African slaves were long known to be immune from the severity of yellow fever and were recruited to help stricken victims during the Philadelphia 1793 yellow fever epidemic [11]. Clearly genetics that determine both acquired and innate immune responses contribute to disease severity or protection from flavivirus infections and can affect the epidemiology of these infections.

6 Mechanism and Route of Transmission

By definition, the flaviviruses as arboviruses must be transmitted by arthropod vectors within which multiplication of the virus is a necessary requirement. Non-arthropod-related transmission to humans can occur such as through goat, sheep, or cow milk in the case of TBEV and respiratory and alimentary tract with Omsk HF [59, 60]. The primary vectors are mosquitoes and ticks. The duration of the necessary period of virus multiplication within the arthropod host before it becomes infectious varies from virus to virus and vector to vector and is also directly temperature dependent. For most viruses under average summer temperature conditions, the extrinsic incubation period falls in the 7- to 14-day range. Once infected, vectors may remain infected and able to transmit for many weeks or months. In the case of ticks, this infection may be years. Transmission of virus transovarially in arthropods, often referred to as "vertical transmission," has been demonstrated (specific examples, tick-borne encephalitis in ticks, DENV in mosquitoes). Venereal transmission of SLE viruses in vector mosquitoes has also been described. These mechanisms may be important for survival of some arboviruses in nature, permitting overwintering or survival over a protracted dry spell. Birds are important reservoir vertebrates for the viruses of JEV, WEE, and SLE.

There are a number of flaviviruses that cause human infections where the principal vector species is not known (Table 16.1). These include the viruses Apoi, Dakar bat, Koutango, Modoc, and Rio Bravo. More research is needed to establish the principal vector or, if not found, to gather details on their mode of transmission.

There are two nonvector modes of transmission to humans of the vector-borne flaviviruses that is essential to know—transfusion mediated and sexual. During the 2002 WNV epidemic in the United States, 23 patients were confirmed to have acquired WNV through transfused contaminated blood products [61]. Of these the majority were immunocompromised due to transplantation or cancer. Sixteen donors with evidence of WNV viremia at donation were linked to the 23 infected recipients. As a result of these findings, the US blood supply is now screened for WNV. Sexual transmission of another normally vector-borne flavivirus was recently documented. Zika virus (ZIKV) is a mosquito-transmitted flavivirus that has been isolated from sick persons in Africa and Southeast Asia. Two American scientists working in Senegal became acutely ill with ZIKV [62]. One of the scientists upon returning to the United States infected his wife who developed confirmed ZIKV symptomatic infection. Because she never traveled outside of the United States, direct person-to-person transmission was thought to be the mode of infection either through saliva or infected semen.

7 Pathogenesis and Immunity

Arbovirus infections are usually transmitted by the bite of the appropriate vector, and the skin is the normal portal of entry. Even before the virus enters the host through the skin, an important cofactor contributed by the vector assists in shaping virus entry. Saliva from arthropods (i.e., ticks and mosquitoes) has been shown to potentiate flavivirus infection and enhance in vertebrate hosts. Virus and arthropod saliva are delivered into the skin of the vertebrate host. Arthropod saliva contains a complex mixture of bioactive molecules that are capable of altering homeostasis, immune response, and dendritic cells and may contribute to the ability of flaviviruses to establish an infection [63]. The potentiation of flavivirus infection

by arthropod saliva has been demonstrated for a number of viruses including WNV and saliva from *Culex tarsalis* which resulted in enhancement of early WNV infection in vertebrate hosts [64].

The first cells that typically come in contact with flaviviruses are skin dendritic cells (DCs). These cells have been shown to be important initial targets of infection that shape the immune response for many of the pathogenic flaviviruses including DENV, YFV, and tick-borne encephalitis virus (TBEV) [50, 65–67]. TBEV targets DCs early in infection and are major inducers of interferon (IFN) and the innate immune response. TBEV modulates DCs and thus shapes the innate response via INF antagonism. DCs become infected and transport the virus to draining lymph nodes promoting virus dissemination. After viral replication in target cells, the virus becomes widely disseminated throughout the host. Viremia in the host is an important determinant for additional infection in noninfected biting arthropods. The degree of viremia determines if the vertebrate host becomes a "dead-end host" where viremia with agents such as JEV and WNV is minimal and no additional biting vectors are infected. In other flaviviruses, high and sustained viremia provides a source of additional vector infection, as is the case for DENV where humans are the primary reservoir for infection.

The site of multiplication of most arboviruses remains undetermined but is presumed to be in the vascular epithelium and the reticuloendothelial cells on the lymph nodes, liver, spleen, and elsewhere. Liberation of virus from these organs constitutes the "systemic phase of viremia," resulting after 4–7 days in fever, chills, and aching joints. A number of arbovirus infections have two phases—this early phase and then a second phase with or without a few days of freedom from symptoms. The second phase may be attended by encephalitis, joint involvement, rash, hemorrhage, and involvement of the liver and kidneys. In most arbovirus infections, only the first phase occurs, and the disease is mild and "nonspecific." In other instances, the early phase may be missed, and only the severe manifestations occur. The early phase is accompanied by leukopenia and the second phase often by leukocytosis. Tissue injury may be the direct effect of viral multiplication in susceptible cells, as is the case with liver involvement in yellow fever.

Humoral antibodies regularly appear early in the course of arbovirus infection and constitute the major basis of immunity. Such immunity may be lifelong. No infection with yellow fever virus has been recorded in an individual who either had antibodies from an earlier infection or had a history of yellow fever vaccination with development of postvaccination antibody. The presence of antibodies in the blood at the time of exposure to an infected arthropod vector provides a primary deterrent to reinfection with the homologous virus.

8 Patterns of Host Response

8.1 Clinical Features

Inapparent and subclinical human host responses predominate in most arbovirus infections. Clinical illness is frequently the exception rather than the rule. This varies from virus to virus. For example, infection with WNV, SLEV, and JEV viruses results principally in mild and inapparent infections, whereas in infection with YFV, the host response is likely to be clinically apparent and often severe (Table 16.2); the reasons for these differences are not known.

8.2 Diagnosis

Cases of arbovirus infections are not likely to be diagnosed unless there is a high degree of clinical suspicion. Outbreaks of encephalitis in horses and dying off of birds may be an early warning sign from some arboviruses. Recognition of the arbovirus infection acquired by the traveler outside the United States also depends on the alertness of the examining physician. Rapid jet transport now permits exposed overseas travelers to reach home and fall sick even within the short incubation period of such infections. The physician must maintain a high degree of suspicion when seeing central nervous system, influenza-like illnesses, or hemorrhage with fever occurring in travelers recently returned from areas endemic for arboviruses. Diagnosis will require specimens (serum, cerebrospinal fluid) to be sent to specific laboratories that have the capacity to perform the required assays such as the state laboratory or the Centers for Disease Control (CDC).

The laboratory diagnosis depends on the isolation of the virus from the blood and/or a fourfold antibody rise in titer or presence of specific IgM in sera taken during the acute and convalescent phases of illness. Often, the suspicion of an arbovirus infection in individual cases arises too late for

Table 16.2 Patterns of host response to arbovirus infections in man

Response	Examples[a]
Asymptomatic infection	WNV, DENV, JEV, SLEV
Mild febrile illness	SLEV, YFV, JEV, DENV
Influenza-like illness with aching and joint pains	DENV, ZIKV
Encephalitis, mild	WNV, TBEV, SLEV
Encephalitis, severe	WNV, TBEV, SLEV
Jaundice, proteinuria	YFV
Rash, sometimes with hemorrhagic manifestations	AHFV, DENV, KFDV
Shock syndrome	AHFV, DENV, KFDV

[a]Certain viruses have been selected for this list particularly to illustrate the range of symptoms that may be seen in populations infected with a single virus

virus isolation or for demonstration of a rise in antibody titer. Often only one acute serum specimen is sent without a convalescent specimen. Under these circumstances, the presence of a high antibody titer in a single serum may be significant if the infection is an uncommon one in that region and particularly if antibody surveys reveal a low antibody prevalence or if prior surveys have demonstrated the absence of antibody in that community. The appropriate procedure in suspected cases is to (1) notify the health department and seek background epidemiologic and clinical data and (2) send acute and convalescent serum samples to the nearest public health laboratory (usually a state laboratory), with a request for antibody tests for arboviruses and other encephalitis-producing viruses. Some state laboratories may not provide this testing, so a request for transshipment of sera to the CDC might be included.

9 Control and Prevention

Major control methods include (1) control of the arthropod vector, which may be by elimination of breeding sites, termed source reduction, or modification of them by application of insecticidal substances or by direct attack on the adult arthropods through residual insecticide treatment of adult resting places; (2) avoidance of exposure to vector bites by screening of houses, by use of protective clothing, and by application of insect repellent sprays or creams when outside in high-risk areas; and (3) immunization, a procedure widely used only for yellow fever, TBEV, and Japanese encephalitis in endemic areas.

Control of vectors through biological approaches ranges from introduction of competing species such as the use of parasites lethal to the vector including protozoa, helminths, bacteria, and viruses to introduction of genes deleterious to the vector population or influencing the vector behavior or capacity to be infected with a pathogen [68].

10 Characteristics of Selected Pathogenic Flaviviruses

Selected flaviviruses are discussed in this section and focused on those that produce human infections and represent emerged or emerging infection or have the potential to become global health problems. This section is organized by vector into tick-borne, mosquito-borne, or viruses with no known vector (see Table 16.1). Under the tick-borne flaviviruses are the mammalian tick-borne virus complex (tick-borne encephalitis, Gadgets Gully virus, Kadam virus, Kyasanur Forest disease, Alkhurma virus, Langat virus, Omsk hemorrhagic fever, Powassan virus, Royal Farm virus, and Louping ill virus). Under the mosquito-borne viruses are the Aroa virus, Japanese encephalitis virus, Kokobera virus, Ntaya virus, Spondweni virus, and the yellow fever virus complexes. Under the viruses with no known arthropod vector are the Entebbe bat virus complex, Modoc virus complex, and Rio Bravo virus complex. For each of the viruses, the epidemiology, vector, human disease, diagnosis, and epidemic potential will be briefly covered.

10.1 Tick-Borne Viruses

10.1.1 Mammalian Tick-Borne Virus Complex

The mammalian tick-borne virus complex, a genetic and antigenically related group of viruses, share similar features and have ticks as their vector and animal vertebrates as a reservoir [69]. These viruses are pathogenic to humans producing a range of diseases to include a mild febrile illness, severe hemorrhagic fever, and encephalitis and contain viruses that have emerged or emerging as global health problems. Some viruses, such as the agent of Kyasanur Forest disease, remain localized to specific geographic areas and have the potential to emerge as regional health threats. The mammalian tick-borne virus group includes Omsk hemorrhagic fever virus (OHFV), Langat virus (LGTV), Alkhurma hemorrhagic fever virus (AHFV), Kyasanur Forest disease virus (KFDV), Powassan virus (POWV), Royal Farm virus (RFV), Karshi virus (KSIV), Gadgets Gully virus (GGYV), and Louping ill virus (LIV) [2].

Gadgets Gully Virus (GGYV)

GGYV was first isolated from *Ixodes uriae* on the penguin rookeries of Macquarie Island, Australia, in 1976 [70]. Six other strains were isolated from the same location in 1976 and 1977. To date GGYV has not been known to be a cause of human disease.

Kyasanur Forest Disease Virus (KFDV)

KFVD, also known as monkey fever, is a disease of man that is transmitted primarily by *Haemaphysalis* ticks in the tropical deciduous forests of the Karnataka State in South India [71]. Its subsequent discovery in the Yunnan Province, China, in 1989, called Nanjianyin virus, is now known as a subtype of KFDV [72]. Results of a 1987–1990 seroepidemiologic investigation in the Yunnan Province demonstrated that residents of the Hengduan Mountain region had been infected with this virus [72]. This suggests that KFDV may have a wider geographic distribution, and migratory birds with attached infected ticks may be responsible. At-risk populations for KFDV include persons with recreational or occupational exposure to the forests in Karnataka, India. Human disease may be accompanied by outbreaks of hemorrhagic disease in forest monkeys maintained in a sylvatic cycle. After an incubation period of 3–8 days, the

symptoms of KFDV infection are fever, headache, severe muscle pain, cough, dehydration, and gastrointestinal symptoms. After 1–2 weeks of symptoms, some patients recover while most patients experience severe hemorrhagic fevers with a 2–10 % fatality [73]. Treatment is supportive care. A formalin-inactivated virus vaccine produced in chick embryo fibroblasts is licensed and available in India [74–77].

Alkhurma Hemorrhagic Fever Virus (AHFV)

AHFV has been isolated several times since 1995 from the blood of patients with severe hemorrhagic fever in Saudi Arabia. It is associated with the camel tick. Among 16 patients, 4 had lethal outcome [78]. Sequence analysis revealed the close genetic relationship of this virus to KFDV [79]. AHFV may be an emerging infection and a risk to travelers based on a report of tourists visiting southeastern Egypt near the border of Sudan who were infected by AHFV and developed hemorrhagic illness [80]. Like KFDV, AHFV may have its origin in Africa and in the case of AHFV transported from Africa and other countries to Saudi Arabia by the transport of camels and other livestock carrying infected ticks [80].

Karshi Virus (KSIV)

KSIV has been isolated from *Ornithodoros papillipes* ticks from Karshi, Uzbekistan. It has been reported in the Russian literature as a cause of encephalitis in humans though the exact numbers are not known (Dr. S. Khodjaev, personal communication). The vector competence of human isolates of KSIV was tested in a variety of mosquitoes and ticks. KSIV replicated in and was transmitted by all three species of Ornithodoros ticks tested (*O. parkeri*, *O. sonrai*, and *O. tartakovskyi*) [81]. Experiments demonstrated that when inoculated with Karshi virus, 90 % of *Ornithodoros* ticks transmitted this virus to suckling mice and transmission continued for at least 1 year [81]. Female *O. tartakovskyi* transmitted KSIV vertically to their progeny. This study suggests that *Ornithodoros* spp. are potential vectors and rodent species a possible reservoir, with the tick responsible for the long-term maintenance of KSIV in the environment.

Royal Farm Virus (RFV)

RFV was first isolated from *Argas hermanni* nymphs from Kabul, Afghanistan, by the US Naval Medical Research Unit in Cairo, Egypt [82]. Its group relation to Powassan and Langat viruses was demonstrated by complement fixation (CF) and neutralization tests (N). By CF test it is closely related to Powassan and by N test to Langat. RFV has never been found to be a cause of human disease.

Langat Virus (LGTV)

LGTV was first isolated in Malaysia and Thailand from pools of ticks of *Ixodes granulatus* and *Haemaphysalis* spp [83]. Although wild-type LGTV has never been found to cause human disease, patients with terminal malignancies were inoculated with LGTV in a clinical study hoping the virus might produce remission. The investigators also thought that data from this study might also support the use of LGTV as a live virus vaccine candidate against other tick-borne flaviviruses in healthy individuals. In this study, LGTV administered subcutaneously was able to produce viremia, fever, and leukopenia [84]. Further studies on this as a viable human vaccine are limited.

Louping Ill Virus (LIV)

Louping ill virus is a zoonotic disease of livestock on the British Isles transmitted by the tick *Ixodes ricinus*. It is also a very rare cause of disease in humans for people who work closely with sheep or the virus. There have been over 30 human cases of disease from LIV and several cases of laboratory-acquired infections [85, 86]. Four clinical syndromes can be seen in human disease to include influenza-like illness, a biphasic encephalitis, a poliomyelitis-like illness, and a hemorrhagic fever [86].

Omsk Hemorrhagic Fever Virus (OHFV)

OHFV is a significant cause of hemorrhagic fever and encephalitis in Western Siberia. While the disease may be transmitted by *Dermacentor* ticks, outbreaks related to direct human contact with the virus from muskrat trapping and skinning may occur [78]. The most marked clinical sign of this disease is hemorrhage, but clinical symptoms also include diffuse encephalitis which disappears during the recovery period [78].

Powassan Virus (POWV)

POWV is a tick-transmitted virus of the tick-borne encephalitis subgroup of flaviviruses. It is antigenically related to TBEV discussed below. POWV is a North American virus and originally isolated from the brain of a child who died in Ontario in 1959 [87]. Powassan virus has been found to be widely distributed in small mammals in Canada and the northern states of the United States including New York, Wisconsin, and Minnesota [88]. POWV was originally thought to be a minor cause of human encephalitis with 27 cases reported in Canada and the northeastern United States during 1958–1998 [89]. Over the past decade there has been an expansion of cases throughout Canada and the United States with four Maine and Vermont residents with encephalitis found to be infected with POWV in 1998–2001. During 1999–2005, there were nine cases of serologically confirmed POWV disease: four from Maine, two from New York, and one each from Michigan, Vermont, and Wisconsin [89, 90]. The Michigan and Wisconsin cases represent the first reported from the north-central United States and suggest that this infection may be underreported in the United States.

POWV encephalitis is characterized as a classic encephalitis with the acute onset of muscle weakness, confusion, and neurologic signs. All patients described in this series recovered but most had long-term neurologic sequelae. POWV is transmitted by hard ticks (*Ixodidae* spp.). Diagnosis can be accomplished by serology and PCR. There is no vaccine available for POWV or specific therapy for illness.

10.1.2 Tick-Borne Encephalitis

Tick-borne encephalitis virus (TBEV) produces a fatal encephalitis in Europe and Asia [91]. The disease has been known under several names, including Russian spring–summer encephalitis and Far Eastern encephalitis. Clinically, TBEV produces an acute febrile illness followed by encephalitis with a high mortality rate. The virus is maintained in an environmental cycle involving small mammals and birds in forested regions and transmitted by the ticks *Ixodes ricinus* and *I. persulcatus*. Humans become infected by the bite of infected ticks. Forest and construction workers, woodsmen, trappers, and farmers are at highest risk. TBEV is an emerging infection spreading geographically and increasing in mortality. From 1974 to 2003 there was a 400 % increase in morbidity in Europe and has spread to regions that were previously unaffected [92, 93]. TBEV is a notifiable disease in 16 European countries. There was an average of 8,755 reported cases of TBEV per year in Europe and Russia between the years 1990 and 2007 as compared to 2,755 cases per year between 1976 and 1989 [94]. The expansion of TBEV may be caused by changes in the number and geographic range of the tick vector due to climate changes and changes in land use. No specific therapy exists for TBEV. Prevention from infection is through control of tick populations, protection against tick bite (repellents, protective clothing), and avoidance of tick habitats. An inactivated vaccine is widely used in central Europe. Diagnosis is made by serology, virus isolation, and PCR.

10.1.3 Mosquito-Borne Viruses

The mosquito-borne viruses represent some of the most pathogenic viruses known to man. They have been historically a cause of large epidemics and have become emerging or emerged pathogens. They produce a range of illnesses from a severe febrile illness to fatal hemorrhagic disease and encephalitis. As mosquito-borne viruses the majority have an intermediate bird or rodent reservoir with humans infected by the bite of infected mosquitoes.

Aroa Virus Complex

Aroa Virus (AROAV)

AROAV was initially isolated from a hamster (*Cricetus auratus*) in the vicinity of the Aroa River, Venezuela, in the early 1970s. Complement fixation (CF) experiments concluded Aroa was a new group B virus most closely related to Bussuquara virus. Antigenic and biological properties categorize AROAV closely with Israel turkey meningoencephalitis, Koutango, and Negishi viruses. AROAV grows in C6/36 cells suggesting vector transmission potential [95]. There are no reports of human infection or disease [3].

Bussuquara Virus (BSQV)

BSQV was isolated in 1956 in Belem, Brazil, from a howler monkey (*Alouatta belzebul*). The virus has been isolated in the Amazon from sentinel and wild *Proechimys* spp. rodents and *Culex* mosquitoes [96]. The virus was known to circulate in Brazil, Colombia, and Panama [97]. In vitro experiments demonstrate infection across a range of murine, chick, and monkey cell lines [98]. Both *Aedes* and *Culex* mosquito species demonstrate a potential for infection. Liver lesions similar to those caused by YFV were noted in an infected howler monkey, *Alouatta belzebul*. There has been one human clinical case reported with fever, headache, profuse sweating, and arthralgia lasting 4 days [3, 99].

Iguape Virus (IGUV)

IGUV was isolated in 1979, from a sentinel mouse, in the rain forest of Iguape county, Sao Paulo state, Brazil [96, 100]. Serosurveys demonstrate wild birds may participate in the virus transmission cycle, as may rodents and marsupials. Defining virus reservoir–vector relationships requires further study [100]. There have been no known human infections.

Naranjal Virus (NJLV)

NJLV was first isolated from a hamster in 1976 in Guayaquil, Ecuador. It is closely related to BSQV, but CF and neutralizing antibody assays clearly demonstrate that it is distinct [101]. Experimental infection of mice produces death and viremia over 5.6–8 days [3]. There are no reports of human infection or disease.

Japanese Encephalitis Virus Complex

Cacipacore Virus (CPCV)

CPCV was first isolated from the *Formicarius analis* (black-faced ant bird) in Oriximina, Para, Brazil, in 1997 [102]. Serosurveys demonstrate evidence of infection in wild birds, rodents, bats, and humans. Injection into mice produces death [102]. A 33-year-old farmworker in the Brazilian state of Rondônia, Brazil, was admitted to intensive care with suspected leptospirosis and yellow fever; however, viral sequencing identified CPCV as the cause of infection [103].

Koutango Virus (KOUV)

KOUV was initially isolated in 1968 from a *Tatera kempi* (gerbil) in the Koutango Village, Saboya region, Dakar, Senegal. KOUV is known to circulate in Senegal, Central

African Republic, Somalia, and Gabon [104, 105]. Natural host range includes rodents, ticks, mosquitoes, and a single case of infection in man (laboratory accident). Mosquito (*Aedes aegypti*) transmission and reinfection experiments have been conducted as well as experimental transovarial transmission which suggest *A. aegypti* is a competent vector [3, 106].

Japanese Encephalitis Virus (JEV)

JEV is the leading cause of viral encephalitis in Asia [107]. JEV is a mosquito-borne zoonotic flavivirus transmitted predominantly between birds and mammals and to humans by mosquitoes. The principal vector of JEV in northern Asia is the rice paddy-breeding *Culex tritaeniorhynchus summarosus* and in southern Asia *C. tritaeniorhynchus* and related *C. vishnui* group. Many species of wild birds function as reservoirs, supporting JEV circulation and transmission. Domestic pigs may serve as amplifying hosts. Horses and humans are dead-end hosts because the virus circulates in blood at low levels and for a short duration, thereby not supporting transmission [108, 109].

Outbreaks of JEV occurred regularly in Japan, Korea, China, and Taiwan during the twentieth century. Seasonal outbreaks now involve portions of Vietnam, Thailand, Nepal, and India. The Philippines, Indonesia, and the northern tip of Queensland, Australia have witnessed smaller outbreaks [108, 109]. Large-scale vaccination programs made it possible for Japan, Korea, and Taiwan to nearly eliminate JEV. More recent vaccine adopters (Thailand, Sri Lanka, and Nepal) have also observed a reduced disease burden [108, 109]. Numerous JEV endemic regions remain at risk despite the availability of safe and efficacious JEV vaccines.

In JEV endemic areas, the incidence of disease is greater in the young; attack rates in the 3- to 15-year age group are five to ten times higher than in older persons; this is likely a herd-immunity phenomenon [108, 109]. Numerous studies and epidemiologic observations document a weak protective effect of prior dengue virus infection on subsequent JEV disease [110, 111]. The ratio of symptomatic JEV cases to infections varies between 1:25 and 1:300 with the lower rates (1:200–1:300) observed in Asians indigenous to areas with enzootic JEV transmission, while higher rates were measured in American military personnel [112–114]. The incidence of JEV for travelers from non-endemic countries to Asia is estimated at <1 case per million travelers. Expatriates and travelers who spend prolonged periods in JEV enzootic areas may share a similar or higher risk than the indigenous population [115–117]. Between 1973 and 2008, 55 cases of JEV were reported in travelers from non-endemic countries. Ten (18 %) of these were fatal and 44 % ($N=24$) recovered but experienced sequelae [115–124].

JEV is a small (50 nm), enveloped virus containing a 10.7-kb, single-stranded, positive-sense RNA genome. The viral envelope (E) protein serves as the cell receptor-binding protein and the fusion protein for virus attachment and entry into host target cells. Antibodies directed against E protein neutralize the virus and play an important role in protection [109, 125, 126]. The JEV was first isolated from the brain of an encephalitis patient in Tokyo (1935) and was virologically and serologically established as the prototype (Nakayama) strain [127]. Antigenic variation among JEV strains has been shown by serologic, virologic, and monoclonal antibody analysis [127–129]. The virus causes cytopathic effect (CPE) in varied cell lines that include chick embryo, human epithelial, mouse, Vero, LLC-MK2, and C6/36. A broad range of hosts experience encephalitis and death following intracranial or intraperitoneal administration of JEV to include mice, hamsters, dogs, foxes, cats, and goats. Asymptomatic viremia is observed in monkeys, guinea pigs, rabbits, chickens, pigs, and bats [3]. *Culex tritaeniorhynchus*, *C. fuscocephalues*, and *C. gelidus* were experimentally infected feeding on viremic pig and chicken and successfully transmitted infection to chicken [130–133]. Transovarial transmission was demonstrated in *Aedes albopictus* and *A. togoi* [134].

Following infection there is a 5- to 7-day incubation period and then a nonspecific viral prodrome. Early clinical symptoms include lethargy, fever, headache, abdominal pain, nausea, and vomiting [135]. Nuchal rigidity, photophobia, altered consciousness, hyperexcitability, masked facies, muscle rigidity, cranial nerve palsies, tremulous eye movements, tremors, involuntary movement of the extremities, paresis, incoordination, pathological reflexes, and meningeal (meningitis), parenchymal (encephalitis), or spinal cord (myelitis) signs may follow [136]. Sensory deficits are rare, and children (50–85 %) experience focal or general seizures more often than adults (10 %) and are associated with poor clinical outcome [136]. In a recently published study of 1,282 adult patients collected from 4 JEV epidemics in India (1978, 1980, 1988, and 1989), altered sensorium occurred in 96 %, convulsions in 86 %, and headache in 85 % [137]. Hyperkinetic movements were noted in 46 %, and most (83 %) were choreoathetoid in nature. Opsoclonus (20 %), gaze palsies (16 %), and pupillary changes (48 %) were observed, but cerebellar signs were not. Dystonia and decerebrate rigidity were observed in 43 and 6 %, respectively, of patients with 17 % having paralytic features and seizures in 30 %. Abnormal breathing patterns, pulmonary edema, and upper gastrointestinal hemorrhage were observations of prognostic importance.

Elevated peripheral white blood cell counts and low sodium may be observed. Cerebrospinal fluid (CSF) opening pressure, white blood cell content, and protein levels may be normal or mildly elevated [136]. Electroencephalogram may demonstrate diffuse delta wave activity and, rarely, seizure

patterns [136]. Imaging studies may demonstrate abnormal findings in the white matter, thalamus, basal ganglia, cerebellum, midbrain, pons, and/or spinal cord.

If the patient improves, it will typically begin about 1 week after symptom onset at the time of defervescence, but recovery of neurologic function may take weeks to years. Seizure disorders, motor and cranial nerve paresis, and movement disorders may persist in up to one third of patients. Persistent behavioral and/or psychological abnormalities occur in 45–75 % of survivors and are more severe in children [138]. JEV is managed with supportive care as there are no specific antivirals demonstrating clear benefit. Fatality rates vary between 5 and 40 % and are often reflective of medical care resources and capabilities.

Diagnosis by viral isolation is infrequent although feasible from brain tissue in fatal cases or CSF in about one third of cases during the acute infection period; this portends a bad prognosis [139]. Serologic diagnosis is possible, demonstrating a fourfold rise in antibody titer using hemagglutinin inhibition, complement fixation, or neutralizing antibody assays with appropriately timed acute and convalescent specimens. Serum IgM antibodies appear early in the course of infection, persist for 3–6 months, and are relatively specific. The IgM-capture ELISA is especially well suited for diagnosis by detection of locally synthesized JEV antibody in the CSF [140]. Earlier and more vigorous antibody responses correlate with improved survival. Serologic assays are plagued by cross-reactivity between JEV and other flaviviruses such as dengue and West Nile virus. Newer serologic, reverse transcriptase polymerase chain reaction and NS1 protein-based assays tout improved sensitivity and specificity.

Individuals can reduce exposure to vectors by use of mosquito repellant, wearing long-sleeved shirts and trousers, avoiding outdoor activities in the evening, and sleeping under permethrin-impregnated mosquito nets or in screened or air-conditioned rooms [141]. Active immunization, in addition to personal protective measures, is the optimal strategy for preventing JEV. JEV vaccines have been available since the 1950s [135]. Early vaccines were produced by inactivating virus grown in mouse brain or in primary hamster kidney (PHK) cells [135]. These vaccine constructs were used in the United States and Europe (BIKEN; distributed as JE-Vax by Sanofi Pasteur, Lyon, France) [142]. Seroconversion rates (i.e., quantitative neutralizing antibody titers following vaccination) and efficacy varied according to the population studied (indigenous vs. nonindigenous to JEV endemic area) and number of doses administered (one, two, or three doses) in the primary immunization series [135, 143]. Prolific vaccine use has contributed to significant decreases in JEV incidence in Thailand, India, Korea, Taiwan, Vietnam, and areas of Malaysia and Sri Lanka [135]. Since 1989, numerous cases of moderate to severe hypersensitivity-type reactions temporally associated with JEV vaccination have been reported, some resulting in hospitalization [144, 145]. The cause of the reactions is unclear, but the presence of murine neural proteins, gelatin, and/or thimerosal in vaccine preparations has been implicated, but not proven. More serious was the case in Japan of acute disseminated encephalomyelitis (ADEM) temporally related to vaccination. ADEM has been reported as a severe drug reaction following administration of inactivated mouse brain vaccine in 5×10^{-4} to 1×10^{-6} administered doses [143]. No definite increased risk of ADEM temporally associated with JEV vaccination has been proven.

To move away from mouse brain-derived vaccines, vero cells were adopted for the development of numerous JEV vaccine candidates [135]. Widespread distribution and use in Asia have demonstrated an excellent safety profile, immunogenicity, and efficacy [146]. Second-generation JEV vaccines with improved safety profiles and lower dosage requirements present new options for immunization [135]. The IC51 (IXIARO®, in Australia and New Zealand available as JESPECT; Intercell AG, Vienna, Austria) vaccine is a purified, formalin-inactivated, whole-virus JEV vaccine. The product is approved in the United States (persons aged ≥17 years), Europe, Canada, Switzerland, and Australia. IC51 is manufactured by Intercell Biomedical (Livingston, United Kingdom) and is distributed in the United States by Novartis vaccines (Cambridge, Massachusetts). The vaccine construct, developed at the Walter Reed Army Institute of Research (Silver Spring, MD), is based on a SA14-14-2 virus strain passaged in primary dog kidney (PDK) cells, cultivated in Vero cells, and formulated with aluminum hydroxide [147]. Numerous clinical trials have demonstrated IC51 safety and immunogenicity [148, 149]. A pooled, 6-month safety analysis of seven phase III trials included 3,558 subjects with at least one IC51 vaccination, 435 subjects with a JE-Vax® vaccination, and 657 with phosphate buffered saline solution with 0.1 % Al(OH)3 (PBS + Alum) control vaccination demonstrated a similar safety profile between the study arms with a superior local reactogenicity profile for IC51 compared to JE-Vax® [150]. Individuals previously given tick-borne encephalitis (TBE) vaccine and then vaccinated with IC51 demonstrated a 17 % higher incidence of at least one adverse event in volunteers with previous TBE vaccination and slightly higher seroconversion rates and higher GMT in the IC51 group who had received TBE vaccination (98 %, 470, compared to 92 %, 182) [151].

Sanofi Pasteur has developed a JEV vaccine based on a construct created at the St. Louis University Health Sciences Center, St. Louis, Missouri, and Acambis Inc., Cambridge, Massachusetts [152]. JEV-CV is produced by inserting prM and E genes from the SA14-14-2 JEV virus into the YFV 17D viral strain "backbone" containing the YF nonstructural genes [153]. The resulting chimeric RNA is electroporated into Vero cells and replicated by the highly stable YFV RNA-dependent replicase. The safety and immunogenicity of a

single dose of JEV-CV at two concentrations (1×5.0 $\log_{10}$PFU versus 1×4.0 $\log_{10}$PFU) was comparable to licensed YFV-VAX (1×5.0 $\log_{10}$PFU) in a phase 1 trial. Low-level viremias were observed in both JEV-CV groups, and the frequency of all adverse events was similar in each group. Overall, 96 % of subjects who received JEV-CV developed neutralizing antibodies to one or more of the wild-type JEV strains tested (Beijing, P3, Nakayama), and all developed antibodies to the homologous strain [154]. There is evidence that JEV-CV induced sterilizing immunity. Safety and immune responses were evaluated in $N = 10$ JEV-CV (1×4.0–5.0 $\log_{10}$PFU) vaccine recipients who, about 9 months later, were given a single dose of JE-Vax. No severe adverse events were noted and seroconversion rates rose from 50 % pre-JE-Vax to 90 % with a GMT greater than 1:150. Phase 3 studies assessed safety and immunogenicity comparing JEV-CV and a mouse brain-derived (MBD)-JEV vaccine. The safety profile of JEV-CV was superior to the MBD-JEV vaccine and similar to placebo. The percent of the cohort seroconverting following a single dose of JEV-CV by PRNT with the homologous strain was non-inferior to three doses of the MBD-JEV vaccine (99.1 % vs. 95.1 %) [155]. Additional studies assessing immunization of toddlers and children, co-administration and sequential administration with yellow fever (YF) vaccine (YF-17D strain; Stamaril((R)), Sanofi Pasteur), and administration of a booster doses demonstrated excellent safety profiles and immunogenicity [156].

Murray Valley Encephalitis Virus (MVEV)

MVEV has caused numerous encephalitis epidemics (Australian X disease) in Australia since the early 1900s. The virus was first isolated in 1951 from the brain of a 19-year-old male who succumbed to infection [3]. Encephalitis outbreaks in 1917, 1918, 1922, and 1925 were initially without an etiologic agent but are now believed to have been MVE [139]. Additional outbreaks occurred in 1956, 1971, 1974, 1978, 1981, and 1984, primarily in the Murray Valley of New South Wales and Victoria [157]. The 1974 outbreak was unique in its geographic expansiveness, infecting people in east central Queensland, Northern Territory, northern and southeastern South Australia, and the Ord River Basin of Western Australia [158]. Between 1978 and 1991 MVE was diagnosed in 26 patients, and 16 were in the Kimberley area of Western Australia [159]. During the 2007–2009 seasons, six human cases of MVEV were reported, at least two fatal, to the National Notifiable Diseases Surveillance System (NNDSS) [160]. Sixteen people are believed to have contracted MVE in 2011 [161]. MVEV is endemic to Papau New Guinea and Irian Jaya [162].

MVEV is a mosquito-borne flavivirus existing in an enzootic cycle involving primarily water birds [161]. *Culex annulirostris* is believed to be the principal vector, preferring to breed in shallow, warm, fresh water. Although MVE outbreaks occur most often during summer (January to May) and following high rain periods, the ecology remains largely unknown [161, 163]. Viral activity is monitored by trapping and testing mosquitoes or testing sentinel chicken flocks for serologic seroconversion (http://medent.usyd.edu.au/arbovirus/index.html, accessed 28 May 2012). Early indicators of MVEV circulation trigger increased public health vigilance and education regarding personal protective measures.

Complement fixation and neutralizing and cross-neutralizing antibody studies demonstrated MVEV was a group B arbovirus in a subgroup containing Japanese encephalitis, West Nile, St. Louis encephalitis, and Kunjin, Usutu, Kokobera, Stratford, and Alfuy viruses [3]. Nucleotide sequencing associates the MVEV most closely with the Japanese encephalitis virus. Phylogenetic studies suggest MVEV emerged in the Malay–Indonesian regions from an African progenitor virus, possibly Usutu. There appears to be persistent homogeneity of MVEV strains from widespread areas throughout Australia different from those circulating in New Guinea. The natural host range of MVEV is broad including humans, chickens, species of water and land birds, horses, dogs, foxes, and opossum. Experimental infection of mice, sheep, chick embryos, and hamsters resulted in death [3]. *Culex annulirostris* and *C. quinquefasciatus* and *Aedes occidentalis* and *A. vigilax* demonstrated competence to infect chicks following ingestion of an infected blood meal, and transovarial transmission following oral infection was achieved with *Aedes aegypti* [49, 164].

Approximately 1 of every 150–1,000 MVEV infections results in clinical disease. Complete recovery is seen in approximately 40 %, long-term neurologic sequelae in 30–50 %, and death in 15–30 % [165]. Following an incubation period of 1–4 weeks, patients will experience 2–5 days prodrome of high fever and headache with anorexia, myalgia, nausea, vomiting, diarrhea, rash, or cough [159, 166]. Neurologic features include lethargy, irritability, and confusion with or without seizures. The clinical spectrum of disease can be broad ranging from fever and headache without encephalitis to flaccid paralysis, cranial nerve and brainstem involvement, encephalitis with complete recovery, or death [161]. The disease appears to progress very rapidly in infants [139].

Magnetic resonance imaging (MRI) is the most sensitive and specific imaging study available to support a diagnosis. T2-weighted images demonstrate bilateral hyperintensity of the deep gray matter and may have findings mimicking herpes simplex virus (HSV) encephalitis involving the temporal lobes, red nucleus, and cervical spinal cord.

Laboratory confirmation of infection is achieved through viral isolation, detection of MVEV RNA, a fourfold rise in IgG between acute and convalescent serum samples, or finding IgM in the serum of cerebral spinal fluid (CSF) (http://www.health.gov.au/internet/main/publishing.nsf/Content/

health-arbovirus-mve-guidelines.htm, accessed 29 May 2012). Cross-reactivity among antibody-based tests complicates distinguishing MVEV from other flaviviruses following infection or vaccination. Neutralization assays or an epitope-blocking ELISA may add diagnostic specificity.

There are no licensed vaccines or therapeutics to protect against or treat MVE. Trials of steroids, ribavirin, interferon alpha-2a, and formulations of intravenous immunoglobulin have been evaluated in the context of Japanese encephalitis and West Nile virus infections but with little success [167, 168]. Treatment is supportive and has reduced mortality and morbidity. Passive immunization studies in animals using gamma globulin from MVE and Japanese encephalitis virus survivors demonstrated prophylactic efficacy. The ability of a live chimeric Japanese encephalitis virus vaccine (ChimeriVax-JE) to cross-protect against MVEV in two encephalitis mouse models suggested a single dose elicited a protective and durable immune response [169]. DNA-based and Semliki Forest virus-vectored vaccines using the MVEV prM and E proteins as immunogens were investigated in a murine model. Both candidates induced durable, MVE-specific neutralizing antibody responses [170].

St. Louis Encephalitis Virus (SLEV)

The first recognized outbreak of SLE occurred in Paris, Illinois, in 1932 followed the next year by epidemics in St. Louis and Kansas City, Missouri. The virus was first isolated from the brain of a fatal encephalitis case following inoculation in mice and rhesus monkeys [171]. Subsequent SLE outbreaks occurred in the Pacific coast states (1940s), Florida (1959, 1961), Texas, Ohio-Mississippi Valley (1970s), Colorado, California, Florida, Texas, and Arkansas [139, 171–175]. Disease has been reported throughout the majority of the United States, southern Canada, Mexico, and in limited areas of Central and South America [68, 173, 175–209]. There is a diffuse endemicity over vast rural areas, with low rates of seropositives in humans, but with occasional epidemics of hundreds to more than a thousand cases. The monitoring of sentinel flocks of birds for infection has proved useful as an early warning system [174].

The virus is transmitted by a number of culicine mosquitoes, including *Culex pipiens* and *C. quinquefasciatus* in the East and urban areas of the West, *C. tarsalis* in rural areas of the western states, and *C. nigripalpus* in Florida and southern areas [210–214]. In rural areas of the West and Southwest, it is closely tied to *C. tarsalis*. Depending on the climate of the region, maintenance of the virus can occur by year-round horizontal transmission from bird to mosquito, overwinter survival of infected mosquitoes, or venereal or transovarian infection [215–221].

SLE virus is in the JEV–MVE–West Nile virus complex or subgroup [139, 222, 223]. The virus produces cytopathic effect and plaques in numerous vertebrate cell lines that include primary chick embryo, hamster kidney, BHK-21, Vero, and LLC-MK2. Cell cultures from human, primate, rodent, swine, and avian cell cultures support replication [224]. Natural host range includes wild vertebrate species of birds as well as raccoons, opossums, and bats in North America and birds, rodents, nonhuman primates, and the three-toed sloth in tropical America. SLE varies in virulence for mice [139, 225, 226].

SLE virus has been responsible for thousands of deaths, more than 10,000 severe illnesses, and no fewer than a million mild or subclinical infections [227, 228]. Only 1–2 % of SLE virus infections are symptomatic [210, 229, 230]. The ratio of asymptomatic to symptomatic infection ranges from 800:1 in children to 85:1 in adults older than 60 years [200, 231]. The infection incubation period ranges between 4 and 21 days. When the disease occurs, it may be classified into three clinical syndromes: constitutional symptoms and headache (febrile headache), aseptic meningitis, and fatal encephalitis [139, 200, 209]. Most cases start with fever, headache, nausea, myalgias, and sometimes respiratory or abdominal symptoms; most recover completely [231]. In those who develop advanced disease, following the acute febrile prodrome, there may be an acute or subacute appearance of meningeal and other neurologic signs and symptoms. Symptoms may include nuchal rigidity, disorientation, unsteady gait, vomiting, and diarrhea. Tremulousness involving the eyelids, tongue, lips, and extremities is observed. Cerebellar and cranial nerve signs are common [231, 232]. Apathy, confusion, and disorientation leading to coma may occur. Nearly one quarter of patients experience urinary symptoms (frequency, urgency, dysuria) [233]. The morality rate is approximately 8 %, ranging from approximately 2 % in the young to 22 % in the elderly who have comorbidities [139]. Advanced age is the most significant risk for developing both symptomatic disease and more severe encephalitis after infection [172]. People older than 60 years of age have the highest frequency of encephalitis [139]. Almost 90 % of elderly SLE patients develop encephalitis [230]. Longer-term sequelae such as asthenia, irritability, tremors, sleeplessness, depression, memory loss, and headaches typically last less than 3 years but may last longer. Persistent symptoms include gait and speech disturbances, sensorimotor impairment, psychoneurotic complaints, and tremors [234–236].

Clinical laboratory findings are nondistinct. Peripheral white blood cell count may be high and urine findings may include microscopic hematuria, proteinuria, and pyuria [231]. Inappropriate antidiuretic hormone secretion and hyponatremia may occur in a third of patients. Fewer than 200/mm^3 white blood cells are typically seen in the cerebral spinal fluid; polymorphonuclear cells predominate early with an eventual shift toward a lymphocytic pleocytosis. Protein levels may be elevated to 1.5–2.0 times normal. Electroencephalogram shows diffuse slowing and seizure

activity. Magnetic resonance imaging may show increased signal in the substantia nigra, while radionuclide brain scans and computed tomography have been normal [139, 237].

Diagnosis should be suspected if the patient presents with a compatible clinical syndrome in the summer or early fall, in an area known to have SLE virus circulation, with reports of similar cases with patients at the extremes of age. Although virus isolation has been documented from the liver, spleen, lung, kidney, and brain, isolation is very unusual from serum or CSF [238]. Polymerase chain reaction assays are being developed for diagnosis and surveillance [239–246]. Diagnosis most often is made using serologic assays testing acute and convalescent serum samples or a cerebral spinal fluid sample [21]. IgM antibodies in serum appear within the 4 days after infection, peak at 7–14 days, and then decline and disappear by day 60 but may persist up to a year in a small percentage of patients [139, 231, 247]. A fourfold change in serum antibody titer confirms infection, and presence of IgM antibody in a single serum is presumptive evidence. Hemagglutinin inhibition detects group-reactive antigens and may be a useful screening test. The presence of complement fixation antibodies in a single serum sample is presumptive evidence of a recent infection, but 20 % of patients with confirmed SLE virus infections do not develop complement fixation antibodies [139]. Detection of IgM antibodies in the cerebral spinal fluid by enzyme-linked immunosorbent assay (ELISA) provides a rapid and early diagnosis.

There is no licensed antiviral therapeutic to treat SLE. The current care standard is supportive focusing on controlling seizures, providing respiratory support, reducing cerebral edema, and managing metabolic derangements [231]. There is no licensed vaccine to prevent SLE. Personal protective measures and other vector avoidance practices are encouraged. Control in rural or an urban epidemics has been attempted by emergency application of mosquito control measures. Long-term control for urban and suburban localities depends on good sanitation with respect to drainage and adequate disposal of waste water. In more specific rural areas under irrigation, much can be accomplished through water management and directed application of insecticides to keep mosquito populations low.

Usutu Virus (USUV)

The USUV is an avian virus discovered in South Africa (1959) in a female mosquito (*Culex neavei*) [3, 248]. The virus has infectivity for numerous primary and continuous animal species and human cell lines [3]. The life cycle is complex, involving host bird species and *Culex* mosquitoes as primary vectors and humans, horses, and other mammals as incidental hosts. Geographic range initially includes Senegal, Central African Republic, Nigeria, Uganda, Burkina Faso, Cote d'Ivoire, and Morocco [249]. The virus emerged in Vienna, Austria, in 2001 associated with avian, mainly blackbird, mortality. Infection has recently been noted in a variety of central European birds in Austria, Hungary, Switzerland, United Kingdom, Spain, Germany, and Italy [250, 251]. USUV was initially discovered to be clinically relevant in 1981 when a man in the Central African Republic developed fever and rash following infection. In 2004, a 10-year-old child in Burkina Faso developed fever and jaundice following infection. In 2009, 2 immunosuppressed people (liver transplant, B-cell lymphoma) in Italy developed encephalitis [252, 253]. A recent serosurvey in Northeastern Italy demonstrated 4 of 359 donors had IgG evidence of USUV infection [254].

West Nile Virus (WNV)

WNV was first isolated in a febrile woman in the West Nile District of Uganda in 1937 [255]. Since its discovery in West Africa, outbreaks of WNV have occurred in many parts of the world, and WNV is now endemic in Africa, Europe, the Middle East, West and Central Asia, Oceania, and North America [256]. In the United States, the westward spread of WNV across the continent from the initial cases in New York over the course of about 5 years was an epidemiologic phenomenon that was closely monitored [257]. WNV now has become the leading cause of neuroinvasive arboviral disease in the United States [258].

Wild birds are the primary vertebrate reservoirs of WNV in endemic regions [259] and WNV has been found to be capable of infecting over 300 different species of birds [260]. In many instances, WNV has spread across continents and regions following bird migration patterns [261]. The virus is transmitted by mosquitoes usually of the *Culex* spp., though various spp. of *Aedes*, *Anopheles*, and Ornithodoros ticks can be infected by WNV [3]. Mammals are less important than birds in maintaining transmission cycles of the virus as viremia is too low in most of the mammal species to reinfect mosquitoes [262].

Nearly all humans are infected with WNV primarily by the bite of infected mosquitoes [257]. However, transmission of WNV to humans has also occurred through transplanted organs, and transfused blood, transplacental transmission [263], and transmission through breast milk may also be likely [264]. Most infections with WNV are asymptomatic. The typical incubation period is 2–14 days, and a self-limited febrile illness without neurologic involvement, known as West Nile fever, may occur in approximately 20–30 % of infected cases. The symptoms of West Nile fever may include malaise, eye pain, headache, myalgia, gastrointestinal discomfort, and rash. A maculopapular rash occurs in about half of the persons with West Nile fever but is less commonly reported in persons with neuroinvasive disease. About 1 in 150 cases may progress to severe disease and develop encephalitis, men-

ingitis, or acute flaccid paralysis. Long-term complications (1 year or more after infection) are common in patients recovering from severe WNV infection. Currently, supportive care is the only treatment available. Advanced age is the most important predictor of death [265]. The mortality rate among neuroinvasive disease is approximately 10 %, with increased risk for patients with compromised immune systems and advanced age and with underlying conditions such as diabetes mellitus.

Diagnosis of WNV is made primarily by the use of indirect antibody-capture ELISA or PCR. In most cases IgM will be produced within 4–7 days and may persist for more than a year [266]. ELISA for WNV may be performed on serum or CSF. Other serologic tests such as neutralization assays generally are less cross-reactive with other flaviviruses, but are not widely available for clinical diagnosis. Antigen detection assays are also being developed but are not widely used in the clinical setting. PCR is widely available and is very specific and sensitive.

There is no WNV vaccine available for use in humans, but a number of WNV vaccine candidates are in clinical trials. There are three WNV vaccines currently licensed for use in horses; two are killed virus vaccines, and one is a chimeric recombinant canarypox virus vaccine [262].

Yaounde Virus (YAOV)

The original source of YAOV was a pool of *Culex nebulosis* female mosquitoes collected in 1968 near Yaounde, Cameroon, Africa [3]. One month later the virus was isolated via intracerebral inoculation in mice. Complement fixation identified the virus as related to Usutu virus. Natural host range includes mosquitoes (*Culex* spp., *Aedes*, *Eretmapodites*), rodent, and bird. Injection in mice produced death. There are no known human cases [3].

Kokobera Virus Complex

Kokobera Virus (KOKV)

KOKV is a mosquito-borne virus that was originally isolated from *Culex annulirostris* at the Kowanyama (Mitchell River Mission) in northern Queensland in 1960 and named after a local Aboriginal tribe [267]. It is isolated throughout Australia and Papua New Guinea [268]. The reservoir for the virus is thought to be kangaroos, wallabies, and horses. Human infections with KOKV result in an acute polyarticular disease.

Stratford Virus (STRV)

STRV has a geographic distribution similar to KOKV from *Culex* species producing a febrile polyarticular illness. Originally classified with KOKV as part of the JEV serocomplex, serologic and sequencing of the virus demonstrated that STRV is closely related to KOKV and its classification into the KOKV complex [3].

New Mapoon Virus

New Mapoon virus was originally isolated as part of viral isolates obtained from mosquitoes in 1998 on Cape York Peninsula (located Far North Queensland, Australia) and in 2000 on Saibai Island (one of the Torres Strait Islands in Australia, between the Australian mainland and the island of New Guinea) and New Mapoon, Queensland [3]. Viruses were characterized by partial genomic sequencing, monoclonal antibody-binding assays, and polyclonal cross-neutralization tests. Two of these isolates were antigenically related to the KOKV complex but distinct. Sequencing demonstrated a distinct novel virus named New Mapoon virus.

Ntaya Virus Complex

Ilheus Virus (ILHV)

Ilheus virus (ILHV) was isolated from *Aedes serratus* and *Psorophora ferox* mosquitoes near Ilheus, Bahia, Brazil, in 1944 [269]. In the original study, *Aedes aegypti* was demonstrated as a competent vector for this virus. After its discovery, the virus was also isolated from a variety of other mosquito species and birds in Latin America. ILHV was described as producing a febrile illness in humans in Central and South America, but the burden of disease is not well studied. In November 2005, a patient in Bolivia was reported with ILHV who developed fever, malaise, asthenia, conjunctival injection, vesicular rash, facial edema, arthralgia, myalgias, bone pain, abdominal pain, headache, and earache [270].

Ntaya Virus (NTAV)

Ntaya virus was originally isolated from mosquitoes in Ntaya, Uganda [3]. Isolation has been made from mosquitoes in Uganda, Cameroon, and the Central African Republic and may be endemic in Nigeria, Kenya, and the Zambia [3]. Serologic surveys suggest that this virus may be widespread with antibodies to Ntaya virus in subjects in Tanzania, Egypt, and the Far East. In a study of returning travelers from Africa, three patients tested positive for Ntaya virus. Illness symptoms reported by the travelers included severe malaise, fever, headache, myalgia, and neurologic manifestations of dizziness, numbness, and weakness of the left leg and arm. In one traveler there was severe amblyopia and restriction of the peripheral visual fields.

Other Viruses Within the Ntaya Complex

Other viruses within this complex are primarily pathogens of poultry causing large epidemics. These include Bagaza virus (BAGV), Israel turkey meningoencephalomyelitis virus (ITV), and Tembusu virus (TMUV). All are spread by a variety of mosquitoes primarily, *Culex* spp. These viruses have the potential to produce human disease with serologic evidence of human infections though the burden of disease within the human population is not known and thus are

briefly described. BAGV which is closely related to ITV has been described as producing a febrile illness in humans with one study demonstrating a possible cause of human encephalitis in India. In an outbreak investigation of a severe duck illness in China, a new virus related to TMUV and BAGV was described named BYD virus [271].

Spondweni Virus Complex

Spondweni Virus (SPOV)

SPOV was first isolated in 1955 from mosquitoes, *Taeniorhynchus uniformis* collected in Tongaland, South Africa. The name was derived from the location they were collected, the district of Spondweni. A serosurvey of residents in Tongaland demonstrated evidence of infection to SPOV. Human infection was demonstrated in laboratory workers working with the virus characterized as a febrile illness with generalized aches and pains, headache rigors, and vertigo [272]. SPOV has been isolated from humans in Mozambique and Cameroon with serologic evidence of infection in humans in Angolal and Botswana [272]. The virus has been isolated from several mosquito species including *Mansonia uniformis*, *Aedes circumluteolus*, *M. africana*, *A. cumminsii*, *Eretmapodites silvestris*, *A. fryeri*, *and A. fowleri* [272]. In 1982 a case of acute SPOV infection was reported in a 40-year-old American man, who had worked in Bitou, located in southeastern Upper Volta (Burkina Faso), near the borders of Ghana and Togo [272]. Symptoms included severe headache, dizziness, nausea, muscle aches, eye pain, and sensitivity to light.

Zika Virus (ZIKV)

ZIKV was originally isolated from a caged febrile rhesus monkey in the Zika Forest, Entebbe, Uganda [273]. Subsequent studies revealed *Aedes africanus* as the primary vector and a serosurvey demonstrated evidence of human infection [274]. Human infection in a field worker and an experimental infection of a volunteer demonstrated that ZIKV could produce a severe febrile illness associated with headache, myalgias, and prostatitis symptoms [275]. Since its original isolation, ZIKV has been isolated from mosquitoes and a cause of human infections throughout equatorial Africa, Indonesia, Malaysia, and Cambodia. *Aedes aegypti* was found to be the primary vector in Southeast Asia [276]. In 2007, a large outbreak of ZIKV infection occurred in Yap Island, Micronesia [277]. In total there were 49 confirmed and 59 probable cases of ZIKV illness. Patients developed rash, fever, arthralgia, and conjunctivitis. The authors estimated that 73 % of Yap residents 3 years of age or older had been recently infected with ZIKV. *Aedes hensilli* was the predominant mosquito species identified. Currently ZIKV is widely distributed outside of Africa with cases or serologic evidence of infection in India, Malaysia, the Philippines, Thailand, Vietnam, and Indonesia. ZIKV was demonstrated to have evolved into an African and Asian lineage [278]. As noted earlier, ZIKV was documented to have been transmitted sexually from a scientist who was infected in Senegal and, upon returning to the United States, infected his spouse [62].

Yellow Fever Virus Complex

Banzi Virus (BANV)

BANV was first isolated in 1956 in a 9-year-old boy in Ndumu, South Africa [279]. The clinical manifestations of this virus was a nonspecific febrile illness. There have only been two acute cases of definitively diagnosed BANV infections, but seroprevalence studies across sub-Saharan Africa suggest BANV infections may be largely subclinical and underdiagnosed [3].

Bouboui Virus (BOUV)

BOUV is a flavivirus that has been isolated in a baboon in Senegal and in mosquitoes in Central Africa and Senegal. While BOUV has never been isolated in humans, seroprevalence studies (employing hemagglutination inhibition assays) involving over 4,000 patients in Central Africa have found 15 patients with serum antibodies to this virus. The clinical manifestations of this disease in humans are not known [3].

Wesselsbron Virus (WESSV)

WESSV is primarily an arboviral disease of sheep, cattle, and goats [280]. However, it has caused nine cases of human febrile disease in sub-Saharan Africa, sometimes with neurologic complications to include disturbances in speech, hearing, and/or vision [281]. While WESSV is primarily located in Africa, it has been isolated in mosquitoes in Bang Phra, Thailand [3].

Yellow Fever Virus (YFV)

YFV is the prototype member of the *Flaviviridae* family of viruses. The origin of YFV is speculated to have been Western Africa, although the disease was first distinguished from other tropical febrile diseases by the Mayans and the Spanish colonists in the Yucatan peninsula in 1648. During the eighteenth and nineteenth centuries, it was one of the great plagues of the world occurring along the eastern US seacoast, in Central America and South America, and in Africa throughout the tropical area [164, 165, 167]. Major epidemics occurred in many seaports of the United States, and the 1905 outbreak in New Orleans was particularly severe. The last US indigenous case occurred in 1911 and the last imported cases in 1924 [13, 214]. It never became established in Europe above the range of the vector, *A. aegypti* mosquitoes. YFV has never been reported from Asia and Australia, despite the endemic presence of *A. aegypti* in tropical Asia [282]. The historical significance of YFV was discussed in a previous section of this chapter.

The development of the YFV vaccine, Theiler's attenuated live virus vaccine introduced in 1937, represents the first successful vaccine against an arbovirus and forms the basis for the present-day product [283]. YFV is maintained in the environment in two cycles: an urban cycle involving human beings and *A. aegypti* mosquitoes and a sylvatic or jungle YFV cycle involving forest primates, principally monkeys, and forest canopy mosquitoes, with human infections tangential to the transmission cycle [284, 285]. In 1901, eradication efforts directed toward *A. aegypti* mosquitoes were launched under the direction of Dr. William Gorgas in Havana. These eradication efforts, with concomitant reduction of YFV, were extended throughout Central and South America in the early 1900s. The chain of urban *A. aegypti-transmitted* YFV was successfully broken by the eradication program. The last endemic focus of *A. aegypti*-transmitted urban YFV was in northeastern Brazil in 1934 [284]. The eradication of the vector, and the concomitant reduction in urban YFV cases in the Americas, historically represents one of the most successful public health campaigns against infectious diseases.

Today, jungle YFV persists in the Western hemisphere and is transmitted chiefly among monkeys, marmosets, and possibly other forest-dwelling animals, commonly causing fatal infections. The vectors are mosquitoes of the forest canopy, chiefly of *Haemagogus* spp. and, to a lesser extent, *A. leucocelaenus*, *Sabethes chloropterus*, and possibly *A. fulvus* in Brazil [285]. For the last few decades in South America, the vast majority of YFV cases were in males over 15 years of age whose occupations increase their exposure to YFV-infected mosquitoes in endemic forest and jungle areas [286, 287]. Up to 500 unvaccinated forest workers were infected in peak years. The majority of cases occur in the first 3 months of the year in South America [287, 288]. *A. aegypti* has now reinfested most of South and Central America and occupies habitats just adjacent to the areas where endemic YFV transmission occurs. A major threat is that this species could transmit YFV in an urban cycle.

In contrast to the endemic–sylvatic circulation of YFV in the Western hemisphere in the late twentieth century, YFV in Africa periodically explodes out of its sylvatic cycle to infect large numbers during major epidemics. During the decade 1980–1990, YFV reemerged as a major health problem in Africa and, as mentioned above, threatens to reemerge in South America. In Africa, two control strategies have been attempted during the last 40 years. The first was routine immunization programs, and the second was emergency control programs after the start of an outbreak. A routine, mandatory YFV immunization program was begun in the early 1940s in French West Africa, and the recurring pattern of epidemics in West Africa was interrupted in those immunizing countries. This strategy was abandoned in 1960, and the program switched to a post-outbreak, emergency immunization and control strategy. Since then, there has been a series of epidemics of varying severity [285]. The period 1986–1990 represented an extraordinarily active period of YFV. The worldwide total of 17,728 cases and 4,710 deaths (i.e., a case fatality rate of 26.6 %) represents the greatest amount of YFV activity reported to the WHO for any 5-year period since reporting began in 1948 [286, 287]. However, in Africa, numerous studies have shown that only a small percentage of African YFV cases are reported [287]. Due to the sylvatic cycle of jungle YFV, worldwide eradication is not considered possible. Despite numerous studies, the question of maintenance of YFV in nature remains somewhat obscure. Although a continual vertebrate–vector maintenance cycle is possible in some environments, in other areas overwintering and maintenance probably occur via other mechanisms. The laboratory and field studies that confirmed that YFV virus can be transovarially transmitted in many of its mosquito vectors suggest that this mechanism might play an important role in nature.

Yellow fever patients have a characteristic but nondiagnostic febrile disease with fever, headache, backache, nausea, variable epistaxis, and a lack of correlation between pulse and body temperature [289]. The clinical course is 2–4 days, followed by uneventful recovery or a remission period before development of severe yellow fever. Many tropical diseases, including a variety of arboviral infections, malaria, and relapsing tick fever, may present similar clinical syndromes, making a clinical diagnosis of suspected YFV difficult unless seen during a recognized epidemic. In a serious complication of the disease, the development of icterus occurs following a remission of the general manifestations. It develops as a yellowish tinge of the sclera, a very important diagnostic sign more easily seen in dark-skinned people, and only rarely becomes marked. Hemorrhagic signs and vomiting of blood characterize this more serious form of disease. They are more common preceding death, which usually occurs within 9 days of onset. Mortality rates vary widely and during epidemics reach from 20 to 80 % of the cases. Icteric YFV must be differentiated from infectious and serum hepatitis, leptospirosis, and poisoning.

Because of these uncertain clinical criteria, laboratory diagnostic tests must be used. Isolation and identification of YFV virus in blood samples or necropsy specimens or demonstration of specific antibody titer rises constitutes definitive diagnosis. While the virus generally grows well in standard cell cultures or suckling mice, identification of YFV virus is now usually performed by PCR. Other rapid diagnostic assays have shown promise for demonstration of YFV-specific antigen or IgG and IgM antibodies. Cross-reactivity with other flaviviruses has been a historical serologic problem. Demonstration of pathognomonic hepatic lesions in necropsy specimens is used when applicable, but needle biopsies of the liver have proved hazardous and the procedure is not generally recommended. No specific

therapeutic regimen is available for YFV, and treatment is chiefly supportive. Prevention is based both on protection from exposure and on vaccination. The 17D YFV vaccine was one of the earliest viral vaccines to be developed, and it has proved to be one of the safest and most efficacious live attenuated vaccines [214, 290].

Yellow fever in urban or jungle form is a continuing threat. The two forms are nonetheless the same virus and the same disease, distinguished on epidemiologic grounds. Human beings can be protected by immunization with 17D YFV vaccine (not advised for infants under 1 year of age). A list of the centers in the United States authorized to give the vaccination can be obtained from the Public Health Service (http://wwwnc.cdc.gov/travel/yellow-fever-vaccination-clinics/search).

Other Viruses in the Yellow Fever Virus Complex Not Pathogenic to Humans

These viruses include Edge Hill virus (EHV) [3, 267], Jugra virus (JUGV) [3], Saboya virus (SABV) [3], Sepik virus (SEPV) [291], and Uganda S virus (UGSV) [3]. These known viruses have been shown to cause illness in humans but by potential exposure as indicated by positive antibody titers.

10.1.4 Viruses with No Known Arthropod Vector

A number of flaviviruses have no known arthropod vector and no documented naturally occurring infection in humans and includes Entebbe bat virus (ENTV) [3], Yokose virus (YOKV) [3], Apoi virus (APOIV) [3], Cowbone Ridge virus (CRV) [292].

Jutiapa virus (JUTV) [3], Modoc virus (MODV) [293], Sal Vieja virus (SVV) [3], San Perlita virus (SPV) [3], and Carey Island virus (CIV) [3].

11 Unresolved Problems

The emergence of WNV in New York and its spread throughout the Americas and the emergence of DENV as a global health problem and recent outbreak in Florida have demonstrated the vulnerability of the United States to emerging viruses. Two studies by the Institute of Medicine dealing with emerging diseases warned that the threat posed by disease-causing microbes may be expected to continue and intensify in coming years [294, 295].

The natural life cycle of many arboviruses is multifaceted and, in addition to the virus, may include one or several reservoir or amplifying hosts and often an arthropod vector. A change affecting the interaction of these fundamental elements might lead to the emergence or reemergence of a viral disease. In some instances, viruses might emerge as the result of selection of new genetic strains and variants with increased infectiousness, virulence, or transmissibility. To control emerging viruses in general, and arthropod-borne viruses in particular, there is a need for expanded (1) basic and applied research that will help formulate coordinated strategies for anticipating, detecting, controlling, and preventing emergence or reemergence of viral diseases and (2) basic and applied research on the virus, the infective process, and the host response to infection, which will be useful in the development of vaccines and antiviral drugs.

Additional unresolved problems are discussed from several points of view, relating to the viruses, the vectors, the vertebrate hosts, and transmission cycles involving virus, vector, and host. The disease in the vertebrate host, which includes the host response to the pathogen, merits independent consideration. Problems relating to the epidemiology of each specific disease require a synthesis of many specific items. Finally, effective control exercised at the level of the virus, the vector, or the vertebrate requires thorough understanding of the epidemiologic background. Specific examples will help to illustrate problems.

11.1 The Viruses

Much progress has been made in recent years in cataloging the several hundred described arboviruses and determining the biochemical, growth, and morphological characteristics in intact vertebrates, in invertebrates, and in cell culture systems of vertebrate and invertebrate cells. However, further work is needed to understand the evolution and emergence of epidemiologically relevant strains. In particular, new research is needed on the nucleic acid homology that may exist among the numerous members of a given arbovirus grouping and on the mechanisms of recombination, reassortment, and selection of new virus strains with important virulence properties.

11.2 The Vectors

The factors that determine specific virus–vector associations are still being elucidated. The role of the vector in genetic conservation of the arboviruses, as genetic bottlenecks, and virus expansion into other vectors due to mutational events, such as in the case of chikungunya virus, is not well understood. Expanded research is needed before the principles governing virus–vector interactions are carefully delineated.

There is a continuing need for taxonomic refinements with respect to arthropods, such as the need for more information on both Old World and New World mosquitoes of the genera *Culex* and *Aedes*. This need is generated by the increasing realization of their involvement with a large number of arboviruses. The same remarks are pertinent for the

tick vectors. The need is equally great for more information on the biology, feeding preferences, longevity, flight range, and distribution of each arthropod species involved. The genetic constitution of each vector species is basic to an understanding of what constitutes a vector, both physiologically and behaviorally, and will become increasingly important as control of vectors through genetic manipulation is considered [118].

Unfortunately, in 1985, the Asian "tiger" mosquito, *A. albopictus*, was introduced in old tires imported from Japan to Houston, Texas. It has now spread to infest over 22 states, mostly in the South and Midwest, and has recently become established in several other countries. This mosquito is an aggressive, opportunistic feeder with a wide host range that includes man. It adapts well to forest or urban settings and it can vector many different arboviruses. In some areas, it already has replaced local mosquito species, and there is a concern it will transmit endemic viruses. Because of its potential as a new vector of endemic or emerging arboviruses in the United States and other countries, research on it should receive high priority.

11.3 The Vertebrate Hosts

For most of the arboviruses, the primary vertebrate host, i.e., the host that serves as the basic unit for propagation of the virus, is not man. For many of the arboviruses, the vertebrate hosts are not yet determined or are recognized on the most tenuous of evidence. Identification of the host(s) is a primary need. Following this, an emphasis should be placed on elucidating a biological profile of the hosts, including the full range of biological and ecological considerations, as well as the degree of host susceptibility to the virus.

11.4 Transmission Cycles Involving Virus, Vector, and Vertebrate

The problem of virus persistence in nature is a particularly baffling one. For example, there are many theories but few facts to explain how a given virus manages to overwinter or survive past a long dry season, when vectors may practically disappear and vertebrate populations decline (or go into hibernation).

Current theories hypothesize that the virus persists in vector populations that overwinter with some members harboring virus or the virus is in vertebrate populations that overwinter and some infected individuals respond to a reactivation stimulus. In other cases, existing data point to an important role for transovarial transmission, permitting passage of virus to generation after generation of a vector without the need for an intercalated vertebrate host. Several of the tick-transmitted viruses apparently utilize mechanisms of long persistence in ticks plus transovarial transmission of virus to exist in an endemic form in defined geographic areas. An excellent review of this topic is presented by Reeves [139] who discusses the epidemiologic problems of overwintering of arboviruses in northern countries and possible transport via infected vectors on migrating birds, with his discussion extending to the Old World as well as the New World viruses. A related paper by Lord and Calisher [112] discusses the transport of arboviruses in infected migrating birds along the Atlantic coast flyway of the United States. Further research in these areas is vitally needed to determine any point in the natural maintenance of the virus where intervention might lead to control or eradication of the endemic disease.

Studies of transmission cycles are tied closely with simulation of cycles by models with carefully defined parameters. Such models may permit computer manipulation and simulation of field conditions by varying the values applied to defined parameters, following which epidemic curves can be generated. Further work on models is needed, with the hope of eventual prediction of emergence and spread of disease.

11.5 Disease in the Vertebrate Host

Studies of the human response to arboviral infections are difficult, since the epidemics that provide numbers of cases for study usually occur unpredictably in time and often far from modern facilities required for detailed clinical investigation. Classically, studies of infection in the vertebrate host (including man) have been part of research programs in the fields of pathology. However, animal models are available for only a limited number of the arboviruses. The development of these models will be crucial for the development modern vaccines and antiviral drugs.

Recognition of disease in the vertebrate host, as well as worldwide surveillance of disease, requires further development of simple diagnostic techniques and standardization of these assays using accepted reference reagents.

11.6 Control

Virus vaccines are a highly cost-effective means of disease control; yet for the arboviruses, only an attenuated yellow fever (17D) vaccine is in use on an international scale. The recent WHO recommendation that this vaccine be included in the Expanded Program of Immunization (EPI) in YFV-endemic countries was a major step toward the control of YFV in Africa [200, 201]. The threat of emergence of YFV in South America has prompted some to call for

consideration of inclusion of YFV vaccine in selected EPI programs in South American countries.

In the case of JEV, an inactivated vaccine has proved highly successful and is in wide-scale use in parts of Asia. An inactivated vaccine for tick-borne encephalitis also has been successfully used for decades in central Europe. However, inactivated vaccines are often costly and need recurring reimmunizations to booster immunity. Advanced testing of the new attenuated JEV vaccine, now in partial use in China, should receive high priority. Research, safety testing, and standardized production studies needed to include such a vaccine in international immunization programs should be strongly supported.

Control at the vector level involves continuing work on methodology for control of arthropods. Development of resistance to various insecticides has impaired many control programs, and recent regulatory actions limiting the use of insecticides have further intensified the need for exploration of alternative methods for vector control.

Limited studies utilizing biological mosquito control (such as *Bacillus thuringiensis*) to control selected arbovirus vectors have been encouraging and may lead to widespread control strategies. Other approaches that need further research include those that introduce genes into a mosquito population relating to (1) increased insecticide susceptibility, (2) reduced capacity to support virus multiplication and/or to transmit virus, (3) alternatives in host feeding preferences, (4) reduction in numbers through mutations leading to reduction in reproductive success (sterile males, conditional lethal mutants), and (5) subversions of host feeding habits.

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Hepatitis A Virus

17

Daniel Shouval

Abbreviation

ACIP	Advisory Committee on Immunization Practices
ALF	Acute liver failure
ALT	Alanine aminotransferase
Anti-HAV	Antibodies to hepatitis A virus
BMI	Body mass index
CDC	Centers for Disease Control (USA)
cDNA	Complementary DNA
EIA	Enzyme-linked immunoassay
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded Program on Immunization
GBD	Global burden of disease
GID	Global incidence of disease
GMC	Geometric mean concentration
HAV	Hepatitis A virus
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Histocompatibility leukocyte antigen
IDU	Intravenous drug use
IEM	Immune electron microscopy
IG	Immunoglobulin
IV	Intravenous
LAK	Lymphokine-activated killer cells
MSM	Men who have sex with men
NHANES	National Health and Nutrition Examination Survey
NK	Cells natural killer cells
PBMC	Peripheral blood mononuclear cells
RIFIT	Radioimmunofocus inhibition assay
RNA	Ribonucleic acid
WHO	World Health Organization

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1 Historical Background

Jaundice or icterus and other diseases of the liver were described in the writings of a number of ancient societies including old Chinese, Greek, Roman, Babylonian, and Talmud literature. Jaundice was generally considered obstructive in origin, although large epidemics associated with military campaigns were described as early as the seventeenth century and continued through World War II [1–6]. Over the years and until discovery of the hepatitis A (HAV) and hepatitis B (HBV) viruses, many synonyms were used to describe viral hepatitis of undetermined origin, later referred to as HAV. These included among others the terms acute catarrhal jaundice, epidemic catarrhal jaundice, epidemic hepatitis, icterus epidemicus, infectious hepatitis, campaign jaundice, and MS-1 hepatitis. From the 1940s through the 1960s, extensive efforts to identify the agent(s) associated with viral hepatitis were unsuccessful [2, 6]. Epidemiologic evidence supported two types of hepatitis viruses: infectious hepatitis (later identified as HAV) and serum hepatitis (later identified as HBV). However, a clear distinction could not be made between the two agents until transmission studies were conducted in primates [1, 7–11]. Studies, conducted between 1940 and 1965, clarified clinical and epidemiologic distinctions and lack of cross-immunity between the two forms of viral hepatitis MS-1 and MS-2, later classified as HAV and HBV, respectively. Clinical studies in the late 1940s and onward established the efficacy of immune serum globulin (IG) in preventing infectious hepatitis A [1, 9, 10, 12].

The discovery of HBV in the early 1970s and development of serological assays further differentiated these two forms of viral hepatitis. However, the critical advance occurred in 1973 when HAV particles were identified through immune electron microscopy (IEM) in stool samples of patients with hepatitis

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A [13]. The development of serological assays to distinguish between acute and resolved HAV infection during the next decade was pivotal in understanding the natural history, the epidemiology, and the pathogenesis of HAV infection [4]. The discovery of the hepatitis E virus (HEV) and the development of diagnostic assay for HEV enabled further distinction between HAV and HEV infections which share similar epidemiology and clinical characteristics [14]. Finally, the identification and characterization of the HAV genome has added power-full tool for the investigation of the phylogenesis and molecular epidemiology of HAV [15–18]. HAV was first grown in cell culture in 1979 [19], and vaccines developed from cell culture-derived attenuated virus have been shown to effectively prevent hepatitis A [20, 21].

2 Methodology Involved in Epidemiologic Analysis

2.1 Mortality

HAV infection has a low case fatality rate, and mortality from hepatitis A is a poor indicator of disease incidence. Although viral hepatitis has been included in the International Classification of Diseases (ICD) for decades, neither serological testing nor appropriate coding to distinguish different viral etiologies was available until 1980. The ICD-coded data on hepatitis A mortality has become more reliable in developed countries where serological testing is widely available. Because accurate serological testing is not widely available in developing countries, mortality statistics are unreliable and remain poor indicators of disease incidence.

2.2 Morbidity

Several factors have limited the usefulness of morbidity data in assessing the impact of HAV infection. In developed countries, the reporting of hepatitis A and hepatitis B as different diseases only started in the late 1970s, and it was not until 1980 that a diagnostic test for hepatitis A became available commercially. Furthermore, although viral hepatitis is a reportable disease in almost all countries, underreporting is assumed to reach >80 % even in developed countries [5, 22]. In less-developed countries, serological testing is not widely available, and each type of viral hepatitis is not reported separately. Consequently, it is difficult to make strict comparisons of reported rates of hepatitis A from different geographic areas.

In spite of these well-recognized limitations, cyclical patterns of increased disease incidence and trends of disease over time are reasonable indicators of the epidemiology of hepatitis A. Data derived from studies using serological testing to define the age-specific etiology of acute cases of viral hepatitis in specific locations can be combined with reported age-specific prevalence data to estimate the overall morbidity resulting from hepatitis A [23, 24].

2.3 Epidemiologic Studies

Epidemiologic studies that used serological assays to differentiate acute from resolved HAV infections have greatly increased the understanding of this disease. Recently, nucleic acid variation within the region of the HAV genome that codes for the capsid (surface) proteins has been used as a marker in epidemiologic studies [5, 17, 25].

The spread of HAV infection can be assessed through monitoring the overall and age-specific anti-HAV (IgG) seroprevalence in selected populations which also enables indirect measurement of incidence rates. Overall prevalence has been classified into high (≥90 % of population by age 10 years), intermediate (≥50 % by age 15 years with <90 % by age 10 years), low (≥50 % by age 30 years with <50 % by age 15), and very low (<50 % by age 30 years) [22].

A new classification is emerging regarding endemicity of HAV worldwide based on reported incidence of serologically confirmed acute HAV cases through detection of anti-HAV (IgM) antibodies [5, 22]. Accordingly, endemicity to HAV may also be classified as very low, with an estimated incidence of <5 cases/10^5; low, 5–15 cases/10^5; intermediate, 15–150 cases/10^5; and high, >150 cases/10^5. Cross-sectional studies of the prevalence of HAV infection in population samples stratified by age, and in some cases by socioeconomic status, have been completed in many parts of the world and provide the most accurate picture of HAV infection [23, 24, 26–28]. Serological testing of representative samples of acute hepatitis cases in children and in adults has been used to define the proportion caused by HAV. In less-developed countries, these data can be combined with reported age-specific rates of acute hepatitis to estimate disease incidence. Studies to define the risk of HAV infection in certain groups or populations have primarily been confined to persons traveling from countries with low HAV endemicity to regions with high rates of infection. Some data are available to define the current risk of infection in men who have sex with men (MSM), drug users, the developmentally disabled, children attending day care, persons working in day-care settings, or health-care workers [5]. Estimates on the national burden of HAV-associated disease should include data on patients with fulminant hepatitis and those with HAV-induced liver failure referred for liver transplantation.

Mechanisms or pathways of disease transmission have been defined by serological testing of persons ill or exposed in outbreaks and in some instances by analysis of genetic variation of HAV isolates [5, 17, 20]. Serological testing, or HAV-RNA detection and sequencing, has improved our

knowledge of the epidemiology of common-source outbreaks (i.e., through contaminated strawberries, shellfish, or lettuce), community-wide outbreaks, outbreaks associated with day-care centers and institutions for the developmentally disabled, and outbreaks occurring in hospitals.

2.4 Laboratory Methods

2.4.1 Isolation and Identification of the Virus

Primary virus isolation from clinical or environmental specimens (i.e., feces, water, shellfish, or strawberries) has become possible using various primary or continuous cell lines. However, because of the slow growth characteristics of HAV (up to 120 days), prolonged adaptation periods are required before either infectious foci or HAV antigen can be detected [19, 29]. HAV generally does not produce a cytopathic effect in cell culture, and detection of virus requires special time-consuming techniques such as the radioimmunofocus assay (RIFA), which uses radiolabeled antibody to detect infectious foci of HAV in fixed cells [29]. The inability to rapidly culture HAV has precluded the use of this method for the diagnosis of infection or for detection of HAV in environmental samples.

Immune electron microscopy (IEM) was used extensively in early studies to identify HAV [13] but has been supplanted by more sensitive techniques. These include immunoassays to detect HAV antigen [30, 31] and detection of HAV-RNA by polymerase chain reaction (PCR), the latter being the method of choice for detection of low levels of HAV in clinical and environmental samples [17, 25, 32–34]. However, the presence of HAV-RNA may not always equate with the presence of infectious virus [35].

2.4.2 Serological and Immunologic Diagnostic Methods

In the 1980s, immunoassays to detect antibodies to HAV structural antigens (anti-HAV) became available commercially, including those that detected both IgG and IgM antibodies (i.e., total anti-HAV often referred to as anti-HAV (IgG)) and anti-HAV (IgM), which allow the differentiation of past and current (within prior 6 months) infections [33, 36]. To better evaluate the effectiveness of hepatitis A immunization, quantitative assays for anti-HAV antibodies (IgG and IgM) with increased sensitivity have been developed. Cell culture assays that detect low levels of neutralizing antibodies have been used in the early phases of HAV vaccine development [31, 37]. An experimental assay for detection of an immune response to nonstructural HAV proteins has raised hopes to establish a method for differentiation between anti-HAV (IgG) response to acute HAV infection and response to immunization [38]. However, in contrast to HBV, no licensed immunoassays are yet available for this purpose.

3 Biological Characteristics of the Virus

HAV is a small (27–32 nm) non-enveloped RNA virus belonging to the family Picornaviridae. The virus has icosahedral symmetry, has a buoyant density of 1.33 g/cc, and contains a single-stranded positive-sense RNA of approximately 7,500 (Figs. 17.1 and 17.2). The virion is composed of at least three major structural viral capsid polypeptides: VP1, VP2, and VP3 (33,000–22,000 Da). Based on the nucleotide sequence, VP4, another viral capsid protein of approximately 2,500 Da, should be encoded but has not been identified in mature virions [20]. The genomic organization and replication of HAV appear similar to that of polio and other picornaviruses; viral RNA encodes a single large polyprotein from which structural and nonstructural proteins are subsequently cleaved [30, 40, 41].

When compared to other enteroviruses, HAV has essentially no nucleotide or amino acid homology, replicates more slowly in cell culture, and is more resistant to heat inactivation. Although HAV was initially classified in the genus *Enterovirus* (*Enterovirus* 72), it has been reclassified in a separate genus designated *Hepatovirus* [42].

HAV is stable in the environment, retaining infectivity in feces for at least 2 weeks and having only a 100-fold decline in infectivity over 4 weeks at room temperature [33, 34, 41, 43, 44]. The virus resists extraction by nonionic detergents, chloroform, or ether and retains infectivity in pH 1.0 at 38 °C for 90 min. HAV is more resistant than poliovirus to heat, being only partially inactivated at 60 °C for 1 h [34]. When suspended in milk at 62.8 °C for 30 min, 0.1 % infectivity remains, suggesting that pasteurization may not completely inactivate HAV and temperatures of 85–95 °C for 1 min are required to completely inactivate HAV in shellfish [34, 45, 46]. HAV is completely inactivated by formalin (0.02 % at 37 °C for 72 h) but appears to be relatively resistant to free chlorine, especially when the virus is associated with organic matter [34]. An outbreak of hepatitis A among swimmers suggested that if a prescribed free chlorine level of 0.3–0.5 ppm existed in the swimming pool, it failed to inactivate HAV in fecally contaminated water [47]. Only sodium hypochlorite, 2 % glutaraldehyde, and quaternary ammonia compound (QAC) with 23 % HC1 have been effective in reducing the titer of HAV by more than 10^4 on contaminated surfaces [48].

HAV exists as a single serotype, and monoclonal antibodies that identify overlapping neutralization epitopes react with virus isolates from all parts of the world [41]. The single immunodominant neutralization epitope appears to be highly conformational and is composed of several sites located on VP1 and VP3 [34, 41, 49, 50]. There are seven known HAV genotypes, defined by sequence of the VP1/P2a junction region of a global collection of viruses [17, 26]. Genotypes are defined by a sequence variability of ~15 % in these regions, while subgenotypes differ by 7.0–7.5 %. Four

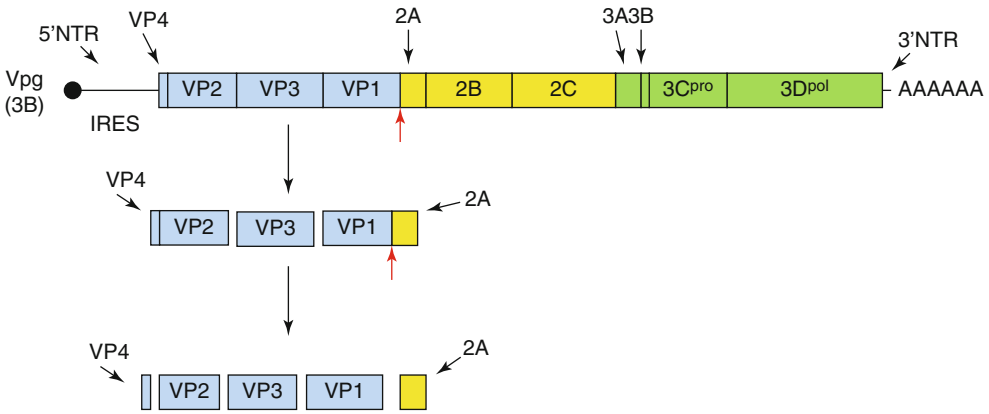

Fig. 17.1 HAV genome organization. On top a scheme of the HAV genome is shown. The positive-strand (messenger-sense) RNA genome contains a single open reading frame encoding a polyprotein that is proteolytically processed by the viral protease $3C^{pro}$. A yet-to-be-identified cellular protease cleavage in VP1-2A is indicated by a *red arrow*. Structural proteins (*P1*) are indicated in blue; nonstructural proteins (*P2* and *P3*) are indicated in *yellow* and *green*, respectively (Reproduced by permission reference Cristina and Costa-Mattioli [39])

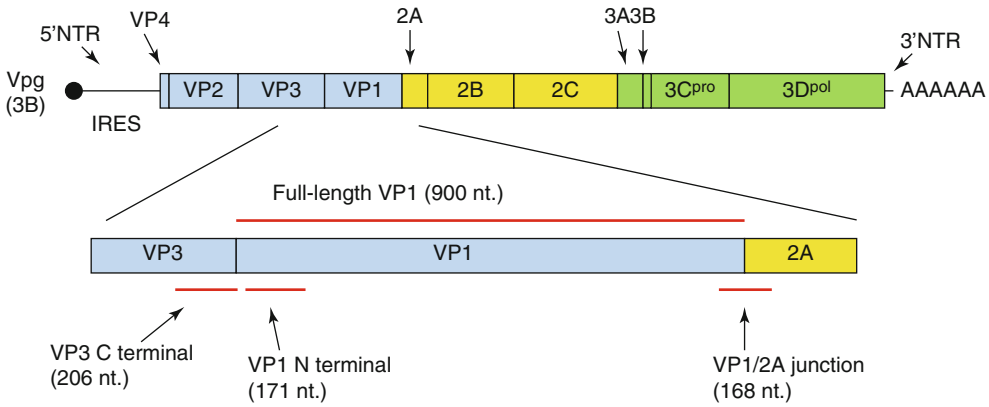

Fig. 17.2 HAV genome regions used for genetic analysis. The *thick red lines* below the genome scheme indicate the different genomic regions used to characterize the different HAV isolates (Reproduced by permission reference Cristina and Costa-Mattioli [39])

genotypes, namely, I, II, III, and VII, were identified in infected humans, while genotypes IV, V, and VI have been found in infected nonhuman primates. Recently, the seven HAV genotypes have been re-classified into six genotypes while genotype VII is now defined as a subgenotype of genotype II [18]. The identification of the various HAV genotypes and subgenotypes has significantly enhanced the ability to investigate the molecular epidemiology of hepatitis A outbreaks and particularly its transmission routes [17, 25].

Besides man, the host range of HAV includes nonhuman primates (marmosets, tamarins, owl monkeys, chimpanzees) that have been infected experimentally and have served as animal models of human HAV infection [51–53]. In addition, several species of Old World monkeys have been shown to be infected with an HAV that is serologically similar to the human virus but genetically distinct and that does not appear to infect humans [7, 34, 54].

In 1979, cell culture of HAV in fetal rhesus monkey kidney cells was achieved only after the virus was passaged multiple times in marmosets [19, 34]. Since then, HAV has been cultivated directly from clinical or environmental samples, but long adaptation periods (4–10 weeks) have been required for detection of significant amounts of HAV antigen in infected cells. The lack of a cytopathic effect allowed serial passage of virus that ultimately produced strains that replicate more rapidly and produce higher virus yields [34]. HAV that has been adapted to grow efficiently in cell culture appears to have mutations in the 2B and 2C areas of the genome that codes for nonstructural proteins [34]. In addition, cytopathic variants of HAV have been isolated, and similar to polio, these variants produce plaques in cell culture and have proven useful in laboratory studies [34, 55].

4 Descriptive Epidemiology

4.1 The Changing Epidemiology of HAV and the Impact on Global Burden of Infection

Prior to identification of its specific viral etiology and the development of diagnostic tests, hepatitis A was presumed to be the cause of most sporadic and epidemic hepatitis worldwide. Large epidemics of hepatitis A occur in developing countries and

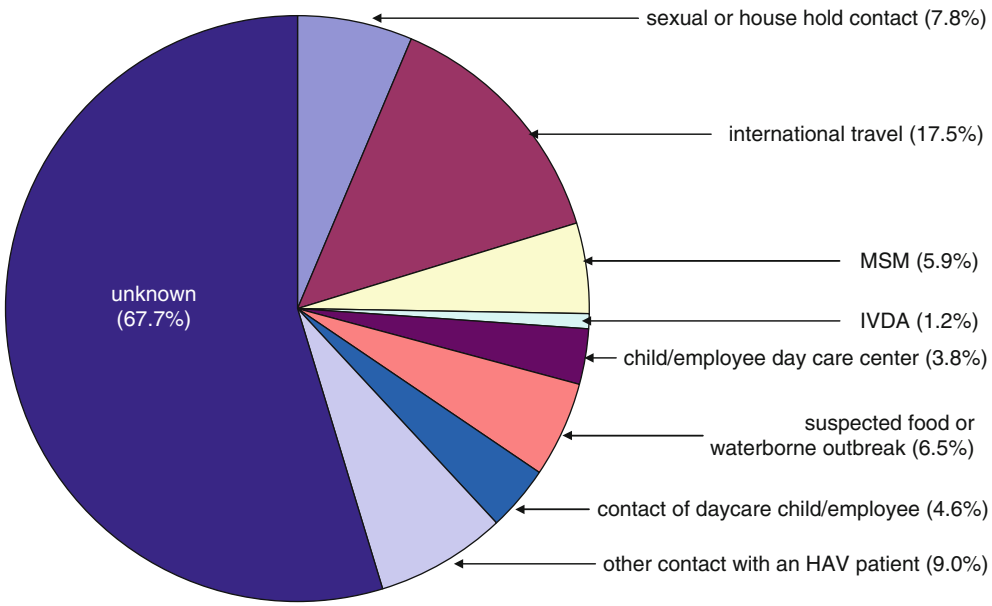

Fig. 17.3 Epidemiologic risk groups for acute hepatitis A virus infection obtained from reported cases in the United States in 2007. *MSM* men who have had sex with men, *IVDA* intravenous drug abuse (Modified from reference Daniels et al. [61])

especially in countries in transition from high to intermediate endemicity of HAV. However, it appears that hepatitis E is also an important cause of epidemics of hepatitis that involved large numbers of people, especially in India and central Asia [3, 56].

Data on the global incidence of disease (GID) and global burden of disease (GBD) of acute HAV infection is incomplete, with an assumed underreporting rate of ≥80 % [28, 57–61]. The WHO has recently completed a preliminary survey for reassessment of the GBD of HAV disease. Estimates suggest an increase in the number of symptomatic acute hepatitis A as well as subclinical cases globally from 177 million in 1990 to 212 million in 2005 and deaths due to hepatitis A to increase from 30,283 in 1990 to 35,245 in 2005 [22, 23]. Increased numbers of cases were estimated to occur in the age groups 2–14 years and >30 years. An epidemiologic shift from high to intermediate endemicity of HAV is now being recognized worldwide. As a result, more adults in such areas of transition escape exposure to HAV in early childhood and become susceptible to infection during outbreaks. This change in susceptibility to HAV infection is paradoxically associated with an increase in disease incidence rates in the presence of improved socioeconomic and sanitary conditions. An example for the potential consequences of such a shift in endemicity was clearly demonstrated during the massive 1988 outbreak of hepatitis A in Shanghai where >300,000 individuals contracted HAV infection within a short period [62, 63].

HAV infection is mainly spread through the fecal–oral route as well as through contaminated water and food. Shellfish are able to ingest and concentrate HAV and as a result become a reservoir for spread of the virus [63, 64]. Transmission occurs mainly through common-source outbreaks (i.e., food- and waterborne) as well as person-to-person contact. HAV is very rarely transmitted through blood products or medical procedures. Epidemiologic risk groups include populations of low socioeconomic status living under crowded conditions, household contacts of infected individuals, children visiting day-care centers and kindergartens, international travelers from countries with low endemicity to areas with intermediate or high endemicity, MSM, intravenous drug users, patients with chronic liver disease, food handlers, caretakers of nonhuman primates, and patients with blood clotting disorders. The clinical expression of HAV infection is highly age dependent. Serological studies have confirmed that less than 10 % of infected children under age 6 will become jaundiced, whereas in adults and children above age 5, infection may cause jaundice in 50–90 % of cases [65–68].

The increased incidence of HAV infection in adults has had an impact on the magnitude and severity of the disease as recently reported from Korea [68, 69] and Brazil [70]. Furthermore, in countries in transition, pockets of intermediate endemicity may exist within the same areas of high endemicity. Despite the observed low attack rates of clinical hepatitis, especially in areas of high but also in countries with intermediate endemicity in transition, HAV infection has been identified as a leading cause of fulminant hepatic failure in a growing number of countries including Korea [71], Argentina [70, 72, 73], and Brazil [70].

The major risk groups for contracting HAV infection reported by the US Centers for Disease Control and Prevention (CDC) for the year 2007 are shown in Fig. 17.3 [61].

4.2 Prevalence and Incidence

The age-specific prevalence of anti-HAV (IgG) antibodies worldwide can be used to define several patterns and risks of infection [23] (Fig. 17.4).

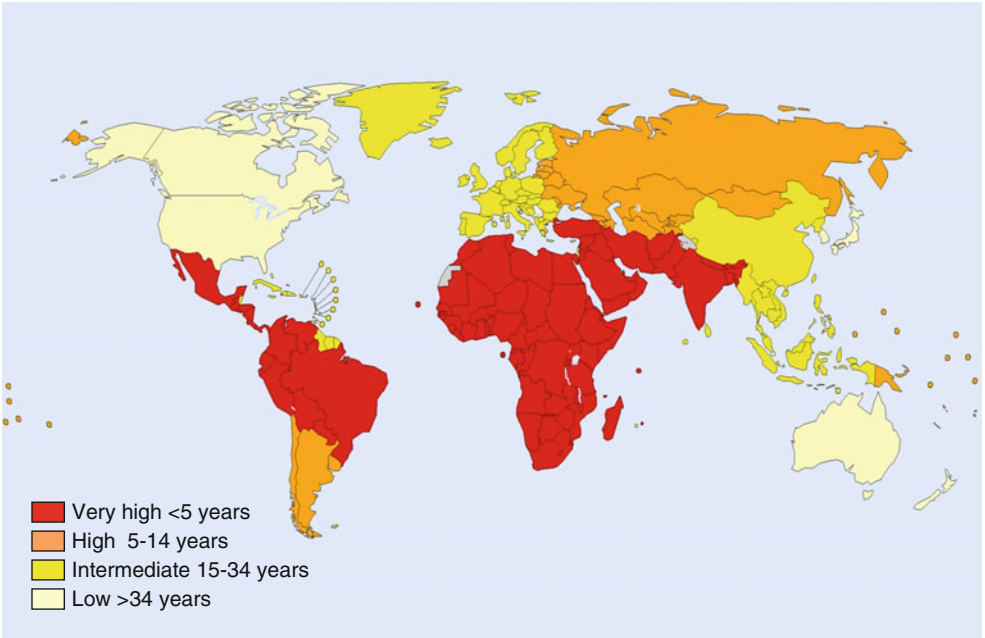

Fig. 17.4 Global risk map of HAV immunity in 2005. Age at midpoint of population immunity to HAV (Reproduced from BioMed Central, reference [74])

Many data on the global prevalence of HAV are outdated and recorded between the late 1970s and early 1990s prior to major shifts in endemicity. Areas with a very high endemicity of infection primarily consist of less-developed and developing nations of Asia, Africa, South and Central America, the Pacific Islands and certain populations in southern regions of Eastern Europe [26]. In these countries and until recently within ethnically defined populations or regions also in the United States, the prevalence of past HAV infection in adults has reached 90 % or higher; almost all older children had serological evidence of prior infection, and most children became infected by the age of 10 years [24, 75, 76]. However, persons of upper socioeconomic status residing in these regions have not become infected until they were adolescents or young adults.

In more developed counties in Western Europe (excluding northern regions) and some countries in Asia, the endemicity of HAV infection was already intermediate to low in the prevaccine era, and the prevalence of anti-HAV (IgG) varied widely [24, 27]. During the 1980s in countries such as Greece, Italy, and Taiwan, prevalence of past HAV infection in adults reached 80–90 %; while in children under age 10, the prevalence was only 20–30 %. The major increase in prevalence of infection occurred between 10 and 19 years [77–79]. In several Western European countries and the United States and prior to introduction of mass vaccination, anti-HAV (IgG) prevalence in young adults varied from 30 to 70 % but was less than 10 % in children under age 10, while low socioeconomic status was associated with high rates (endemicity) of past infection [80].

In some northern European countries, endemicity of HAV infection is very low. In several regions in the United States and in Japan, HAV infection is disappearing, and the endemicity of infection is considered low or very low. Until recently, in some US states, 30–60 % of US adults >40 years old had serological evidence for past HAV infection. In contrast, the prevalence of infection is less than 10 % in young adults and almost nil in children. In both very-low- and low-endemicity areas, there is evidence that a cohort effect accounts for the high prevalence of infection among older adults, indicating that HAV infection was much more common in the past, especially among persons who were born prior to World War II [24]. The epidemiology of HAV infection is rapidly changing in countries and regions which introduced mass or universal vaccination against HAV in babies and young children [5, 23, 81, 82]. Consequently, in such regions and countries, assessment of prevalence and incidence of HAV infection should be divided into pre- and postvaccination periods. For example, in the United States, endemicity of HAV is shifting from intermediate to low and very low prevalence of infection in populations previously considered at risk [83] as also shown in Israel [84] and some regions in Spain, Italy, and Australia [5]. The impact of rising socioeconomic conditions, improved sanitary conditions, and vaccination of children against hepatitis A is clearly shown in surveillance of incidence which has markedly dropped in age groups 5–39 years according to data obtained by the US Centers for Disease Control (CDC) [61, 83] as shown in Fig. 17.5.

4.3 Epidemic Behavior and Contagiousness

Rates of hepatitis A are not inherently stable, and periodic widespread epidemics may occur in high, intermediate, and

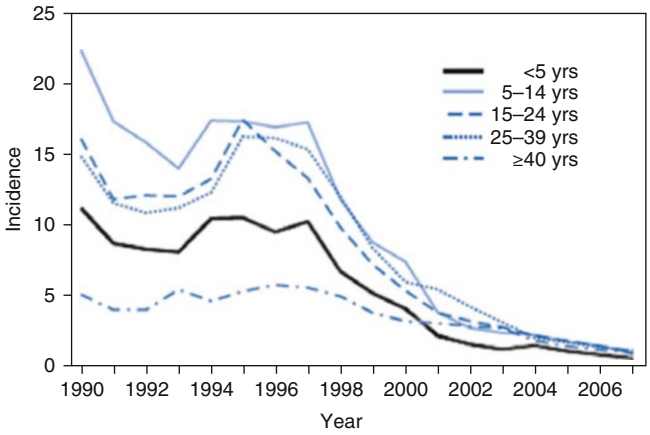

Fig. 17.5 US CDC report on decline of hepatitis A incidence between 1990 and 2007 [85]

even low endemicity areas and produce cyclical patterns of infection. However, over the past 20–40 years, nationwide epidemics of hepatitis A have gradually declined in most countries with a low endemicity of infection, while epidemic cycles continue to occur in countries with either a high or intermediate endemicity in transition. Determinants of the cyclical recurrence of epidemics include the age-specifc proportion of the population susceptible to infection, the interepidemic rate of HAV infection and the likelihood that HAV will be introduced into the susceptible population. Several patterns appear to produce these epidemic cycles: (1) high rates of asymptomatic infection in children under age 5 who serve as a reservoir for spread of infection to susceptible older children, a pattern observed in American Indian populations [86]; (2) periodic introductions of HAV into largely susceptible isolated populations where epidemics occur due to living conditions that facilitate virus transmission and where the high attack rate results in the disappearance of hepatitis A during interepidemic periods, a pattern observed in Alaskan Native villages and some Pacific Island populations [87, 88]; and (3) the periodic introduction of HAV through vehicles such as food in populations where the standard of living has improved and a large proportion of the young adult population remains susceptible, a pattern observed in Shanghai, China, during the 1988 hepatitis A epidemic [63]. Smaller outbreaks continue to play a role in disease transmission in the United States and elsewhere [89]. Common-source outbreaks related to contaminated water, shellfish harvested from sewage-contaminated areas, or food contaminated by an infected food handler have been recognized for many years [34, 90–95]. Outbreaks among MEM and drug users have been recognized in recent years, with infections in the latter probably occurring from person-to-person spread or occasionally from common-source contamination of illicit drugs, although HAV has rarely been transmitted parenterally [96–99]. In addition, outbreaks may occur among adult contacts (parents, caretakers) of diapered children in day-care settings [100, 101].

4.4 Geographic Distribution

As described previously, HAV infection occurs worldwide with endemicity patterns defined by the socioeconomic development of the individual nation and impact of vaccination campaigns. Published world maps on the prevalence of HAV infection have been useful to convey public health messages as shown in Fig. 17.4. Yet most data on the global prevalence of HAV infection are decades old. Consequently, as the socioeconomic status of the population increases, endemicity declines and the median age of infection increases, such maps require frequent updating. Recently, it was suggested that world maps should also use the age at midpoint of population susceptibility as a standard indicator for the level of HAV endemicity within the world regions [74]. In the least-developed countries, HAV transmission occurs exclusively during early childhood, almost the entire population becomes infected before age 10 years, and clinical disease is recognized only in older children. As hygiene/sanitation standards improve, the age of infection shifts progressively to include older children and young adults, particularly in upper socioeconomic groups. This may result in higher rates of clinical disease, and common-source outbreaks and/or nationwide epidemics appear if a substantial proportion of adults remain susceptible to infection. As sanitation and socioeconomic conditions improve further, the infection frequency in all age groups and overall rates of disease decrease; clinical disease is recognized in older children and young adults, and localized outbreaks may occur. Finally, in some developed countries, infection rates decline in all age groups, and cases of disease occur primarily as importations from areas having a high endemicity of infection.

4.5 Temporal Distribution

Long-term follow-up data from Europe and the United States have shown a marked change in patterns of reported incidence of disease in the past 50 years. Prior to and immediately following World War II, hepatitis rates were high, and nationwide epidemics, presumably of hepatitis A, occurred at 6- to 10-year intervals. In addition, seasonal fluctuations of the disease occurred, with peaks in the late summer and early fall. Following World War II, disease rates began to decrease in northern Europe and epidemic cycles disappeared, as did seasonal variation in disease rates. In the United States, improvement in overall socioeconomic conditions, sanitation, and hygienic standards and introduction of universal vaccination in states at risk have led to a reduction

of epidemic cycles which gradually are waning. The reported incidence of acute hepatitis A in the United States has declined by 92 %, from 12.0 cases per 100,000 population in 1995 to 1.0 case per 100,000 population in 2007 [61]. In the European Union (EU), though figures may vary among countries, the overall incidence of hepatitis A has decreased from 15.1 per 100,000 population in 1996 to 3.9 per 100,000 in 2006 [89].

4.6 Age

In the United States and prior to introduction of HAV vaccination, children aged 5–14 years have had the highest attack rates as shown in community-wide outbreaks affecting specific ethnic or low socioeconomic groups [86, 102, 103]. A bimodal distribution has been seen in day-care outbreaks, with children aged 5–9 years and adults 25–35 years most commonly affected [65]. In recent years, the overall incidence of hepatitis A declined among all age groups in the United States, but the greatest decrease recorded between 2001 and 2007 was among children <5 years of age. However, asymptomatic infection is common among young children, and symptomatic cases in children aged <5 years represent only a limited proportion of infections that occur in this age group [61]. In 2007, rates were highest for persons aged 25–39 years (1.3 cases per 100,000 population) [61].

During 1999–2006 and following the introduction of universal vaccination in 17 US states, the overall seroprevalence of anti-HAV (IgG) was 34.9 % as reported by the National Health and Nutrition Examination Survey (NHANES) [83]. During this period, US-born children living in vaccinating states had a higher seroprevalence (33.8 %) than children in non-vaccinating states (11.0 %). Seroprevalence among children increased from 8.0 % during 1988–1994 to 20.2 % during 1999–2006. For US-born children aged 6–19 years, the strongest factor associated with seroprevalence was residence in vaccinating states. Among US-born adults aged >19 years, the overall age-adjusted seroprevalence of anti-HAV (IgG) was 29.9 % during 1999–2006, which was not significantly different from the seroprevalence during 1988–1994 [83].

Similar age-specific prevalence patterns occur in regions which initiated mass vaccination campaigns in Europe and Australia [104]. In countries with a low or very low endemicity of infection, the disease and the prevalence of anti-HAV(IgG) are largely limited to adults, since the majority of infections are imported. Seroprevalence rates vary considerably in various African, Asian, and South American countries where HAV endemicity is either high or in transition [26]. In populations with a high endemicity of infection, cases occur primarily in children, as all adults are immune as a result of infection in childhood.

4.7 Sex

Until 2002, rates of acute hepatitis A had consistently been higher among males than among females at a ratio of almost 2:1; however, by 2007, overall rates had declined more among males than among females, and the reported incidence in males was 1.1 cases per 100,000 population, compared with 0.9 cases per 100,000 population among females [61].

4.8 Race

Hepatitis A occurs in all racial groups, and race per se is not believed to predispose to infection except when related to socioeconomic status or country of origin [101]. Hepatitis A rates in the United States have differed historically by race; the highest rates occurred among American Indian/Alaska Natives and the lowest rates among Asians/Pacific Islanders. In 2007, rates among American Indian/Alaska Natives dropped from >60 cases per 100,000 population before 1996 to 0.5 cases per 100,000 population. Rates for Hispanics decreased 94 %, from the peak of 24.1 cases per 100,000 population in 1997 to 1.4 cases per 100,000 population in 2007 [61].

4.9 Occupation

There does not appear to be an increased risk of HAV infection associated with a particular occupational group, although outbreaks of hepatitis A have occurred in persons working with certain nonhuman primates and sewage workers. Employment in a day-care center is a reported source of infection, but no studies have shown that this occupational group has an increased risk of infection compared to the general population. Susceptible persons from low endemic countries who work in or travel to countries with a high endemicity of HAV infection are at risk of infection, and this risk increases the longer they reside in the country [105, 106]. Outbreaks of hepatitis A in health-care workers are rare [107, 108]. Studies of health-care workers have shown that they are not at increased risk of infection [109, 110].

4.10 Military and Other Settings

Hepatitis A among military personnel stationed in low endemicity countries does not appear to differ from that in the

surrounding community. Outbreaks caused by contaminated food, exposure to children in day-care centers, and sporadic disease from person-to-person contact have been reported. However, prior to the availability of HAV vaccines, hepatitis A posed a risk among troops deployed to parts of the world where HAV infection is of high or intermediate endemicity. However, in recent years, American troops sent to regions with high or intermediate HAV endemicity are immunized prior to deployment. Consequently, the crude incidence of HAV in the US military is very low at 1.37/100,000 person-years, and the rate of hospitalization for HAV infection has declined sharply [111].

5 Mechanisms and Routes of Transmission

Studies in experimentally infected nonhuman primates and in humans have shown that HAV is excreted in large quantities (up to 10^8 infectious units per ml) in feces during the late incubation period and first week of clinical illness. Some studies suggest that children may shed virus longer than adults, but there is no evidence of chronic virus excretion [112–114]. Fecal shedding of HAV has been reported in patients with relapsing HAV infection [115]. Viremia occurs from the middle of the incubation period into the early clinical illness; infectivity titers of serum are 10^3- to 10^6-fold lower than those of feces. Virus is also present in saliva with an infectivity that may be 10^4 lower than serum [114, 116]. These physical data are firmly supported by epidemiologic data that have implicated the fecal–oral pathway as the predominant route of HAV transmission and occasionally demonstrated blood-borne transmission. Ingestion of infectious feces or vomits transferred from person to person or ingestion of contaminated food or water is the major route of HAV transmission. Blood-borne transmission occurs rarely and can lead to secondary outbreaks among health-care workers due to inapparent fecal–oral spread of the virus [117, 118]. Transmission among MSM has been well documented; whether this occurs through sexual contact or simply by non-sexual intimate contact is not certain [96, 119]. Finally, HAV-RNA was identified in breast milk of infected nursing mothers but without evidence for HAV transmission to their babies [35].

Common-source outbreaks have occurred from contamination of food and various sources of water, including streams, individual wells, or community supplies [34, 41, 120]. Food-borne outbreaks usually result from contamination by a food handler during the disease incubation period, and poor hygiene and diarrhea enhance the risk of transmission [90]. Implicated foods are usually handled extensively after cooking and/or are eaten uncooked. Such foods have included salads, sandwiches, glazed or iced pastries, and in the past some dairy products [34, 90, 121]. Outbreaks have been reported throughout the world when filter-feeding bivalve mollusks (clams, oysters, mussels) harvested from sewage-contaminated waters have been eaten with little or no cooking [34, 91, 122]. In addition, outbreaks have occurred from foods contaminated at the time of harvest or processing that are subsequently served raw and have included lettuce, strawberries, and raspberries [34, 92, 93]. Nevertheless, during the 1990s, in the United States, disease traceable to contaminated food or water constituted less than 5 % of the overall disease burden [102].

Direct person-to-person transmission by the fecal–oral route accounts for most infections in all parts of the world. Susceptible household contacts have a 10–20 % risk of acquiring infection from a family member with acute illness [123, 41]. Person-to-person transmission is the predominant pathway in the day-care setting, in community-wide outbreaks, and among homosexual men. The common denominator of close contact in settings with less than optimal hygiene accounts for the ease of HAV transmission.

Transmission in the day-care setting has received close study and provides a model for person-to-person disease transmission [65]. In centers enrolling children in diapers, hepatitis A outbreaks may be common and involve transmission not only among young children but also among adult contacts in the center, at home, or in the community. Infected children are rarely jaundiced, but they transmit HAV to adult contacts who are more likely to become symptomatic and comprise 70–80 % of recognized cases. Adult contacts of 1- to 2-year-old children are at highest risk of infection; risk of transmission decreases with increasing age of the child, and contacts of older children (age 5–6 years) are not commonly affected. In centers not enrolling children in diapers, outbreaks are uncommon, and spread following the introduction of infection is limited. HAV spreads rapidly but silently among mobile, fecally incontinent children in day care and subsequently to their contacts.

The day-care model indicates that asymptomatic HAV-infected children are highly infectious and efficiently spread disease. Among persons without an apparent source for their HAV infection, almost 50 % have a child less than 5 years of age residing in the household. This suggests that much community-acquired hepatitis A may be acquired from inapparently infected young children.

Transmission of HAV by blood or blood products occurs infrequently and represents a very rare cause of posttransfusion hepatitis, i.e., in patients with blood coagulation disorder receiving multiple blood products [97, 117, 118]. Outbreaks of hepatitis A in hospitals have been traced to an index case (often an infant) who acquired infection through transfusion. Transmission to hospital staff exposed to feces

through breaks in infection control practices has occurred in neonatal intensive care units, with silent transmission among infants and high attack rates among hospital staff [117, 118].

6 Pathogenesis and Immunity

6.1 The Viral Life Cycle

The virological, immunologic, and clinical events that occur during HAV infection have been characterized in experimentally infected nonhuman primates and naturally infected humans [124, 125]. The incubation period may range from 14 to 45 days, with a median of 28 days observed in common-source exposures. Studies in experimentally infected nonhuman primates suggest that the incubation period is lengthened with the inoculation of a lower dose of virus or shortened when inoculated parenterally but that the clinical severity of disease is not dependent on dose and route of inoculation [114, 126]. Infection usually occurs through ingestion of HAV-contaminated food or fluid when HAV penetrates the gut mucosa reaching the liver through the portal circulation. HAV was demonstrated in intestinal crypts by immunofluorescence but replication was not verified. HAV has a specific tropism for hepatocytes which are the primary site of replication [125, 127]. HAV entry into hepatocytes is mediated most probably through a surrogate putative mucin-like glycoprotein receptor. Another hypothesis suggests that HAV enters the liver cell as a virus–IgA complex through the asialoglycoprotein receptor [128]. The virus replicates in the liver and is then shed into the bile and feces and to a lesser degree into the bloodstream. Upon hepatocyte cell entry, host cell ribosomes bind to the viral uncoated RNA. HAV-RNA is then translated into a major protein of 2,225 amino acids. This large polyprotein is divided into three regions: the P_1 region encoding for the structural proteins VP_1, VP_2, and VP_3 and the P_2 and P_3 regions encoding for the nonstructural proteins involved in viral replication (Fig. 17.1). HAV-RNA can be detected in body fluids and feces, using nucleic acid amplification and sequencing techniques (Fig. 17.2). Such methods, mainly used by research laboratories, have been utilized for studies on the genetic organization of HAV infection [4, 17].

HAV antigen is found primarily in the cytoplasm of hepatocytes but can also be found in liver macrophages (Kupffer cells). HAV appears in hepatocytes prior to detection in feces and prior to the onset of liver enzyme elevations. HAV is excreted via the biliary system into the feces where it appears in high concentrations from 1 to 2 weeks prior to onset of clinical illness. Virus excretion begins to decline at the onset of clinical illness, and in most persons, it has decreased substantially 1 week later. A modest proportion (< 33 %) of those infected may continue to excrete virus during the second and third week of illness, and children may excrete virus for longer periods than adults [112, 113, 118]. Between 5 and 20 % of cases may experience a relapse of symptoms and ALT rise, 2–8 weeks after the initial illness, and virus excretion may occur during this time [112, 115, 129]. Viremia is present from the middle of the incubation period until early in the clinical illness and during relapse [129].

In tissue culture, HAV can replicate and be released without cell damage. No cellular injury occurs during the high rate of viral replication that occurs early in the infection. The mechanism of hepatic cell injury induced by HAV is not fully understood, but available evidence suggests that it is immune mediated. The immune response to wild-type HAV infection involves the cellular, humoral, and innate limbs of the immune system.

6.2 Cellular Immune Response in Acute Hepatitis A

Infection with HAV leads to a cellular immune response which is involved in the immunopathogenesis of HAV infection and the induction of hepatocyte injury [130–138]. Despite the proven tropism of HAV for liver cells, the virus is not cytopathic, and liver cell injury occurs through activation of HAV multi-specific cytolytic T cells [137]. Inflammatory cell infiltrates isolated from liver biopsies of patients with hepatitis A contain CD_8-positive T cells which can specifically lyse hepatitis A virus-infected target cells in an histocompatibility leukocyte antigen (HLA) class I-restricted manner [130]. Although there is only limited information on involvement of the innate immune system in HAV infection, there is evidence that secretion of interferon gamma by activated T cells may facilitate the expression of HLA class 1 determinants on the surface of infected liver cells. Cytolytic T-cell epitopes residing on the structural protein of HAV may be involved in cytolysis of HAV-infected hepatocytes [134, 135, 137]. Little is known about the role of T-helper cells in mounting an immune response to HAV. One putative CD_4 T-cell helper lymphocyte epitope was identified on the VP3 102–121 sequence [132]. There is also some evidence that nonspecific immune mechanisms, including natural killer cells (NK) and lymphokine-activated killer cells (LAK), are involved in the induction of hepatocellular injury even before the initiation of cytotoxic T lymphocyte injury [133]. Finally, impaired function of CD4+/CD25+ regulatory T cells has been linked to the

frequent resolution of acute hepatitis A with spontaneous recovery [136].

6.3 Humoral Immune Response in Acute Hepatitis A

HAV infection generates a humoral immune response directed mainly against structural HAV proteins (Fig. 17.6). Diagnosis of acute hepatitis A is established through detection of anti-HAV (IgM) antibodies. Postinfection and postvaccination immunity is established through detection of total anti-HAV consisting mainly of IgG antibodies [17, 67, 139]. Presence of total anti-HAV antibodies in the absence of anti-HAV (IgM) antibodies signifies immunity against HAV and exclusion of acute HAV infection. Commercially available enzyme-linked immunoassays (EIA) are used for detection of anti-HAV (IgM) antibodies (directed against HAV capsid proteins). EIAs are also used for detection of total anti-HAV antibodies which consist mainly of anti-HAV (IgG) antibodies and referred to as such in this chapter. Anti-HAV (IgM) assays utilize the principle of direct binding of anti-HAV (IgM) in the test sample to anti-human IgM-coated matrix or particles. Qualitative competitive inhibition assays for measurement of total anti-HAV (IgG) antibodies are used in routine clinical practice. Experimental quantitative sensitive assays containing calibrators standardized against a WHO reference serum have been used in the assessment of anti-HAV (IgG) response to immunization.

Symptoms and signs of the acute infection usually occur within 2–4 weeks of exposure. IgM, IgG, and IgA anti-HAV antibodies appear shortly before or during the onset of symptoms [17, 126]. Anti-HAV (IgM) antibodies are detectable in symptomatic and asymptomatic patients alike. In symptomatic patients, anti-HAV (IgM) antibodies appear within 5–10 days before symptoms, or at the early phase of alanine aminotransferase (ALT) elevation, and persist for a period of about 4 months (range 30–420 days) (Fig. 17.6) [129, 140, 141]. In patients with relapsing hepatitis A (3–20 % of patients), anti-HAV (IgM), viremia, and shedding of HAV in the feces may reappear intermittently for up to 6 months and occasionally even longer [33, 126, 129, 140]. False-positive anti-HAV (IgM) may rarely be present >1 year postinfection or in patients with hyperglobulinemia [33, 67].

Anti-HAV (IgG) antibodies are usually detectable at the onset of symptoms, and their titer rises slowly parallel to the decrease in titer of anti-HAV (IgM) antibodies (Fig. 17.6). Anti-HAV (IgG) antibodies generated through natural infection provide protection against rechallenge with HAV and signify long-term immunity against hepatitis A apparently for life, irrespective of whether the infection was symptomatic or subclinical [17, 142]. Immunity to HAV is established by convention once anti-HAV (IgG) antibodies rise to a titer above 10–20 mIU/ml, depending on the immunoassay used for detection. However, the absolute lower limit of protective antibody level has not been determined [143].

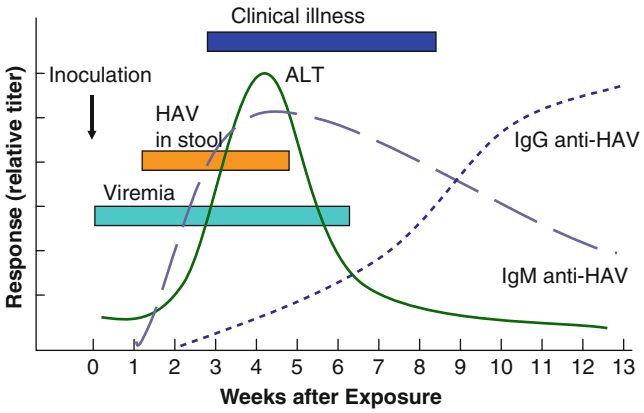

Fig. 17.6 Timeline of clinical and laboratory manifestations of acute hepatitis A. The sequence of events includes HAV viremia (*green-blue*) and shedding of infectious HAV in feces (*orange bar*), followed by increase in serum alanine aminotransferase (*ALT*) (*green line*) and the appearance of anti-HAV (*IgM*) (*gray*) and total, mainly anti-HAV (*IgG*) antibody responses (*blue dotted line*). IgM antibody declines within 3 months but can be detected in some patients as late as 6–12 months postinfection as well as in relapsing patients by sensitive assays. Data were obtained during the course of experimental infection in chimpanzees inoculated IV with human strain HLD2 (Modified from references Nainan et al. [17, 126])

Qualitative assays for total anti-HAV antibodies are used for prevalence studies and may be used for assessment of immunity pending and following vaccination. However, this method does not enable a distinction between immunity generated by "natural," wild-type HAV infection and vaccine-induced immunity. Such a differentiation may be possible in part through quantitative IgM and total anti-HAV measurements using modified, more sensitive (and experimental) immunoassays, since the humoral response to immunization is generally weaker compared to the response to wild-type HAV infection [139, 144]. The inability to distinguish clearly between these two situations led to an attempt to develop an antibody assay against nonstructural proteins of the P_2 and P_3 regions of the HAV genome, for differentiation of the humoral immune response against replicating virus from the response to a killed-inactivated HAV vaccine [145, 146]. However, at present, these tests remain a research tool only.

There are also other sensitive but also more time-consuming methods, compared to the commercially available assays for identification of neutralizing antibodies against hepatitis A. These assays, which are mainly used as a

research tool, include the radioimmunofocus inhibition assay (RIFIT), HAVARNA, and radioimmunoprecipitation assay [139].

The role of secretory immunity in hepatitis A remains unclear. Anti-HAV (IgA) antibodies have been detected in the saliva and feces of experimentally infected animals and humans [145, 146].

7 Patterns of Host Response and Clinical Outcome

7.1 Clinical Features

Acute HAV infection causes an acute necro-inflammatory process in the liver which normally resolves spontaneously. The clinical spectrum of acute disease is comparable to that of other types of viral hepatitis and ranges from few or no symptoms to fulminant hepatitis [147, 148]. However, in contrast to HBV and hepatitis C virus (HCV) infection, HAV infection does not lead to chronic liver disease. An important feature of HAV infection includes the relationship of age to clinical expression. Children under age 6 generally have mild, often nonspecific, symptoms that may include nausea and/or vomiting, malaise, diarrhea in 50–70 %, and fever or dark urine in 30–50 % [65]. Among those under age 3, fewer than 5 % become icteric, compared to 10 % of those aged 4–6 years. Data are less complete for children aged 6–14 years but suggest that symptom patterns are comparable to those of adults.

In adults, symptoms include malaise, fatigue, anorexia, vomiting, abdominal discomfort, diarrhea, pruritus, and less commonly fever, headaches, arthralgia, and myalgia [148]. Five clinical patterns are recognized: (1) asymptomatic HAV infection, often present in children under the age of 5 years; (2) symptomatic HAV infection with the appearance of dark urine and sometimes clay-colored stools, often accompanied or followed by jaundice; (3) cholestatic hepatitis characterized by pruritus, prolonged elevation of alkaline phosphates, gamma-glutamyl transpeptidase, bilirubinemia, and weight loss; (4) relapsing hepatitis A infection manifested by reappearance of the clinical, biochemical, and virologic markers of acute hepatitis A after initial resolution; and (5) fulminant hepatitis, which frequently resolves spontaneously under conservative care but which occasionally may require liver transplantation.

Extrahepatic manifestations of acute hepatitis A may occur and include skin involvement, vasculitis, pancreatitis, neuritis, encephalitis, carditis, glomerulonephritis, pneumonitis, hemolysis (especially in patients with glucose-6-phosphate dehydrogenase (G6PD) syndrome), cryoglobulinemia, and aplastic anemia [5, 41, 149]. Other post-icteric manifestations may occur in a minority of patients including prolonged fatigue, right upper quadrant discomfort, fat intolerance and indigestion, weight loss, emotional instability, and prolonged indirect bilirubinemia. Acute HAV infection resolves spontaneously in >99 % of infected individuals. Relapsing hepatitis A with subsequent complete resolution has been reported in 3–20 % of patients with clinical hepatitis A [129].

Fulminant hepatitis A is rare, with a wide range of estimated rates as low as 1:10,000 or more in immunocompetent individuals. About 70 % of fulminant hepatitis A patients experience a relatively high spontaneous survival rate; the remaining patients either die or require emergency liver transplantation. In the United States, hepatitis A accounted for 3 % of cases of fulminant hepatitis referred for liver transplantation. The Acute Hepatic Failure Study group reported in 2008 that only 29 % of 31 US patients with fulminant hepatitis A underwent liver transplantation [59, 150]. Recent reports from South America and Korea have raised concern that the current incidence of fulminant hepatitis A may be rising [59, 70, 71, 73]. Immunosuppressed patients and patients with chronic liver disease such as chronic hepatitis C and B are at an increased risk of developing severe or fulminant hepatitis [151]. Mortality in fulminant hepatitis is rare. Following the massive outbreak of hepatitis A in Shanghai, there were 47 death among >300,000 infected patients. Fatality in HBsAg+Chinese patients reached 0.05 % as compared to 0.015 % in non-HBV carriers [62]. In Western countries, reports suggest a case fatality ratio in age groups <40 years of ~0.3–0.6 %. In age groups >50 years and older, case fatality may rise to 1.8–5.4 % (these data were obtained prior to introduction of liver transplantation for HAV-associated liver failure) [11, 66, 81, 148, 152–154]. Risk factors associated with development of fulminant HAV infection are poorly understood [155]. Familial clustering of fulminant HAV cases and putative sequence variations have been suggested as potential risk factors [156–158] but await confirmation.

Hepatitis surveillance in the United States has shown the fatality rate among reported cases to be age dependent. The highest rates were recorded among children under age five (1.5 per 1,000 cases) and in persons over age 40 (27.0 per 1,000 cases), especially among those with underlying chronic liver disease [24].

7.2 Diagnosis of Acute Viral Hepatitis A

Diagnosis of hepatitis A requires demonstration of hepatocellular liver injury by standard biochemical tests (e.g., ALT, AST, bilirubin) and the presence of anti-HAV (IgM) antibodies. The intensity of hepatocellular injury (and clinical symptoms) is variable and ALT levels may rise to thousands of units accompanied by bilirubinemia and jaundice or remain very low, unicteric and undetected, especially in young children. Historically, in situation of major outbreaks,

documentation of hepatocellular injury and bilirubinemia has been used to classify such events as HAV induced provided one or more index cases were serologically positive for anti-HAV (IgM). Because of the higher frequency of nonspecific symptoms or anicteric infection in young children, the index of suspicion should be high in particular circumstances (i.e., those exposed in day-care centers), and testing for liver enzymes or anti-HAV (IgM) should be considered when an adult contact of a child has hepatitis. Clinical symptoms are inadequate to distinguish hepatitis types, and virus-specific serological testing must be done for all cases of presumed viral hepatitis. Commercially available anti-HAV (IgM) tests remain positive in virtually all cases tested within 2 months of the disease, often remaining detectable for up to 6 months after onset, and thus allow for the diagnosis based on a single acute or early convalescent serum specimen. Testing for anti-HAV (IgG) will not differentiate acute from past infection, but is useful for identifying immunity as a result of prior infection or immunization. Measurement of plasma HAV-RNA levels by PCR is not used in routine clinical diagnosis but remains an important tool for investigation of the molecular epidemiology of HAV and used especially in situations of outbreaks [15, 17].

8 Control and Prevention

There are three measures for protection against HAV infection: (1) improvement in personal hygiene, sanitation, and socioeconomic status; (2) passive prophylaxis with immunoglobulin (IG); and (3) active immunization with formaldehyde-inactivated or live-attenuated vaccines.

8.1 Personal Hygiene and Sanitation

Personal hygiene and environmental sanitation to prevent transmission through fecal contamination of food and water or by personal contact have been the primary means to control hepatitis A in any setting. Improved sanitation is presumed to have lowered the incidence of infection and disease in developed countries.

8.2 Passive Prophylaxis Against Hepatitis A with IG

Since the late 1940s and until recently, administration of human immunoglobulin (IG) prepared from pooled human plasma through ethanol fractionation [159] and containing high concentration of anti-HAV (IgG) antibodies has been used as an efficient means for pre- and postexposure prophylaxis against HAV infection [12, 143, 160]. The protective efficacy of IG is well documented [143, 161, 162], but duration of protection is limited to 12–20 weeks following intramuscular injection of 0.02 and 0.06 ml/kg weight, respectively. Preexposure prophylaxis is achieved within hours of injection. Postexposure administration is ~85 % effective when administered as close as possible to exposure and no later than 14 days [143]. The mechanism of protection against hepatitis A conferred by IG is not fully established, but most probably involves neutralization of circulating virus and possibly prevention of uptake of virus through the gut mucosa and hepatocytes.

Administration of IG is considered very safe, although contraindicated in patients with IgA deficiency. Interference with live-attenuated vaccines such as measles, mumps, rubella (MMR), and varicella requires special caution. Coadministration of IG with an active hepatitis A vaccine may blunt the initial quantitative anti-HAV (IgG) antibody response after the first vaccine dose [163, 164]. This effect, which is similar to the effect of passively transferred maternal anti-HAV (IgG) antibodies, is of minor significance, and such vaccines respond well to a booster dose given 6 months after the primary immunization. Finally, although administration of IG for pre- and postexposure short-term prophylaxis is highly efficacious, the use of immunoglobulin worldwide is now declining for a number of reasons: (a) nonspecific IG preparations increasingly fail to contain adequate amounts of anti-HAV (IgG) [165–168]; (b) cost of specific HAV IG preparations is high [167, 169]; (c) duration of IG-mediated protection against HAV infection lasts only several months as compared to hepatitis A vaccines [143]; and (d) hepatitis A vaccines have already been shown to induce very rapid pre- as well as postexposure protection against HAV following the first out of the two recommended doses [20, 37, 162].

8.3 Active Pre- and Postexposure Prophylaxis Against Hepatitis A

8.3.1 Serological Measurement of Protection

A positive (qualitative) test for anti-HAV (IgG) antibodies signifies immunity to hepatitis A. The lowest protective level against challenge with HAV is unknown. The reported minimal serum levels of anti-HAV (IgG) antibodies required for protection against HAV in humans vary between 10 and 20 mIU/ml depending on the immunoassay used for detection and regardless of whether these antibodies emerged following "natural" wild-type HAV infection or following vaccination [31, 139]. Clinical experience suggests that protection against hepatitis A following passive immunization with IG or active vaccination may still be present even in the absence of detectable anti-HAV antibodies using standard immunoassays [81]. Low levels of anti-HAV (IgM) antibodies may be detectable by a conventional assay for a few

weeks in ~20 % of recipients of HAV vaccines [37]. Therefore, anti-HAV (IgM) antibody assays cannot be used for reliable distinction between acute hepatitis A and anti-HAV response to vaccination.

8.4 Active Immunization

All HAV vaccines contain HAV antigens derived from cell cultures of attenuated HAV strains adapted to grow in human and nonhuman mammalian cells. Viral attenuation is associated with a number of mutations generated through serial passage of wild-type HAV [4, 5, 17, 170]. Comparison of the nucleotide sequence of complementary DNA (cDNA) cloned from wild-type virus with attenuated HM-175/7 MK-5 HAV strain revealed a small number of nucleotide changes distributed throughout the genome [170]. Most attenuated virus strains used for vaccine production are grown in human diploid MRC-5 fibroblasts, and the nucleotide and amino acid sequences of the virus are about 95 % identical among different strains. Cell culture-derived HAV antigen is purified, inactivated by formaldehyde, and adsorbed to aluminum hydroxide for the following vaccines: HAVRIX®, VAQTA®, and AVAXIM®). The HAV antigen in EPAXAL® is formulated in influenza-reconstituted virosomes. VAQTA®, HAVRIX®, EPAXAL®, HEALIVE®, and the Chinese Lv-8 inactivated HAV vaccine are at present preservative-free (Table 17.1). In addition to monovalent HAV vaccines, formaldehyde-inactivated combination vaccines have been developed in Europe against HAV and HBV or HAV and typhoid [5, 174–179].

Two live-attenuated hepatitis A vaccines were developed in China containing the H2 and LA-1 strains [172, 180, 181] used in a single-dose immunization schedule. Both these vaccines as well as two formaldehyde-inactivated vaccines, administered in two doses, were tested in clinical trials and integrated into the Chinese EPI in 2008 [172, 181] (http://www.who.int/wer/2010/wer8530/en/index.html).

Formaldehyde-inactivated hepatitis A vaccines are highly immunogenic and safe, providing rapid, protective immunity to hepatitis A within 2–4 weeks after primary immunization. A second dose is usually administered within 6–12 months after the priming injection, but the interval may be extended to 18–36 months depending on vaccine type. Long-term, most probably lifelong immune memory and protection after a complete vaccination has been predicted [104, 173, 182] and already documented for at least 17 years [182, 183]. Consequently, booster doses are not recommended in vaccinees who completed a two-dose vaccination schedule [104, 182, 184].

The immunogenicity of inactivated hepatitis A vaccine is blunted somewhat by preexisting antibodies, which occurs when vaccine is coadministered with IG [185, 186] or when given to infants of previously infected HAV immune mothers [187]. Yet, the clinical consequences of this phenomenon are most likely negligible in view of a recent report that vaccine-induced anti-HAV (IgG) seropositivity in children vaccinated <2 years of age persists for at least 10 years regardless of maternal anti-HAV (IgG) status [188].

8.4.1 Preexposure Prophylaxis Through Active Immunization

Inactivated hepatitis A vaccines have been licensed in Europe, Asia, Australia, and the Americas. The high efficacy of these vaccines in preventing hepatitis A in children has been shown in two pivotal placebo-controlled clinical trials conducted in Thailand and the United States demonstrating a 99 % efficacy after three doses and 100 % after two doses of aluminum hydroxide-formulated vaccines, respectively [18, 19]. In a third study a single dose of an inactivated vaccine formulated in virosomes was shown to provide complete protection against the disease [189]. Several factors have a marginal impact on blunting of anti-HAV (IgG) antibody levels following immunization. These include overweight, older age, smoking, as well as passively transferred anti-HAV (IgG) antibodies from pregnant mothers to their newborns. Lower sero-protection rates following vaccination have been reported in human immunodeficiency virus-infected patients as well as solid organ and stem-cell transplant recipients. As observed with other vaccines, anti-HAV (IgG) levels were reported to be higher in females as compared to males, following a priming and booster dose of the vaccine [5]. HAV vaccines have an excellent safety and tolerability record and are interchangeable [5, 81].

As more information has become available on the extraordinary immunogenicity, effectiveness, and safety of hepatitis A vaccines, immunization strategies have shifted from vac-

Table 17.1 Attenuated hepatitis A virus strains used for production of formaldehyde-inactivated hepatitis A virus vaccines

Attenuated HAV strain	Trade name	Adjuvant	HAV antigen dose/injection		Manufactures	Reference
			Pediatric	Adult		
HM-175	HAVRIX®	Alum hydroxide	720 EU	1440 EU	GSK	[21]
CR-326	VAQTA®	Alum hydroxide	25 U	50 U	MSD	[20]
GBM	AVAXIM®	Alum hydroxide	80 U	160 U	Aventis Pasteur [171]	[171]
TZ84	HELIVE®	Alum hydroxide	250 U	500U	Sinovac Biotech Ltd	[172]
RG-SB	EPAXAL®	Virosome	24 U	24 U	Crucell/Berna Biotech	[173]

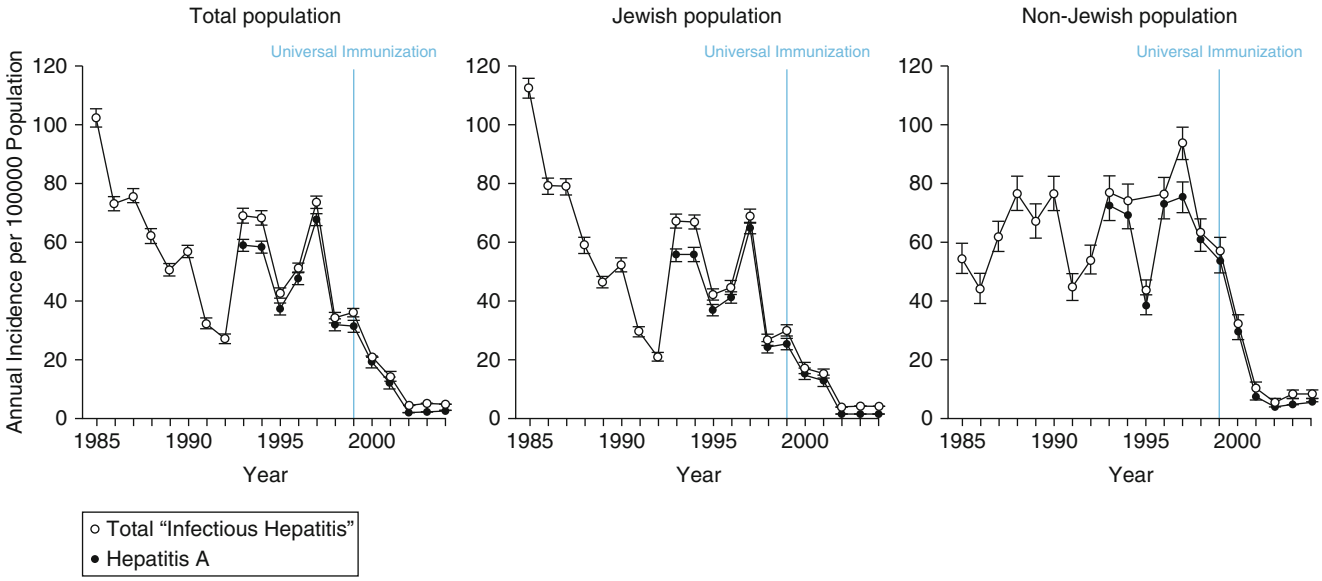

Fig. 17.7 The impact of universal vaccination against hepatitis A of 18-month-old toddlers on the incidence rates in the Jewish, the Arab, and the overall population in Israel. Data obtained by passive and active surveillance. Between1985 and 2004, acute hepatitis cases were classified as "infectious hepatitis" (including A, B, C, and nonspecified). Afterward, between 1993 and 2004, data include only serologically confirmed hepatitis A cases (Reproduced by permission from reference Dagan et al. [84])

cination of individuals belonging to specific risk groups to mass vaccination campaigns and then to universal vaccination, which is however still restricted to a limited number of countries [61, 81, 82, 84, 104, 190].

Following the licensure of HAV vaccines, four immunization strategies have been evaluated for preexposure prophylaxis. First, early efforts were aimed at immunization of individuals at increased risk, which included international travelers to areas of HAV endemicity, MSM, intravenous drug users, patients with chronic liver disease, food handlers, day-care center staff, caretakers of nonhuman primates, and patients with blood clotting disorders. This policy is still valid but has no or little public health impact on the herd immunity at large.

The second strategy included regional mass vaccination of pediatric populations at risk. Three demonstration projects conducted in the United States in native Americans in Alaska, in native American Indians, and in Butte County, California, led to a 94–97 % reduction in incidence of symptomatic acute hepatitis [82]. Consequently, in 1999, the US Advisory Committee on Immunization Practices (ACIP) issued a recommendation to introduce universal hepatitis A vaccination into routine childhood vaccination (two doses in children >2 years old and catch-up at the age of 10–12 years) in 17 states in the United States with an annual incidence of >20 cases/100,000. Postimmunization surveillance revealed that, despite variable first vaccine coverage of 50–80 %, a progressive decline in reported incidence of hepatitis A was observed from 21.1 cases/100,000 to 2.5 cases/100,000, which represents an 88 % drop (Fig. 17.5) [61, 82, 83]. Similar projects were introduced in Puglia, Italy, in 1997 [191]; in Catalonia, Spain, in 1998 [192]; and in North Queensland, Australia, in 1999 [193], leading to a 90–97 % decline in the reported incidence in these regions. The results of these highly successful vaccination projects in selected geographic regions worldwide suggested that mass vaccination of children in communities at risk is effective and will lead to herd immunity even under moderate coverage.

These early projects paved the way for the introduction of the third immunization strategy, namely, universal vaccination against HAV in selected countries with intermediate endemicity in transition. At present, 11 countries have embarked on the road toward universal HAV vaccination in babies. In 1999, Israel, a country with intermediate HAV endemicity in transition, became the first country to introduce universal HAV vaccination given in two doses to toddlers at 18 and 24 months of age. At a vaccination coverage of 90 and 85 % for the first and second dose, respectively, the annual incidence of hepatitis A dropped sharply within 2–3 years of program initiation. An overall decline of 95 % in incidence was documented not only in babies but also in the unvaccinated adult population (Figs. 17.7 and 17.8) [84]. Thus, immunization of ~3 % of the population annually led to a marked decrease in attack rates of HAV infection in all age groups until the age of 44 years and a shift from a state of intermediate HAV endemicity to very low endemicity with an annual incidence of ~2.5 cases/100,000.

In the United States, the public health objective of hepatitis A vaccination is the reduction of disease incidence and possibly eradication of HAV infection through routine infant

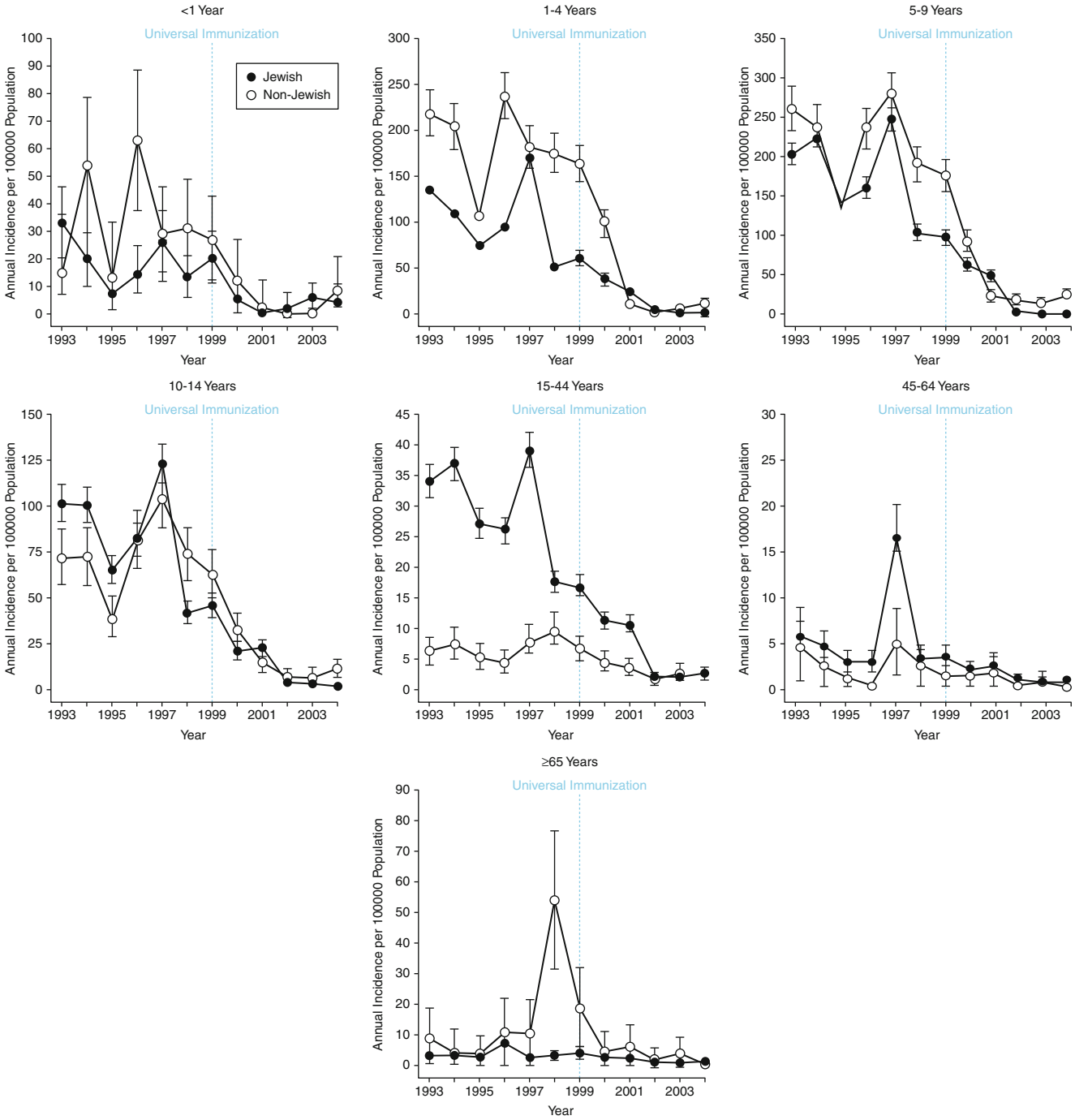

Fig. 17.8 Annual age-specific incidences of reported hepatitis A disease among the Jewish and non-Jewish populations in Israel, 1993–2004. Error bars indicate 95 % confidence intervals (Reproduced with permission from reference Dagan et al. [83])

immunization [187]. Major progress in this direction has already been achieved [83] (Fig. 17.5).

The fourth strategy for preexposure prophylaxis includes immunization with only a single dose of an inactivated HAV vaccine. The rationale for this strategy is based on the cumulative experience that anti-HAV (IgG) sero-protection rates may reach 88 % in vaccinees within 2 weeks after a single vaccine dose rising to 97–100 % at weeks 4–6 [37, 194, 195]. In 2005, public health authorities in Argentina began an experimental universal immunization program in 12-month-old babies [196]. The original baseline incidence of HAV in Argentina dropped from 70.5 to 173.8 cases/100,000 between 1995 and 2004 to ~10 cases/100,000 in all age groups within a few years, representing a >80 % decrease in incidence. These results also confirmed the experience gained in mass and universal immunization programs

elsewhere that effective immunization of toddlers will lead to widespread herd immunity. It remains, however, to be seen if a single-dose immunization strategy will indeed provide long-term protection against HAV or whether a booster dose will be required after all [197].

The ultimate success of routine infant immunization to eliminate HAV transmission is dependent on the availability of an affordable vaccine that potentially can be combined with other antigens and provides long-term protection. Such a strategy would also be appropriate for countries with an intermediate and high endemicity of infection where most persons become infected during childhood.

8.4.2 Postexposure Prophylaxis

Evidence obtained in a number of clinical trials suggested that postexposure immunization against hepatitis A may also have similar effectiveness as IG, provided that immunization is started within 2 weeks of exposure. Support for this impression was initially obtained from the hepatitis A efficacy trial conducted in the United States where the HAV vaccine was administered during an HAV outbreak and no new cases of acute hepatitis were identified from day 17 onward after vaccination [20]. A similar experience was obtained in Slovakia [198], in Israel, and in Italy where a 79 % protective efficacy of postexposure immunization was documented in Italian household contacts of acute hepatitis A cases [199]. In Israel, as in Slovakia, prompt intervention with an active vaccine in a community outbreak of hepatitis A led to effective control of an epidemic within 2 weeks of starting the intervention, in contrast to the relatively poor performance of IG [200]. Final proof for the use of inactivated HAV vaccines for postexposure prophylaxis was obtained in a pivotal study conducted in Kazakhstan [162]. In this controlled clinical trial, 1,090 household and day-care contacts (2–40 years old) of index cases with acute hepatitis were randomized to receive hepatitis A vaccine or IG. Transmission of HAV, confirmed by anti-HAV (IgM) testing, occurred in 4.4 and 3.3 % of the study groups respectively (RR 1.35; 95 % CI = 0.70–2.67). Consequently, the US ACIP is now recommending postexposure immunization with an active HAV vaccine in 2–40-year-old individuals at risk [201]. Altogether, the use of hepatitis A vaccines instead of IG for postexposure prophylaxis has a number of advantages, including induction of long-term protection against HAV, ease of administration, and acceptance at a similar cost per dose [169].

9 Unresolved Problems

Despite the availability of efficacious and safe hepatitis A vaccines for more than 20 years, hepatitis A still remains a frequent and debilitating disease affecting millions of individuals annually. The continuous improvement in sanitary and socioeconomic conditions in many countries and in regions within individual countries has led to a shift in prevalence of HAV infection from high to intermediate and even low endemicity. Consequently, a growing population of adolescents and young adults becomes susceptible to HAV infection. Hepatitis A is a vaccine-preventable disease, and vaccination of babies is the most efficient means for the control of the infection. Yet, at present only a few countries at risk have embarked on the route toward implementation of universal vaccination. The demonstrated effectiveness of hepatitis A immunization holds the potential for the eventual eradication of HAV infection. Elimination of HAV transmission occurs when a critical level of herd immunity is present in the population. As recently stated in a 2012 WHO position paper on hepatitis A, "Countries should collect and review the information needed to estimate their national burden of hepatitis A" [22]. In addition to surveys estimating age-specific prevalence of anti-HAV (IgG) antibodies, this may require examining vital registration systems, acute disease surveillance, and health information systems capturing fulminant hepatic failure cases and/or causes for liver transplantation. Economic evaluation, including cost-effectiveness analyses of relevant immunization strategies, can serve as a useful additional element for decision making.

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Hepatitis E Virus

18

Xiang-Jin Meng

1 Introduction

Although evidence for the existence of a new form of enterically transmitted non-A, non-B viral hepatitis came from serological studies of waterborne epidemics of hepatitis in India in 1980 [1, 2], the identity of the virus was not known until 1990 when the genomic sequence of the hepatitis E virus (HEV) was determined [3]. The diseases caused by HEV are characterized by explosive outbreaks of acute hepatitis in developing countries and sporadic and clustered cases in industrialized countries [4, 5]. The identification of various animal strains of HEV has broadened the appreciation of the host range and diversity of the virus [6] and also provided unique homologous animal model systems to study HEV replication and pathogenesis. Hepatitis E is now a recognized zoonotic disease, and pigs and likely other animal species are reservoirs for HEV [7]. A vaccine has recently been licensed for use in China, although it is not yet available in other countries [8]. Promising antiviral agents have also been identified.

2 Historical Background

The epidemiological features of the large waterborne outbreaks of hepatitis in India in the 1950s and 1970s such as the 1955–1956 Delhi, the 1975–1976 Ahmadabad, and the 1978 Kashmir epidemics resembled hepatitis A in terms of the mode of spread, incubation period, and clinical and biochemical features [9]. In fact, the 1955–1956 Delhi epidemics were initially reported as due to hepatitis A [10]. However, diagnostic markers of acute hepatitis A were not detected in the 1978 Kashmir epidemic [1] or retrospectively in the earlier outbreaks [2]. In addition, these outbreaks displayed unique clinical features that were distinct from that of hepatitis A, such as the high attack rate among young adults and high mortality rate in pregnant women [11]. This led to the recognition of a new form of epidemic non-A, non-B, or enterically transmitted non-A, non-B hepatitis [1, 2]. The term hepatitis E was designated only after the etiological agent responsible for these outbreaks was identified in 1990 [3].

In 1983, Mikhail Balayan (a Russian virologist) successfully transmitted the disease to himself. He had experienced prior infection with the hepatitis A virus (HAV) and thus was immune to HAV, when he voluntarily ingested a suspension of the stool samples collected from non-A, non-B hepatitis patients [12]. About 36 days after ingestion of the stool suspension, he developed severe acute hepatitis but acute diagnostic markers for hepatitis A and B were not detected in his sera, thus indicating a new agent was responsible for the hepatitis. Immune electron microscopy identified 27–30 nm viruslike particles in samples of his stool; they provided the first direct evidence for the existence of a new enterically transmitted viral hepatitis agent. Acute viral hepatitis was further transmitted to cynomolgus macaques by intravenous inoculation of monkeys with the stool suspension collected from the infected volunteer, and viruslike particles were visualized from the feces of the experimentally infected monkeys [12]. A nonhuman primate model was subsequently established for the study of the disease [13, 14].

In 1990, the agent responsible for the enterically transmitted non-A, non-B hepatitis was successfully cloned and sequenced and designated as hepatitis E virus (HEV) [3]. Subsequently, the development of diagnostic tests based on recombinant HEV antigens or peptides enabled the study of the HEV epidemiology [15]. In 1997, the first animal strain of HEV, swine hepatitis E virus (swine HEV), was discovered and characterized [16], leading to the recognition of hepatitis E as a zoonotic disease [6, 7, 17] and significantly expanding our understanding of the natural history and ecology of this pathogen [4–6, 18].

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3 Methods for Epidemiologic Analysis

3.1 Mortality

Prior to the identification of the causative agent, the sources of mortality data were primarily studies of large waterborne outbreaks of acute hepatitis that were negative for hepatitis A and B diagnostic markers [19]. With the development of serological and molecular diagnostic assays specific for HEV, the mortality data are now based on patients that are positive for anti-HEV antibodies or HEV RNA [20, 21]. The mortality associated with HEV infection is typically less than 1 % in the general population, but it can reach up to 25 % in infected pregnant women especially during the third trimester [4, 5, 22]. Among the recognized hepatitis viruses, the high mortality observed during pregnancy is unique to HEV.

3.2 Morbidity

The main sources of morbidity data have been investigations of outbreaks and sporadic cases of acute viral hepatitis [19]. Due to the lack of accurate etiology-specific data for acute viral hepatitis, the morbidity data for hepatitis E are not very reliable [23]. Investigations of acute viral hepatitis disease outbreaks using specific serological assays to detect IgG and IgM anti-HEV and PCR-based assays to detect HEV RNA help produce more accurate morbidity data regarding hepatitis E. Unfortunately, many sporadic cases of acute viral hepatitis are not routinely tested for hepatitis E and thus are not reported. Due to the lack of an FDA-approved diagnostic assay, underdiagnosis of hepatitis E in the United States and many other industrialized countries has further limited the available morbidity data.

3.3 Serological Survey

The seroprevalence of HEV infection has been primarily inferred from serological studies of archived samples and from reported outbreaks of the disease worldwide [24–27]. The seroprevalence data from the retrospective studies should be interpreted with caution due to the potential sampling bias of the convenient samples, the duration of IgG anti-HEV response in infected individuals, and the variations of different serological assays. Age- and geography-matched seroprevalence studies in special high-risk populations such as pig handlers, swine veterinarians, organ transplant recipients, and HIV-infected patents have also been reported [28–31]. The prevalence of HEV infection has been investigated prospectively in the placebo groups in conjunction with vaccine clinical trials [32–34], which provided a more accurate estimate of HEV seroprevalence.

3.4 Laboratory Diagnosis

Immune electron microscopy is insensitive for the detection of HEV particles and thus is of little value in the laboratory diagnosis of HEV infection [4, 35]. Specific serological assays to detect IgA, IgG, and IgM anti-HEV have been developed [36, 37] and are commercially available in some countries in Asia and Europe and recently in the United States. However, a considerable variation in the specificity and sensitivity of these assays has been reported [38, 39]. The available serological diagnostic assays primarily use the recombinant ORF2 capsid protein expressed in bacterial or baculovirus expression system as the antigen, although a mixture of both ORF2 and ORF3 proteins is also used as the antigen in some assays. Some commercial serological assays are approved for HEV diagnosis by regulatory agencies in Asia and Europe, although none has been approved by the FDA. When serum samples are collected during the acute stage of HEV infection, IgM anti-HEV is detectable in up to 90 % of the acute cases within 1–4 weeks after the onset of the disease [4, 15, 40, 41], but in <50 % of cases 3 months after the onset of the disease [15, 42]. A rising IgG anti-HEV titer in samples collected 2–4 weeks apart after the onset of the disease can also aid in the diagnosis of hepatitis E [4, 15, 40]. The duration of IgG anti-HEV persistence in infected individuals varies from <1 year in some pediatric patients to 2–14 years in some adult patients [40, 41, 43].

RT-PCR has been successfully used to detect HEV RNA from outbreaks and sporadic cases of hepatitis E [44–48]. Real-time PCR assays have also been developed to detect and quantify HEV genomes [49, 50]. By using a panel of known HEV-containing plasma samples, the performance of 20 conventional or real-time RT-PCR assays from different laboratories worldwide was evaluated for the detection of HEV RNA [51]. Significant variations in the sensitivity (100–1,000-fold difference) and specificity of these PCR-based assays were observed, indicating the need for a standardized PCR-based diagnostic assay for HEV. The existence of at least four recognized and two putative genetically distinct genotypes of mammalian HEV [18, 52] further stresses the need for a standardized universal RNA-based detection assay for HEV.

4 Biological Characteristics

4.1 Physical and Biochemical Properties

The buoyant density of HEV virion is 1.35–1.40 g cm^{-3} in CsCl and 1.29 g cm^{-3} in glycerol and potassium tartrate gradients. The HEV virion is sensitive to low-temperature storage (between −70 °C and +8 °C) and iodinated disinfectants [4, 18]. It is more heat labile than is HAV: HEV was about

50 % inactivated at 56℃ and almost totally inactivated (96 %) at 60℃ for 1 h [53]. Liver suspensions containing HEV remained infectious after incubating at 56 °C for 1 h, although the virus is completely inactivated by boiling or stir-frying the HEV-contaminated livers for 5 min [54].

4.2 Morphology

The HEV virions are nonenveloped icosahedral particles with a diameter of approximately 27–34 nm [52] (Fig. 18.1). The capsid is formed by capsomers consisting of homodimers of a single capsid protein forming the virus shell. Each capsid protein contains three linear domains forming distinct structural elements: S (continuous capsid), P1 (three-fold protrusions), and P2 (two-fold spikes). Each domain contains a putative polysaccharide-binding site that may interact with cellular receptors [56, 57]. Native $T=3$ capsid contains flat dimers, with less curvature than those of $T=1$ viruslike particles [58].

4.3 Antigenic Properties

It is thought that there exists only a single serotype of HEV, with antigenic cross-reactivity among strains in different genotypes [52]. Antibodies cross-reactive to human HEV strain capsid proteins have been reported in various animal species, but the viruses responsible for the antigenic cross-reactivity were genetically identified only in swine, chickens, deer, mongoose, rats, and rabbits [6, 18]. Common antigenic epitopes in the capsid protein between avian and mammalian HEV strains and among different animal strains of HEV have been identified [59–61].

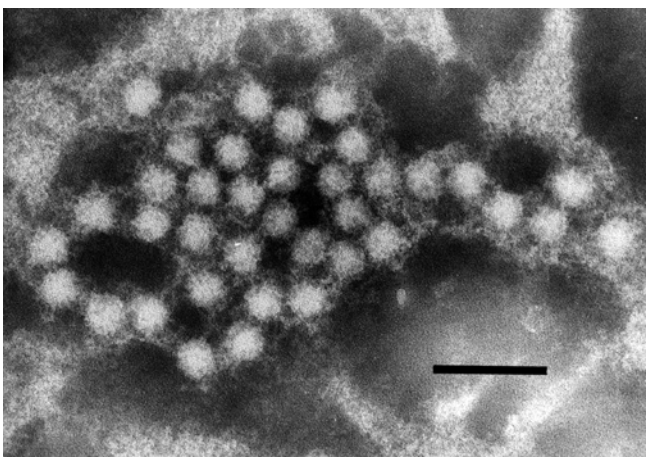

Fig. 18.1 An electron micrograph of a strain of hepatitis E virus particles (30–35 nm diameter). Negative staining, bar = 100 nm (Reproduced with permission from Haqshenas et al. [55] the Society for General Microbiology)

4.4 Genome Organization and Genotypes

The HEV genome is organized into a short 5′ noncoding region (NCR), three open reading frames (ORFs 1, 2, and 3), and a 3′ NCR [62]. ORF2 overlaps ORF3, but neither overlaps with ORF1 [63]. A cap structure has been identified in the 5′ end of the viral genome and may play a role in the initiation of HEV replication [64]. A bicistronic subgenomic mRNA encoding both ORF2 and ORF3 proteins has been identified [65]. The 5′-NCR is only about 26 nt long and may play a role in the initiation of HEV replication. The 3′-NCR contains a *cis*-reactive element. ORF1 encodes the nonstructural polyprotein, ORF2 encodes the major capsid protein, and ORF3 encodes a small phosphoprotein with a multifunctional C-terminal region [62].

Currently HEV is the only species in the genus *Hepevirus* within the family *Hepeviridae*, and avian HEV from chickens is classified as a floating species within the family [52]. There exist at least 4 recognized and two putative genotypes of mammalian HEV (Fig. 18.2): genotype 1 (Burmese-like Asian strains), genotype 2 (a single Mexican strain and some African strains), genotype 3 (strains from sporadic human cases mostly from industrialized countries, and animal strains from pig, deer, rabbit and mongoose), and genotype 4 (strains from sporadic human cases in Asia and swine strains) [52]. The strain of HEV recently identified from rats in Germany [67] and the United States [68] appears to be a new genotype, as does the recently identified HEV strain from wild boars in Japan [69]. The HEV strains from chickens [55] and cutthroat trout [70] likely represent different genera.

4.5 Gene Expression and Replication of HEV

The mechanisms of HEV replication and gene expression are largely unknown. The recent reports on the development of improved cell culture systems for HEV may aid in understanding the mechanisms of HEV replication in the future [71, 72]. The ORF1 encodes a nonstructural polyprotein that is important for viral replication, although it remains unknown if the polyprotein functions as a single protein or as individually cleaved smaller proteins [5, 52]. Functional activities such as methyltransferase [64, 73], guanylyltransferase [73], deubiquitination [74, 75], NTPase and 5′ to 3′ RNA duplex-unwinding activities [76], and RNA 5′-triphosphatase [77] have been experimentally demonstrated in the ORF1 protein. A proline-rich hypervariable region in the ORF1 protein is dispensable for viral replication both in vitro and in vivo and is functionally exchangeable between HEV genotypes [78, 79]. The ORF2 encodes the viral capsid protein that contains a typical

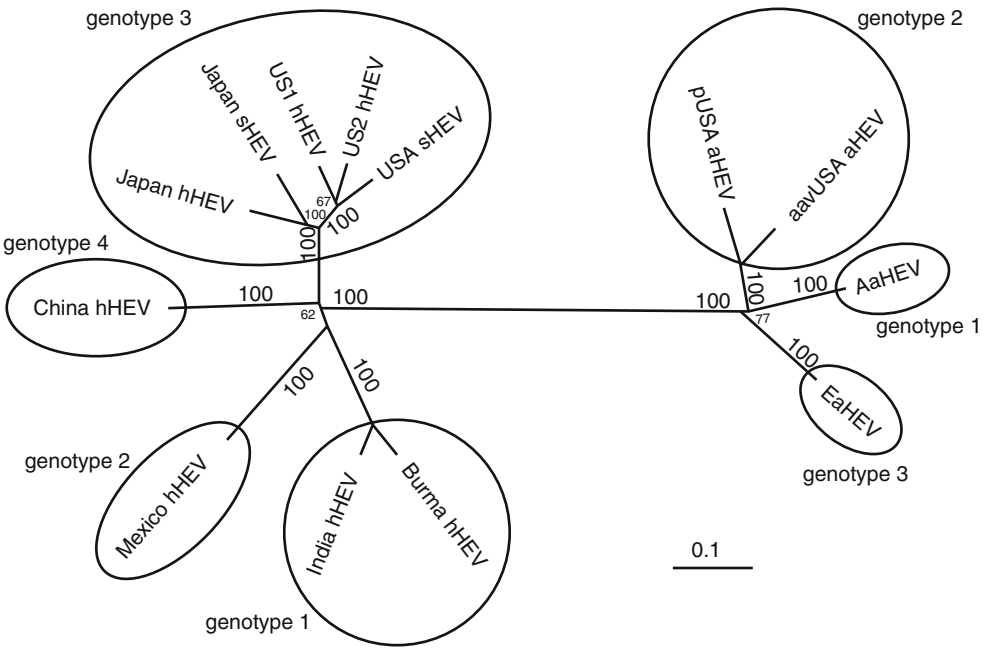

Fig. 18.2 A phylogenetic tree of mammalian and avian strains of hepatitis E viruses. The four recognized genotypes of mammalian H*epeviruses* and the three recognized genotypes of avian hepatitis E virus are indicated (Reproduced with permission from Bilic et al. [66] the Society for General Microbiology)

signal peptide sequence and three potential glycosylation sites. Mutations within the glycosylation sites prevent the formation of infectious virus particles [80–82]. The ORF2 capsid protein contains neutralizing epitopes and is the target for vaccine development [82]. The ORF3 encodes a small cytoskeleton-associated phosphoprotein [83]. The N terminus of ORF3 binds to HEV RNA and forms a complex with the capsid protein, whereas the C terminus is multifunctional and may be involved in virion morphogenesis and pathogenesis [62, 84].

HEV is thought to enter the host through the gastrointestinal epithelial cells. HEV replication in small intestines has been experimentally demonstrated [85, 86]. The capsid protein attaches to cell surface heparan sulfate proteoglycans (HSPGs), specifically syndecans, in Huh7 human liver cells [87], although a specific cellular receptor for HEV is unknown. The capsid protein is co-translationally translocated across the ER membrane [88]. After uncoating, viral genomic RNA is released to cytoplasm where transcription, translation, and virus replication occur [62, 89]. During HEV replication, an intermediate negative-sense genomic RNA is produced to serve as the template for the production of positive-sense progeny viral genomic RNA as well as the subgenomic RNA for the translation of ORF2 and ORF3 proteins [62, 89]. Negative-sense, replicative, viral RNA has been detected in the livers and gastrointestinal tissues of experimentally infected animals [85, 86]. A conserved double stem-loop RNA structure identified in the ORF1 and ORF2 junction region of HEV genome is critical for virus replication, and both the sequence and the stem-loop structure in the junction region play important roles in HEV replication [90]. The mechanisms of HEV assembly and release remain largely unknown. The ORF3 protein is responsible for virion egress from infected cells [84, 91].

4.6 Host Range and Cross-Species Infection

Genetic identification of HEV strains from several animal species including pig [16], chicken [55], mongoose [92], deer [93], rabbit [94, 95], rat [67, 68], and fish [70] and the demonstration of cross-species infection by some animal strains of HEV [61, 96, 97] have significantly expanded the host range and genetic diversity of HEV (Table 18.1). The strains of human HEV genotypes 1 and 2 have a more limited host range and are restricted to humans, whereas genotype 3 and 4 strains can infect across species barriers. Genotype 3 and 4 strains of swine HEV infected nonhuman primates [96, 97], and conversely genotypes 3 and 4 strains of human HEV infected pigs [98, 99]. Cross-species infection of HEV has also been reported in other animal species. Wistar rats were reportedly infected by human HEV isolate [100]. Avian HEV from chickens infected turkeys but failed to infect rhesus monkeys [101], suggesting that avian HEV may have a more limited host range and may not infect humans. The HEV strains from rabbits infected pigs [61], and genotypes 1 and 4 human HEV also reportedly infected rabbits [102].

Table 18.1 Animal reservoirs and host range of the hepatitis E virus (HEV)

Genotypes	Natural hosts	Epidemiological features in humans
Mammalian HEV		
Genotype 1	Humans	Epidemic outbreak
Genotype 2	Humans	Epidemic outbreak
Genotype 3	Humans, domestic and wild pigs, deer, mongoose, rabbits	Sporadic cases
Genotype 4	Humans, domestic and wild pigs, cattle[a], sheep[a]	Sporadic cases
Putative genotype 5	Rats	Non-transmissible to humans
Putative genotype 6	Wild boars	Unknown
Avian HEV		
Genotype 1	Chickens (Australia, Korea)	Non-transmissible to humans
Genotype 2	Chickens (USA, Canada)	Non-transmissible to hum ans
Genotype 3	Chickens (Europe, China)	Non-transmissible to humans
Fish HEV		
Cutthroat trout virus	Brown, Apache, and Gila trouts	Non-transmissible to humans

[a]Not independently confirmed

5 Descriptive Epidemiology

5.1 Incidence and Prevalence

The incidence of hepatitis E in the general population is unclear as there are few such studies to determine the disease incidence [103]. A prospective study of a cohort of 1,134 randomly selected individuals with 1,172 person-years of follow-up in southern Bangladesh revealed that the overall incidence of HEV infection was 64 per 1,000 person-years over an 18-month period [104]. Based on surveys of clinical diseases, the incidence of hepatitis E during outbreaks was estimated at 1,400–1,650 per 100,000 population during large outbreaks in India and Nepal [19]. In 2005, a large outbreak of 1,611 cases of hepatitis E was reported in Hyderabad, India, with an attack rate of 40 per 100,000 population. A significantly higher attack rate (203 per 100,000 population) was found in neighborhoods supplied by water lines that crossed open sewage drains than in neighborhoods supplied by non-crossing water pipes (38 in 100,000 population) [105]. Another large outbreak of 2,621 cases of acute hepatitis E was reported in refugee camps in Darfur, Sudan, with an attack rate of 3.3 % and a case fatality rate of 1.7 % [106]. In industrialized countries, the incidence of sporadic or clustered cases of acute hepatitis E is difficult to determine but appears to have been on the rise in recent years [107, 108]. Whether the increased incidence of sporadic cases of acute hepatitis E is due to increased awareness of the disease and availability of diagnostic tests or due to actual increase in disease incidence remains debatable. The majority of the sporadic or clustered cases are caused by zoonotic genotypes 3 and 4 strains of HEV [6, 18].

The prevalence of anti-HEV antibodies is very high in some developing countries: for example, more than 70 % of the general populations in Egypt are positive for IgG anti-HEV antibodies [20]. Interestingly, IgG anti-HEV prevalence in some industrialized countries is much higher than expected: for example, in some regions of the United States, up to 30 % of the blood donors were tested positive for IgG anti-HEV antibodies [28, 30, 109].

5.2 Epidemic Behavior

The sources of infection appear to be different for epidemics and sporadic cases. HEV-contaminated drinking water is the main source for epidemics [4], which typically occur in small towns and villages, refugee camps, as well as in cities of developing countries lacking modern sanitation [9, 19, 110]. The outbreaks are often associated with inadequate water treatment, contamination of water supplies with fecal materials, and poor sanitary conditions such as washing hands in a group basin and storage of drinking water in large-mouthed vessels [105, 106, 111]. Person-to-person spread of hepatitis E, although uncommon, does occur during an epidemic [112]. The epidemic cycle is believed to be similar to hepatitis A with an occurrence of 7–10-year cycles [19]. It has been reported that a second epidemic of hepatitis E occurred in a village that had experienced a prior outbreak 30 years earlier [113], suggesting a loss of protective immunity over time in the community. Most outbreaks are associated with contamination of well water or leakage of untreated sewage into city water treatment plants [19]. Attack rates are higher in villages that rely on river water as compared to villages that rely on wells or ponds [114]. For villages relying on river water, the peak of epidemics is associated with the rise in the level of the river during rainfall [19, 114]. HEV RNA has been detected in sewage samples from an HEV-endemic region of India

during a time when no HEV outbreak was being reported, suggesting that HEV infection and fecal viral excretion may be common in HEV-endemic regions throughout the year even during nonepidemic periods. [115].

The sources for sporadic cases of hepatitis E appear to be contaminated animal meats, shellfish, and direct contacts with infected animals [6, 18, 116]. In the United States, having a pet in the home and consuming liver or other organ meats more than once per month were significantly associated with increased odds of HEV seropositivity [109].

5.3 Geographic and Temporal Distribution

Seroepidemiological studies revealed that HEV infection is distributed worldwide [34, 117] (Fig. 18.3). Large explosive epidemics of hepatitis E occur primarily in the developing countries of Asia, Africa, and Latin America [111, 118–122]. Outbreaks are more common in geographic regions with hot climates but are rare in temperate climates [34, 117]. Sporadic or clustered cases have been reported in developing countries as well as in many industrialized countries [4]. As

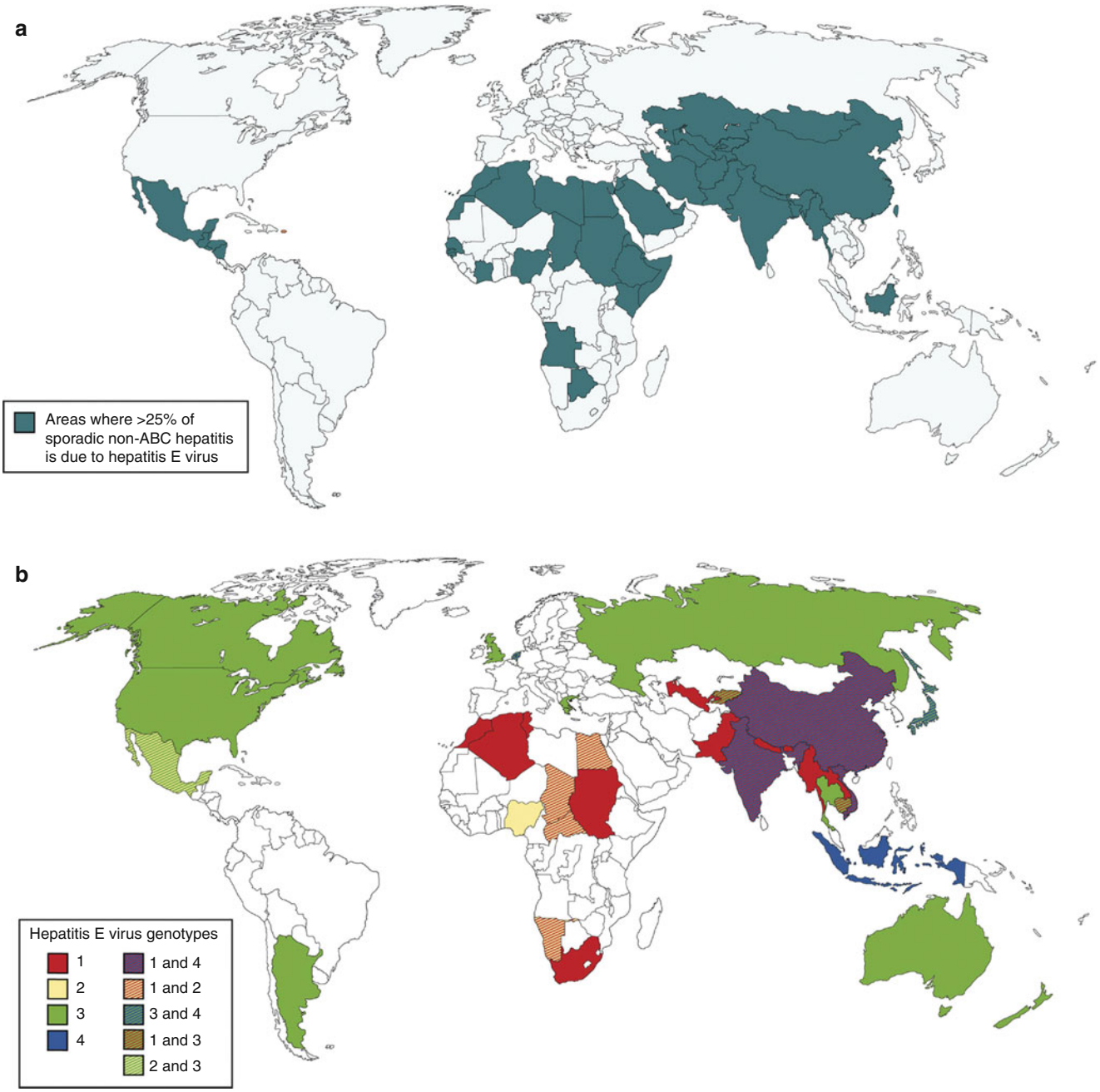

Fig. 18.3 Worldwide prevalence of hepatitis E virus (HEV) (**a**) and the global geographic distribution of the different HEV genotypes (**b**) (Reproduced with permission from Wedemeyer et al. [117] the American Gastroenterological Association)

a fecal-orally transmitted disease primarily through contaminated water or water supplies, the frequency of hepatitis E outbreaks rises in seasons with monsoon rains and flooding. A unique riverine ecology of HEV transmission has been reported in Southeast Asia [123]. Significantly higher prevalence of HEV infection has been associated with the use of river water for drinking and cooking, personal washing, and disposal of human excreta [123, 124]. In Indonesia, unusually dry weather led to decreased dilution of HEV in river water and thus contributed to the risk of epidemic HEV transmission. However, in Vietnam the risk of HEV infection increased with river-flooding conditions and contamination [123, 124]. In the United States, individuals from traditionally major swine states appear more likely to be seropositive for IgG anti-HEV than those from traditionally non-swine states: for example, subjects from Minnesota, a major swine state, are approximately five to six times more likely to be seropositive for IgG anti-HEV than those from Alabama, which is traditionally not a major swine state [30].

5.4 Occupation

Pigs are reservoirs for HEV [7], and therefore pig farmers, swine veterinarians, and other pig handlers in both developing and industrialized countries have been shown to be at an increased risk of HEV infection [28, 30]. An investigation of the 295 swine veterinarians from 8 US states with available age- and geography-matched normal blood donors revealed that swine veterinarians were 1.51 times more likely to be IgG anti-HEV positive than normal blood donors [30]. Veterinarians who reported having needle sticks while performing procedures on pigs were about 1.9 times more likely to be seropositive than those who did not. Similarly, swine workers in North Carolina had a 4.5-fold higher IgG anti-HEV prevalence rate (10.9 %) than the control subjects (2.4 %) [125]. In Moldova, approximately 51 % of pig farmers were positive for IgG anti-HEV, whereas only 25 % of control subjects with no occupational exposure to swine were seropositive. In Thailand, the prevalence of IgG anti-HEV in swine and poultry farmers was significantly higher than that in government officers [126]. Human populations with occupational exposure to wild animals have also been found to have an increased risk of zoonotic HEV infection. For example, field workers from the Iowa Department of Natural Resources had significantly higher seroprevalence of IgG anti-HEV than normal blood donors [127].

Sewage workers in India were found to have a significantly higher seroprevalence of IgG anti-HEV (56.5 %) than the control subjects (19 %), and the IgG anti-HEV seropositivity significantly increased in sewage workers with >5 years in the occupation [128]. However, a cross-sectional study in Switzerland did not clearly identify sewage work as a high-risk occupation for HEV infection [129].

5.5 Age, Sex, Race, and Other Factors

The highest attack rate of clinical disease is in young adults of 20–29 years of age [15], although the seroprevalence of IgG anti-HEV is age dependent [130]. The seroprevalence of IgG anti-HEV and the clinical attack rate are low in young children <15 years of age with the exception in epidemic settings [4, 42, 130, 131]. In Pune, India, an HEV-endemic region, HEV infection is rare in children and the prevalence does not peak (33–40 %) until early adulthood [130]. In Japan, an investigation of sera of 1,015 individuals collected in 1974, 1984, and 1994 showed that age-specific profiles of IgG anti-HEV prevalence remained unchanged, with a peak at 40–49 years, suggesting ongoing silent HEV infection during the 20-year period studied [132] (Fig. 18.4). Age, socioeconomic status, and well water were significant independent variables for HEV infection in India [133]. In

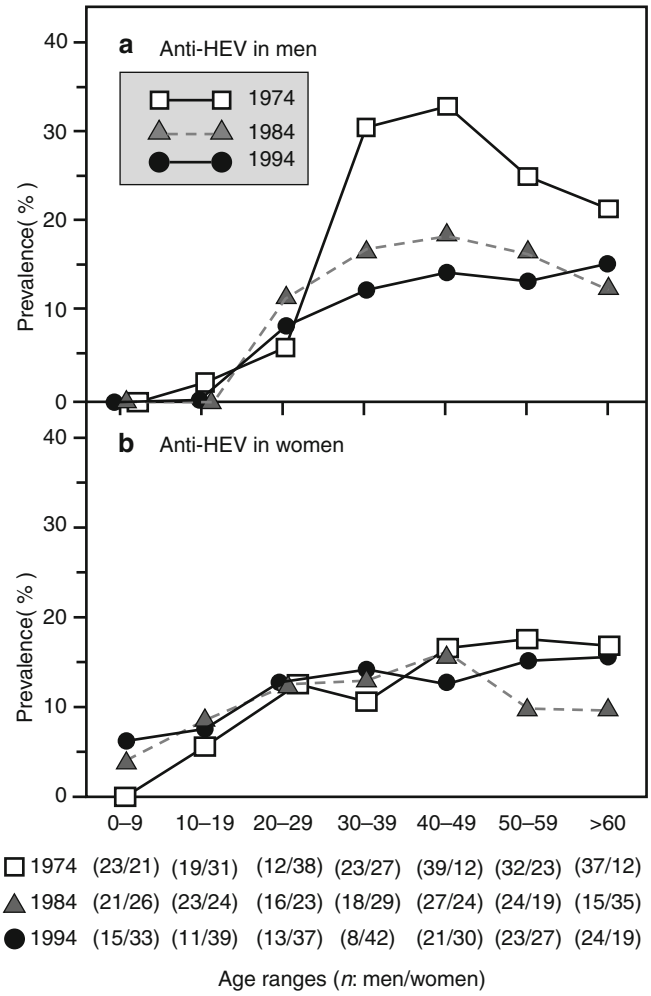

Fig. 18.4 Age- and sex-specific prevalence of IgG anti-HEV at three different times in an industrialized country. Numbers of men (**a**) / women (**b**) tested in each age group and year are indicated below in parentheses (Reproduced with permission from Tanaka et al. [132] John Wiley and Sons)

Pakistan, the attack rate was significantly higher in individuals 11–30 years of age (15.3 %) than in children <11 years of age (1.4 %) [134]. In a large hepatitis E outbreak in 2004 in refugee camps in Darfur, Sudan, the risk factors included age of 15–45 years (OR = 2.13) and drinking chlorinated surface water (OR = 2.49) [106].

No significant differences in IgG anti-HEV prevalence between males and females have been observed [4, 42, 130, 131], although individuals with symptomatic HEV infection had male-to-female ratios ranging from 1:1 to 3:1 [19, 132]. The higher rate of disease in men in certain outbreaks may reflect the fact that men from rural areas worked outside the village whereas women stayed at home in local villages [19, 132] (Fig. 18.4). A higher mortality of up to 28 % was observed in infected pregnant females [22, 114, 135, 136]. Racial difference in HEV infection is not evident. However, in a large seroepidemiological survey among US-born individuals, it was found that males, non-Hispanic whites, and individuals residing in the Midwest and/or in metropolitan areas had the highest IgG anti-HEV seroprevalence [109].

6 Mechanisms and Routes of Transmission

The main route of HEV transmission is fecal-oral. Under experimental conditions, however, infection of nonhuman primates or pigs with HEV via oral inoculation proved to be difficult [137], even though the animals could be readily infected by HEV via the intravenous route of inoculation. Chickens were successfully infected via the oral route of inoculation with an avian strain of HEV. Other routes such as vertical, blood-borne, foodborne, and zoonotic transmissions have also been reported.

6.1 Fecal-Oral Waterborne Transmission

Contaminated water supplies are major sources of HEV infections. Historically, waterborne transmission is characteristic of hepatitis E outbreaks in humans. Raw sewage water contamination of drinking water and contaminated well or river water used for washing and drinking purpose are the main sources of HEV transmission in developing countries. Zoonotically infected pigs and other animals may excrete large amounts of HEV in feces [18]; thus, HEV-containing animal manure and feces could contaminate irrigation or coastal water with concomitant contamination of produce or shellfish. Strains of HEV of both human and swine origins have been detected in sewage water [138, 139].

6.2 Foodborne Transmission

Sporadic and clustered cases of acute hepatitis E have been linked to the consumption of raw or undercooked pig livers and pork products. HEV RNA was detected in approximately 2 % of the pig livers sold in local grocery stores in Japan [46] and 11 % in the United States [140]. The contaminating virus in commercial pig livers remains infectious [140]. The virus sequences recovered from pig livers in grocery stores are closely related, or identical in some cases, to the viruses recovered from human hepatitis E patients [46]. Sporadic cases of hepatitis E including a fulminant hepatic failure case were linked to the consumption of raw or undercooked wild boar meats [141, 142]. In France, raw pig liver sausages (figatelli) were the source for some sporadic cases of hepatitis E [44]. In Japan, a cluster of 4 cases of acute hepatitis E were linked to the consumption of raw deer meats in two families [93, 143]. The viral nucleotide sequence recovered from the leftover frozen deer meat was 99.7–100 % identical to the viruses recovered from the four human patients [93, 143, 144]. Additionally, cases of hepatitis E have been linked to the consumption of shellfish [145, 146]. Taken together, the available data provided compelling evidence of foodborne transmission of HEV.

6.3 Zoonotic Transmission

The increased risk of HEV infection to persons in pig-handling occupations was noted above. In addition, potential transmissions of hepatitis E from a pet cat [147] and a pet pig [148] to human owners have been reported. Zoonotic transmission of hepatitis E to individuals who consume infected pig and deer meats and pork products has also been documented [44, 93, 142].

6.4 Vertical Transmission

There have been reports of HEV transmissions from the mother to the fetus leading to premature birth, increased fetal loss and acute hepatitis in the newborns, and high neonatal mortality [135, 149–151]. An investigation of 62 pregnant women with jaundice in the third trimester of pregnancy showed that vertical HEV transmission occurred in 33.3 % of the cases [135]. In a study of mother-to-child HEV transmission, 3/6 (50 %) of the cord blood samples tested were positive for HEV RNA [150]. Although HEV RNA has been detected in the colostrum of HEV-infected mothers, breast-feeding appears to be safe for the infants, but transmission may occur postpartum through close contact with infected mothers [152].

Although vertical HEV transmission has been reported in patients, experimental evidence of vertical HEV transmission in animal models is still lacking. For example, infectious HEV was detected in egg whites from eggs of chickens experimentally infected with an avian strain of HEV, but evidence of complete vertical transmission is absent [153]. In another study, gilts (female pigs pregnant with their first litter) inoculated with a genotype 3 HEV became infected and shed virus in feces; however, vertical transmission was not detected in the fetuses [154]. Similarly, pregnant rhesus macaques experimentally infected with HEV failed to transmit the virus to the offspring [155].

6.5 Blood-Borne Transmission

Numerous reports have documented the transmissions of hepatitis E through blood transfusions in patients from both developing and industrialized countries [156–161]. A small proportion of blood donors in Japan are viremic for HEV and thus pose a potential risk for transfusion-associated hepatitis E [162, 163]. Among the 41 blood donors with elevated ALT levels in Japan, HEV RNA was detected in 8 serum samples (20 %) [164].

7 Pathogenesis and Immunity

7.1 Pathogenesis

The pathogenesis of HEV is not well understood. After oral ingestion of the virus, HEV is believed to first replicate in the gastrointestinal tract and then spread to its target organ, the liver, through viremia. Evidence of HEV replication in the liver has been documented in nonhuman primates, pigs, and chickens experimentally infected with human and swine HEVs. After replication in the liver, the virus is released to the gallbladder from hepatocytes and then is excreted in feces. In addition to livers, HEV replication has also been identified in extrahepatic tissues including small intestines, colon, and hepatic and mesenteric lymph nodes [85, 86], although the clinical and pathological significance of these extrahepatic sites of virus replication remains unknown.

Earlier studies in human volunteers provided some clues on the course of HEV infection in humans [12, 165]. Fecal virus shedding and viremia precedes the onset of clinical and biochemical hepatitis, and virus shedding ends when the level of serum liver enzyme returns to baseline [4, 117] (Fig. 18.5). Pregnancy increased the severity and mortality of the disease, although the mechanism of fulminant hepatitis E during pregnancy remains unknown. Histological changes include focal necrosis with minimal infiltration and moderate inflammation of Kupffer cells and leukocytes [4, 19]. In pigs experimentally infected with a genotype 3 human HEV [98], mildly-to-moderately enlarged hepatic and mesenteric lymph nodes were observed from 7 to 55 days postinoculation. Mild-to-moderate multifocal lymphoplasmacytic hepatitis and focal hepatocellular necrosis were observed. In chickens experimentally infected with an avian strain of HEV, gross lesions include subcapsular hemorrhages and slightly enlarged right intermediate lobe of the livers. Foci of lymphocytic periphlebitis and phlebitis were the characteristic histological lesions in livers [166].

7.2 Immunity

The immune response to HEV infection is characterized by a transient appearance of IgM HEV antibodies followed by long-lasting IgG antibodies [4, 40] (Fig. 18.5). The HEV capsid protein is immunogenic and induces neutralizing antibodies and protective immunity. The capsid proteins of mammalian and avian HEV strains share common antigenic epitopes, and all HEV strains identified thus far appear to belong to a single serotype. Protection against hepatitis E has been demonstrated in passively or actively immunized cynomolgus monkeys [167]. It has also been demonstrated that passive transfer of anti-HEV immunity from immunized mothers to their offspring occurs both by transplacental and lactation routes [168]. The characteristics of cell-mediated immune response against HEV infection are largely unknown. The HEV-specific IFN-γ responses were found to correlate strongly and significantly with the presence or absence of IgG anti-HEV in experimentally infected chimpanzees as well as in seroconverted human subjects [169].

8 Patterns of Host Response

8.1 Clinical Features

The incubation period ranges from approximately 2 weeks to 2 months. The clinical presentation of acute hepatitis E is often indistinguishable from other types of acute viral hepatitis. Patients generally have jaundice, anorexia, dark urine, and hepatomegaly, and approximately 50 % of the patients also have abdominal pains and tenderness, nausea, vomiting, and fever [4, 19]. Not all HEV-infected individuals develop overt clinical disease, and in industrialized countries a significant proportion of individuals are seropositive for HEV antibodies with no known history of hepatic disease [28, 30]. Hepatitis E is a dose-dependent disease: exposure to a higher dose of virus may lead to manifestation of clinical symptoms of hepatitis E, whereas patients exposed to a lower virus dose generally had only subclinical infections [4].

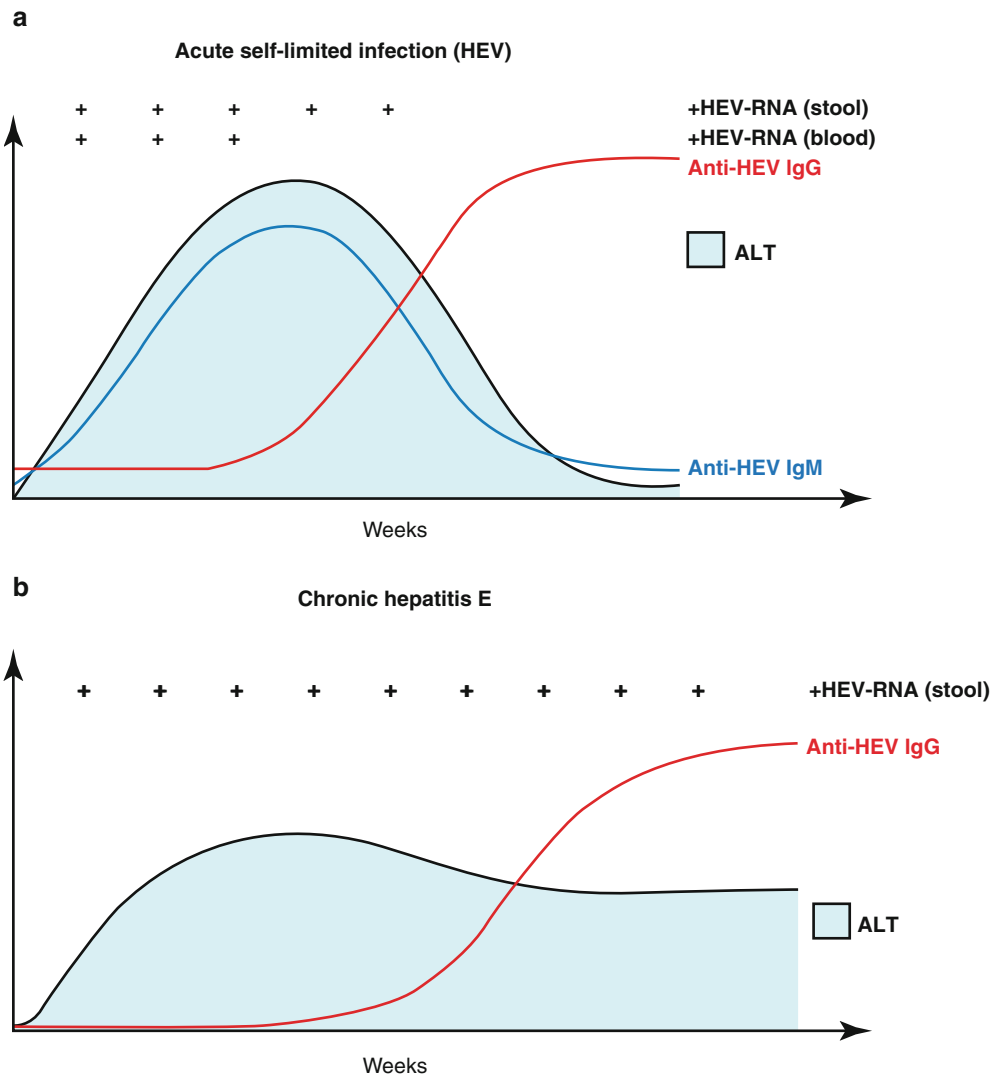

Fig. 18.5 A diagram of serological, virological, and clinical courses of acute and chronic hepatitis E in humans. The detection and appearance of viral RNA in blood and stool samples, serum IgG anti-HEV antibodies, and levels of alanine aminotransferase (ALT) levels during the course of acute (**a**) or chronic (**b**) HEV infection are schematically depicted (Reproduced with permission from Wedemeyer et al. [117] the American Gastroenterological Association)

8.2 Hepatitis E and Pregnancy

An important feature of HEV infection is the observed high mortality during pregnancy, particularly in certain regions in India, where HEV infection causes severe hepatitis and acute liver failure (ALF), leading to death in a significant proportion of infected pregnant women [22, 151]. For example, it was reported that the case fatality among the HEV-positive pregnant women was 27 % and that approximately two-thirds of the infected pregnant women had preterm deliveries in India [135]. In Pakistan, the hepatitis E attack rate among the 162 pregnant females was 22 %, compared to only 11 % in nonpregnant females of childbearing age [134]. Other studies, however, question the mortality data during pregnancy since the severity of hepatitis in pregnant women was found to be similar to that in nonpregnant women. For example, a study of 2,428 pregnant women in Egypt did not link HEV exposure to liver disease history, although the authors of the study concede that the predominant HEV strain(s) in Egypt could be less virulent than those in South Asia [20]. In India, a large retrospective study showed that HEV-related ALF was independent of the sex or the pregnancy status of the patients: case fatality was 51 % in HEV-related ALF in pregnant patients and 54.7 % in non-HEV-ALF in pregnant patients [21]. Aggarwal has suggested that, in aggregate, pregnant women appear to be at an increased risk of developing HEV-associated ALF but that once ALF develops, the risk of death is similar in pregnant and nonpregnant women and boys and men [151].

Under experimental conditions, pregnant sows experimentally infected with a genotype 3 HEV at various stages of gestation did not develop clinical signs of hepatitis or elevation of liver enzymes [154]. Similarly, pregnant rhesus monkeys experimentally infected with a genotype 1 HEV did not develop more severe hepatitis than the nonpregnant

monkeys [155]. The socioeconomic status, hormonal changes and immunological status during pregnancy, and the existence of coinfecting agents may explain the observed geographical difference of HEV-associated mortality during pregnancy.

8.3 Chronic Hepatitis E in Immunocompromised Individuals

Hepatitis E is generally regarded as a self-limiting acute viral hepatitis that does not progress to a chronic infection [4]. However, recent studies revealed that chronic HEV infections do occur, more often than previously thought, especially in immunocompromised individuals such as organ transplant recipients and HIV-infected patients. For example, eight of the 14 HEV-infected patients who received organ transplants developed chronic hepatitis E with persistently elevated aminotransferase levels, viremia, and chronic hepatitis lesions [170]. Numerous other studies also documented chronic hepatitis E in liver, kidney, heart, and kidney-pancreas transplant recipients [161, 170–177]. Additionally, chronic HEV infections have also been reported in individuals with HIV infection [178–181]. Under experimental conditions, prolonged viremia and fecal virus shedding were also observed in a few animals experimentally infected by HEV [99, 166, 182]. Taken together, these recent studies indicated that acute HEV infection in immunocompromised individuals can progress to a chronic infection and can rapidly progress into cirrhosis as well.

8.4 HEV Infection and Neurological Diseases

Increasing evidence indicates that neurologic disorders are an emerging clinical manifestation of HEV infection. In a kidney transplant patient chronically infected by HEV, peripheral nerve involvement with proximal muscular weakness in the four limbs, with central nervous system involvement and bilateral pyramidal syndrome, was reported [183, 184]. HEV RNA was detected in the serum and cerebrospinal fluid of the patient. In the United Kingdom and France, among the 126 patients with genotype 3 HEV infection, 7 patients (5.5 %) developed neurologic symptoms including 3 with inflammatory polyradiculopathy, 1 with Guillain-Barré syndrome, 1 with bilateral brachial neuritis, 1 with encephalitis, and 1 with ataxia/proximal myopathy [183]. In France, meningitis with diffuse neuralgic pain or polyradiculoneuropathy was associated with HEV infection in 2 adults [185]. Additionally, several cases of Guillain-Barré syndrome in different countries have been linked to HEV infection [186, 187].

9 Control and Prevention

Protection of water system from fecal material contamination, practicing good hygiene, and avoiding drinking water of unknown purity or consuming raw or undercooked animal meats are important preventive measures [4–6, 18, 19, 188]. Washing hands thoroughly after handling infected animals is also an effective measure to prevent zoonotic HEV transmission [18].

A recombinant vaccine against HEV has recently been licensed for use in China, although it is not yet available in other countries [8, 33]. Other experimental recombinant HEV vaccines based on the recombinant capsid protein of HEV also appear to be very promising [32]. The efficacies of the recombinant HEV vaccines were 100 % for a bacteria-expressed recombinant vaccine [33] and 95.5 % for a baculovirus-expressed recombinant vaccine [32]. However, the efficacies of these vaccines against the novel strains of HEV including the emerging animal strains with zoonotic potential will need to be evaluated.

Pegylated interferon and ribavirin have been shown to clear HEV infection and improve liver histology in patients [173, 179, 189]. Ribavirin monotherapy inhibits HEV replication and induces a sustained virus inhibition in patients with chronic HEV infections [173, 177, 190, 191]. Pegylated-interferon-α-2a also induced a sustained virological response in an HEV-infected hemodialysis patient [189]. In addition, in vitro treatment of A549 cells persistently infected with a genotype 3 HEV with IFN-α for 72 h showed a dose-dependent reduction in the levels of HEV RNA [192]. Other experimental antivirals such as short hairpin RNA (shRNA) [193], RNAi [194], and proteasome inhibitors such as MG132 [75] have also been shown to inhibit HEV replication in vitro and thus could serve as potential therapeutic agents against HEV.

10 Unresolved Problems

Hepatitis E is an important but extremely understudied disease. The life cycle of HEV remains largely unknown in large part due to the lack of a robust cell culture system for HEV. However, several recently identified cell lines that support more efficient HEV replication should aid in future studies of the HEV life cycle. Identification of a specific cellular receptor for HEV will help establish a more efficient cell culture system. The mechanisms of HEV transcription, translation, and genome replication need to be delineated. One of the most pressing issues regarding HEV epidemiology is the elucidation of the now poorly understood ecology and natural history of this virus. The existence of IgG anti-HEV in various animal species suggests that more animal species are likely infected by HEV. Unfortunately, the virus

has been genetically identified only from a few animal species. Therefore, genetic identification and characterization of animal strains of HEV will help our understanding of the epidemiology, transmission routes, host range, and reservoirs of this important pathogen.

The relatively high prevalence of IgG anti-HEV in individuals from industrialized countries suggests that the occurrence of hepatitis E in those locations is underestimated, since sporadic cases of hepatitis E may go undiagnosed. The lack of an FDA-approved diagnostic assay for HEV further contributes to the underdiagnosis of hepatitis E in the United States and likely other industrialized countries. Therefore, there is an urgent need for standardized serological and molecular diagnostic assays with improved sensitivity and specificity. The association of HEV infection with chronic hepatitis in immunosuppressed individuals opens new avenues for research into the mechanism of chronic HEV infections. The mechanism of extrahepatic disease manifestation of neurological symptoms in HEV-infected patients also requires in-depth research in the future. The relationship between pregnancy and severe hepatitis E remains controversial. Although the current licensed and experimental vaccines appear to be promising, it will be important to evaluate the efficacies of the vaccines against the emerging novel strains of HEV including the zoonotic strains. Development of vaccines against the animal strains of HEV in their respective animal hosts may minimize the risks of zoonotic transmission and increase food safety.

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Influenza Viruses

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John J. Treanor

1 Introduction

More than 80 years after the recognition of influenza virus as the cause of the syndrome of influenza, or grippe, influenza continues to be a major cause of acute respiratory illnesses. These illnesses rival acute gastroenteritis as causes of morbidity and mortality throughout the world. Although the majority of cases of influenza are not identified by laboratory methods, a variety of direct and indirect methods have attributed a substantial proportion of the overall burden of acute respiratory illness directly to influenza. This burden includes both deaths and hospitalizations, which occur most commonly at the extremes of age and in persons with other underlying heart or lung diseases and in pregnancy, as well as an enormous burden of transiently disabling illness in all ages that result in substantial economic and productivity losses.

Infection with influenza virus stimulates a coordinated response of the innate, and cellular and humoral adaptive, immune response that leads to effective and long-lived resistance to reinfection with the same strain of virus. However, influenza viruses uniquely subvert this immune response through rapid evolution of the viral surface glycoproteins resulting in antigenic changes that allow infection in the presence of immunity to prior strains. Relatively minor antigenic changes, traditionally referred to as "antigenic drift," typically result in new viruses that cause the familiar seasonal epidemics of acute influenza. In addition, influenza A viruses occasionally and unpredictably undergo major changes in antigenicity referred to as "antigenic shift" that lead to worldwide, more severe epidemics, or pandemics of influenza. In many cases, it appears that pandemics viruses emerge from the enormous gene pool of influenza A viruses in migratory waterfowl. Surveillance for these viruses in birds, and their transmission to domestic poultry, swine, other mammals, and ultimately man, has therefore become an object of intense interest in recent years.

Two specific forms of influenza control approaches have been developed in the 80 years since the discovery of these viruses, vaccines, and antiviral agents. In theory, developing effective vaccines for influenza should be straightforward. However, the same antigenic variation that circumvents natural immunity also has confounded efforts to develop effective influenza vaccines, and currently available live or inactivated vaccines must be reformulated and readministered annually to keep pace with these antigenic changes. Despite intense effort over many decades, a truly universal influenza vaccine has never been successfully developed, although recent progress in influenza immunology has increased optimism along these lines. Nevertheless, the current vaccines do provide a substantial measure of protection and are important tools for reducing the overall disease burden of influenza.

The same high rate of evolution of influenza viruses has also complicated efforts to develop antiviral agents. Two viral proteins, the influenza A virus M2 protein and the neuraminidase (NA) protein of influenza A and B viruses, are the targets of the current classes of antiviral agents, the M2-inhibitors (amantadine and rimantadine) and NA-inhibitors (zanamivir and oseltamivir), respectively. Both classes of drug have been shown to reduce the duration of illness and viral shedding in acutely infected individuals and to reduce the rate of hospitalization and death when used relatively early in the course of illness. However, the rapid development of antiviral resistance has significantly impacted the utility of these agents. At the moment, essentially all current influenza A viruses are completely resistant to the M2 inhibitors, and there has been frequent development of resistance to the NAI oseltamivir, particularly in the N1 neuraminidases. The use of multiple antiviral agents with synergistic activities may represent a strategy to reduce the emergence of resistance.

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In this chapter, we will briefly review the history of influenza throughout. We will then describe the classification and the basic virology of these agents and consider the data used to estimate their yearly impact on human health. We will review the basics of the immune response to influenza and the characteristics of illness induced by these viruses and then discuss the current approaches to prevention and control.

2 Historical Background

The characteristics of influenza epidemics, such as the high attack rates, explosive spread of disease, and characteristic cough and fever, have allowed identification of past influenza epidemic throughout history. Older studies identified probable influenza epidemics occurring at an average interval of 2.4 years between 1173 and 1875 [1]. The greatest pandemic in recorded history occurred in 1918–1919 when, during three "waves" of influenza, 21 million deaths were recorded worldwide, among them 549,000 in the United States [2].

William Farr introduced the concept of "excess mortality" in his vivid description of the London epidemic of 1847 [3]. Frost [4] first used this concept in the United States to describe the 1918 influenza epidemic, but it was Selwyn Collins who systematically used excess mortality as an index for recognition of influenza epidemics [5, 6]. Collins estimated baseline mortality by calculating weekly arithmetic means, and Serfling refined the baseline estimate by deriving a regressin function to describe seasonal variation in baseline mortality [7], the basic methodology which is still in use today for assessing excess mortality.

The first influenza virus was isolated from chickens with fowl plague in 1901, but it was not recognized that this was an influenza A virus until 1955. Shope isolated the swine influenza virus in 1931 [8, 9]. Influenza A virus was first transmitted from humans to ferrets and recognized as the cause of influenza in 1933 [10]. Influenza B virus was isolated by Francis in 1939 [11] and influenza C virus by Taylor in 1950 [12]. The discovery by Burnet in 1936 that influenza virus could be grown in embryonated hens' eggs allowed extensive study of the properties of the virus and the development of inactivated vaccines [13]. Animal cell culture systems for the growth of influenza viruses were developed in the 1950s [14]. The phenomenon of hemagglutination, which was discovered by Hirst in 1941, led to simple and inexpensive methods for the measurement of virus and specific antibody [15].

Evidence of the protective efficacy of inactivated vaccines was developed in the 1940s [16]. The use of live vaccines for influenza was first suggested shortly after the virus was discovered [17], but the first live vaccine was not licensed in the United States until 2003, approximately 70 years later. Finally, four antiviral agents in two classes have been approved for prevention and treatment of influenza. These include the so-called M2 inhibitors, amantadine in the mid-1960s, rimantadine in 1993, and the neuraminidase inhibitors zanamivir and oseltamivir in 2000.

3 Biological Characteristics

Influenza viruses are members of the Orthomyxoviridae family of viruses and are enveloped, single-stranded, negative-sense RNA viruses. The viruses are further divided into types A, B, and C based on substantial differences in proteins and genomic structure. Types A and B influenza are causes of seasonal epidemics of acute respiratory disease in children, adults, and elders. Type C influenza is primarily a minor cause of mild respiratory illness in children.

Type A influenza viruses are further subdivided into subtypes based on antigenic and sequence differences in the two major surface glycoproteins: the hemagglutinin (HA) and neuraminidase (NA). To date, a total of 16 distinct HA subtypes have been identified, designated H1 to H16, and nine distinct NA subtypes, N1 to N9. Recently, a new, unique influenza virus was detected in yellow-shouldered bats in Guatemala [18]. Preliminary characterization of this virus suggests that its HA and NA should be designated H17 and N10, although crystal structure suggests that the N10 neuraminidase does not actually have neuraminidase activity [19].

While influenza A viruses of the H1N1, H2N2, and H3N2 subtypes have caused substantial disease in humans, the natural hosts of influenza A viruses are probably migratory waterfowl, in which a genetically diverse ecology of all known HA and NA subtypes have been found. These avian species thus are thought to act as a reservoir of genetic diversity of influenza A viruses and as the source of emerging pandemic influenza viruses (discussed below). Avian influenza viruses are frequently transmitted from waterfowl to domestic poultry, where they can cause widespread epidemics of severe or fatal disease. Transmission of influenza A viruses also commonly occurs to pigs, horses, mink and ferrets, and marine mammals, in which the viruses can become established and maintained for years. Influenza in each of these populations is typically limited to certain subtypes, with H1 and H3 viruses in pigs, H7 and H3 viruses in horses, and H7 in marine mammals. Recently, influenza A has also been described in felines and dogs.

Influenza B viruses do not exhibit the same type of antigenic and genetic variation in the HA and NA, and therefore do not have subtypes. However, since 2001, two antigenically lineages of influenza B viruses, termed the "Victoria" lineage and the "Yamagata" lineage, have cocirculated in humans [20]. In contrast to influenza A viruses, influenza B

viruses appear to be limited to humans, although isolation of influenza B from seals has been described [21]. Possibly for this reason, influenza B viruses have not been responsible for pandemics of influenza.

An important feature of influenza viruses is that the genome is segmented. Each gene segment is responsible for the synthesis of one or more viral proteins. Both influenza A and B viruses contain eight gene segments, although the specific proteins assigned to each gene segment differ between these two types. The consequences of this are that when a cell is infected with two different influenza viruses, the resulting progeny virus can in theory contain any combination of gene segments from the two parent viruses, meaning that 256 possible combinations of genes can be derived from reassortment event between two influenza viruses. However, some combinations may not be compatible [22], and all combinations are probably not equally likely. Genetic reassortment has been demonstrated to play an important role in the generation of pandemic influenza A viruses and has also been taken advantage of for the construction of attenuated live influenza vaccines.

Influenza viruses enter the cell by attachment of the viral hemagglutinin to sialic-acid-containing receptors on the cell membrane, followed by internalization of the virus into an acidic endosome. In the acidic environment of the endosome, the HA undergoes a conformational change that liberates a fusion peptide and results in fusion of the viral envelope with the endosomal membrane. At the same time, the third envelope protein, the M2 protein, acts as an ion channel allowing H+ions to enter the virion from the endosome. This in turn allows the viral gene segments to leave the virion and enter the cytoplasm, a process known as uncoating.

Viral gene segments are transported to the nucleus, where the viral polymerase complex, comprised of the proteins PB1, PB2, and PA, directs the synthesis of the plus sense messenger RNA as well as synthesis of negative-sense copies that will serve as progeny genomic RNA. The polymerase proteins also may play a role in disruption of host cell protein synthesis. Because replication takes place in the nucleus, some mRNA are spliced, giving rise in the case of influenza A viruses to the M2 protein from the M gene segment, and the NS2, or NEP (nuclear export protein) from the NS gene segment. Alternative start codons also give rise to a number of additional proteins from the polymerase genes of influenza A viruses including PB1-F2 [23] and PA-X [24], which may play roles in pathogenesis.

Negative-sense daughter virion RNAs are encased in nucleoprotein, associated with one copy of the polymerase complex and transported to the cytoplasm for assembly at the cell surface. Envelope proteins are glycosylated and transported to the cell surface. Virions bud from selected lipid rafts at the cell surface, acquiring an envelope derived from the cell membrane and decorated with HA, NA, and small amounts of M2 protein. Finally, the NA removes sialic acid from receptors on the cell surface or on the viral envelope, allowing the progeny viruses to leave the infected cell.

4 Descriptive Epidemiology

The epidemiology of influenza is characterized by seasonal epidemics of acute respiratory disease, punctuated, in the case of influenza A, at random intervals by worldwide epidemics of varying levels of severity, referred to as pandemics. Both of these events are felt to be driven by antigenic variation. In the case of seasonal epidemics of influenza, it is felt that population immunity drives the selection of mutations in the immunologically critical HA and NA proteins that result in sufficient antigenic differences from previously circulating viruses to allow the selected antigenic variant to efficiently replicate and infect individuals who have developed immunity to previous viruses of the same subtype. Because these antigenic changes are relatively minor and are generally the result of a few mutations in critical antibody epitopes, this process is frequently referred to as antigenic drift.

In contrast, radical changes in the HA and NA result in the emergence of viruses that are able to spread rapidly in populations with little or no effective immunity, resulting in pandemics with high attack rates and, usually, high levels of morbidity and mortality (Fig. 19.1).

Classically, pandemics involve the replacement of influenza A viruses of one subtype with influenza A viruses of a different subtype. For example, the most severe pandemic of influenza in recently recorded history was the so-called Spanish flu pandemic of 1918, due to a virus of the H1N1 subtype (the pandemic occurred before influenza virus was discovered and before the recognition of subtypes but was classified as H1N1 retrospectively). Subsequent seasonal epidemics of influenza were due to H1N1 viruses with various degrees of antigenic change, most remarkably in 1947 when a pseudopandemic was experienced due to an H1N1 virus (A/Fort Monmouth/47) with enough antigenic change from previous viruses that it was initially characterized as a new influenza A virus, so-called A' influenza [25]. H1N1 viruses continued to cause seasonal epidemics in man until 1957, when an influenza virus with an entirely new H and N subtype, A/Japan/57 (H2N2), emerged to cause a second pandemic of the twentieth century, the Asian flu. Initially, these viruses were referred to as strain A2, until the modern typing system was developed and they were categorized as H2N2 viruses. For reasons that are unclear, H1N1 viruses ceased to circulate in man after the emergence of H2N2 viruses.

H2N2 viruses then underwent antigenic drift resulting in seasonal influenza until 1968 when these viruses were

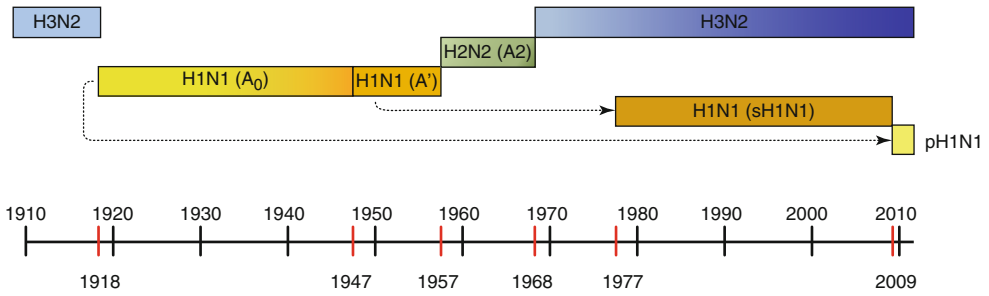

Fig. 19.1 Schematic diagram of the subtype circulation of influenza A viruses in man. The pandemic of 1918 was caused by a virus of the 1918 subtype, which may have been introduced from an unknown animal reservoir. Pandemics of 1957 and 1968 were reassortment events between previous human and avian viruses (see text). In 1977 and 2009, novel H1N1 viruses which were antigenically related to previous H1N1 viruses (*dotted lines*) were introduced into the population

replaced by the new subtype H3N2 viruses or the so-called Hong Kong flu. This virus represented a slightly different circumstance in that while the HA was a new subtype, the NA was retained from the previous H2N2 virus. Several studies have suggested that residual immunity to the retained N2 component of the H3N2 viruses substantially ameliorated the severity of this pandemic worldwide [26]. Again, for unclear reasons, the emergence of H3N2 viruses coincided with the disappearance of the previous H2N2 viruses, which have not circulated in man since then. Studies of the serologic reactivity of banked sera (so-called seroarcheology) have suggested that an H3 virus, possibly of the H3N8 subtype, may have also caused a pandemic in 1889–1891 [27].

H1N1 viruses reemerged in 1977 and resulted in a relatively more mild pandemic referred to as the Russian flu (A/USSR/77). This virus was genetically and antigenically identical to influenza A viruses that had circulated in man in 1950, and the mechanism that led to the preservation of this virus over the subsequent 27 years has never been fully explained. As expected, disease was largely restricted to younger persons born after 1957 who had not been previously exposed to H1N1 viruses, and the overall impact of this pandemic was much less severe than previous pandemics. In addition, the emergence of the H1N1 viruses did not result in the disappearance of the previous H3N2 virus. These H1N1 viruses cocirculated with the H3N2 viruses until 2009, when a new variant of H1N1 virus emerged from swine and replaced the previous H1N1, but not H3N2 viruses.

4.1 Emergence of Pandemic Viruses from Birds

Extensive surveillance studies have identified influenza A viruses of all 16 HA subtypes and all 9 NA subtypes in migratory waterfowl. In these birds influenza A causes mild illness or may be shed asymptomatically at high levels and for long duration in the feces. These birds may transmit influenza to other animals, including domestic poultry, horses, swine, and marine mammals, which may in turn transmit these viruses to man. Comparisons of sequence data from animal and human influenza viruses isolates have suggested that the 1918 virus was introduced into humans from such an animal population. In contrast, the 1957 and 1968 pandemic influenza viruses were reassortant viruses that derived some genes from previously circulating human viruses, while deriving the HA and sometimes NA genes from an avian influenza virus [28].

Because of the likely role of avian influenza viruses in the generation of emerging pandemics, there has been intense interest in recent outbreaks involving transmission of avian influenza viruses to man, with resulting disease. Most of these transmission events have been quite limited, with small numbers of persons affected, relatively mild disease, and little or no evidence of person-to-person transmission. In most cases, virus has been transmitted to humans from infected domestic poultry, but cases have also occurred in association with marine mammals and possibly wild birds.

Subtype H7 viruses have been responsible for several small outbreaks. Human infections with H7 AI viruses have generally been sporadic and mild in nature, with infected individuals presenting with mild flu-like illness and/or conjunctivitis [29, 30]. Human cases have typically been associated with outbreaks in birds, although one case of human H7 infection was reported in a laboratory worker who was sneezed upon by a seal that was infected with an H7N7 influenza virus [31]. The largest known cluster of H7N7 infections of humans occurred in 2003 in association with an outbreak of highly pathogenic avian influenza in commercial poultry farms in the Netherlands [32, 33]. While almost all cases in this outbreak were mild or subclinical, there was one confirmed fatal case in a 57-year-old otherwise healthy veterinarian, who developed pneumonia [33].

A new outbreak of influenza illness due to viruses of the H7N9 subtype has been recognized in western China since the spring of 2013 [34]. In contrast to previous H7 disease which has been predominantly mild respiratory illness with conjunctivitis, cases in this outbreak have been more severe, with hospitalizations and an approximately 20% case fatality rate [35]. Cases have been mostly recognized in older adults, for unknown reasons, and fatalities have largely occurred in individuals with underlying heart of lung disease , somewhat similar to seasonal influenza [36]. Almost all cases have direct contact with poul-

try, mostly in live bird markets. Because H7N9 viruses are not highly lethal in poultry, outbreaks in markets are much harder to recognize and control, which may be contributing to the persistence of this outbreak in affected areas.

Subtype H9 viruses have rarely caused human disease. H9N2 virus was isolated from two children in Hong Kong with mild febrile pharyngitis in 1999 [37]. Retrospective serologic cohort studies of individuals exposed to these two H9N2-infected children did not suggest person-to-person transmission [38]. Subsequently, H9N2 infection has been detected from five individuals with typical influenza in China [39] and from a child with a relatively severe influenza in Hong Kong.

The greatest concern has been for H5N1 viruses, which were first recognized in humans in 1997 [40] and which have continued to cause substantial numbers of human cases since that time. From 2003 to October 1, 2012, a total of 608 laboratory-confirmed human cases of H5N1 infection had been reported to the WHO, of which 359 cases were fatal. Cases have ranged in age from 3 months to 75 years with the median age being 20 years. Half of all cases have been in people aged less than 20 years and 90 % of cases have been in those less than 40 years of age. The median duration from onset of illness to hospitalization has been 4 days (range of 0–18 days). The case fatality rates have been the highest for those in the 10–19-year age group, lowest for people 50 or older, and in between for children aged <10 years [41].

Most cases have had close contact with ill poultry in the week before the onset of illness. Activities like plucking and preparing diseased birds, playing with birds, especially asymptomatically infected ducks, and handling fighting cocks are risk factors for infection [42]. Other apparent modes of acquisition have included eating undercooked poultry or drinking raw duck blood or exposure to contaminated water [43]. However, instances of person-to-person transmission have been rare. Fifteen family clusters of infection involving ≥2 family members were documented between January 2004 and July of 2005 [44], with the largest cluster identified thus far involving seven confirmed cases in family members of a woman who died of an acute respiratory illness [45]. In addition, there is one well-documented transmission of virus from an ill child in Thailand to her mother [46].

4.2 Emergence of Pandemic Viruses from Swine

Domestic swine have also been recognized as a potential source of pandemic influenza viruses in man. Genomic data have suggested that influenza A (H1N1) viruses were introduced into swine populations at around the same time that H1N1 viruses emerged in man in 1918 [47]. Since that time, these H1N1 viruses, or classic swine viruses, continued to be maintained in domestic swine where they caused minor illnesses and underwent relatively little antigenic evolution. During this time, swine were also occasionally infected with influenza A viruses from humans and from birds and have always been considered to represent a potential "mixing vessel" in which reassortment between human and avian influenza viruses could occur. This concept was strengthened by the recognition that the swine respiratory tract contains abundant receptors of both the α2-3 and α2-6 types favored by avian and human viruses, respectively [48].

In the late 1990s, one such reassortment event has been recognized leading to a unique virus containing polymerase genes of both swine and avian influenza virus origin. This unique combination of genes, referred to as the triple reassortant cassette, apparently increased the frequency with which these viruses underwent reassortment with other variants in swine populations. These viruses were also occasionally transmitted to humans, typically in the context of state agricultural fairs. While most of the resulting disease was relatively mild, occasional severe disease and deaths were reported, primarily in pregnant women. However, person-to-person transmission was not observed.

This situation changed in early 2009, when cases of swine-origin influenza A H1N1 viruses were first recognized in the United States and rapidly spread throughout the world [49]. The virus responsible for this pandemic was determined to be a quadruple reassortant virus derived from the triple reassortant virus by the addition of M and NA genes from a swine virus of Eurasian lineage (Fig. 19.2). The exact circumstances that led to this event remain mysterious, but it appears that the M1 protein derived from the Eurasian M gene conferred on these viruses an enhanced ability to transmit from person to person [50].

The age distribution of cases in the resulting pandemic displayed the relative sparing of older adults that has been observed in previous pandemics and might be explained by the exposure of older adults to antigenically similar viruses in their childhood [51]. Thus, the bulk of the disease occurred in adolescents and young adults. As a result, the estimated number of excess deaths due to the pandemic in 2009 was estimated to be only 12,000 in the United States, which, while substantial, was considerably less than often experienced during seasonal influenza, especially in years predominated by H3N2 viruses. However, as many of these deaths occurred in young people, the impact on years of life lost was much greater and overall more representative of the impact of the pandemic.

Since the emergence of the pH1N1 viruses, influenza surveillance activities have increased their focus on domestic swine, and a new potential pandemic threat in the form of quadruple reassortant viruses with the same internal genes as the pH1N1 but containing variant H3 HA genes has been recognized [52]. Antigenically, these viruses resemble human H3 viruses that circulated in the early 1970s [53]. Approximately 300 cases of human disease have been identified, almost all as the result of transmission from swine to humans during agricultural fairs. As expected, almost all of these cases have occurred in children. There has been little evidence of person-to-person spread, but there remains a need for continued vigilance regarding this possibility.

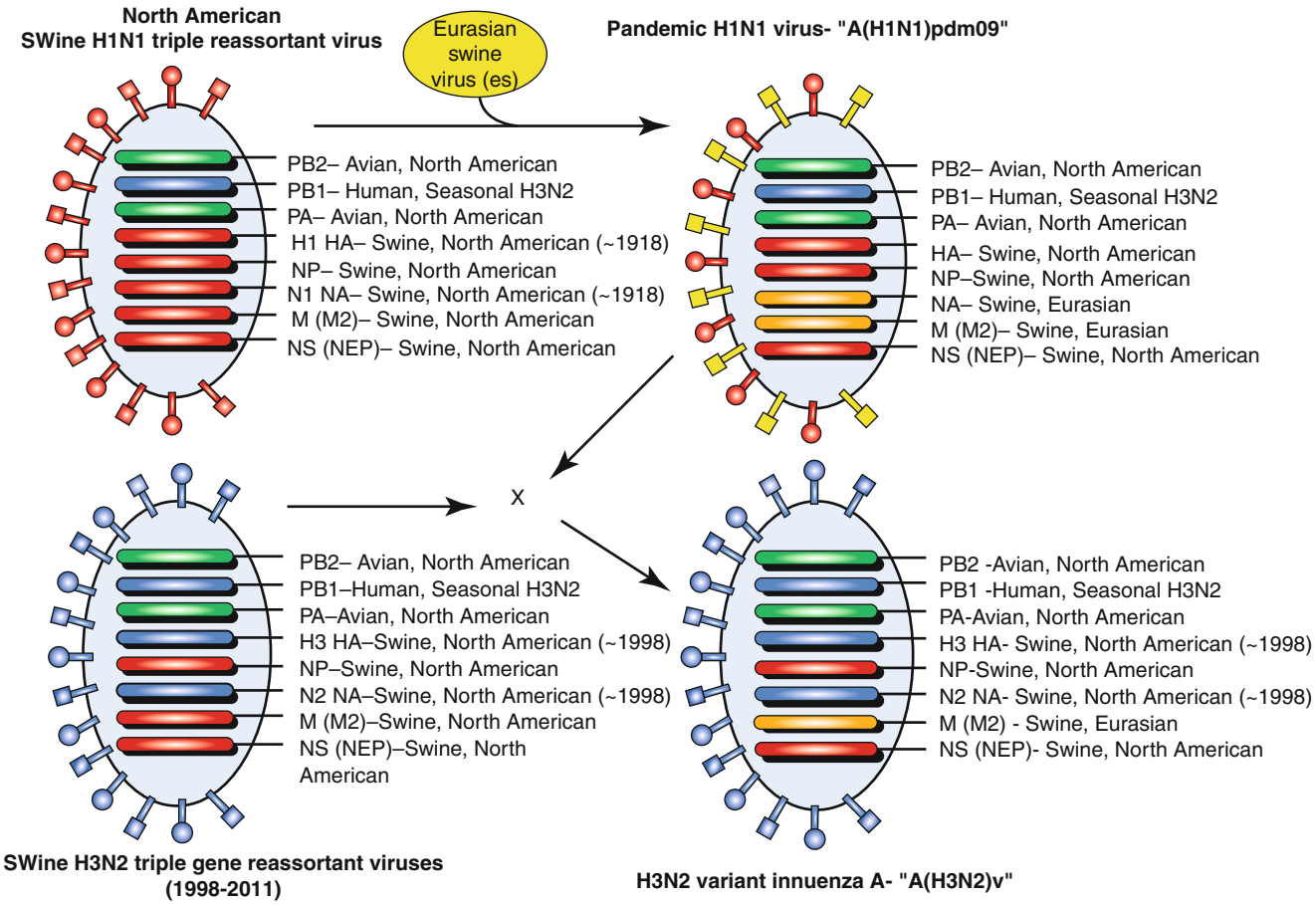

Fig. 19.2 Current swine-origin viruses are the result of a complex series of reassortment events. Both current pH1N1 viruses and H3N2v viruses derive the M gene from a Eurasian swine lineage

4.3 Seasonal Influenza

Influenza epidemics during the interpandemic period generally display a marked seasonal periodicity in regions with temperate climates, with the majority of disease activity occurring between November and April in the Northern Hemisphere and between May and September in the Southern Hemisphere (Fig. 19.3).

Seasonal periodicity is also observed in tropical climates, with increased activity during periods of low absolute humidity, although influenza can occur throughout the year and seasonal fluctuations are not as marked [54]. The reasons for these seasonal changes are not entirely clear but might be the result of more favorable environmental conditions for virus survival [55]. Studies in a model of transmission of influenza in guinea pigs have also supported a role for conditions of cold temperature and dry humidity in facilitating transmission [56]. Colder temperatures are also associated with behavioral changes that may increase transmission, such as indoor crowding or school attendance.

4.3.1 Disease Impact

Influenza epidemics are regularly associated with excess morbidity and mortality, usually expressed in the form of excess rates of pneumonia- and influenza-associated (P&I) hospitalizations and deaths during epidemics. In order to estimate the disease burden of influenza, observed P&I events during periods of influenza epidemic activity are compared with an expected seasonal baseline derived from a time-series regression model, and the excess event rate attributable to influenza is calculated. Because not all influenza-related deaths are manifested as pneumonia, P&I mortality statistics may underestimate the true impact of influenza on the population [57]. Although less precise, seasonal excess all-cause mortality is probably a more accurate reflection of the total burden of influenza.

From 1979 to 1991, these methodologies have led to estimated rates of influenza-associated hospitalizations ranging from 55,000 to 431,000 annually, with an overall average of 226,000 hospitalizations attributable to influenza [58]. Estimates of influenza-associated deaths have increased in

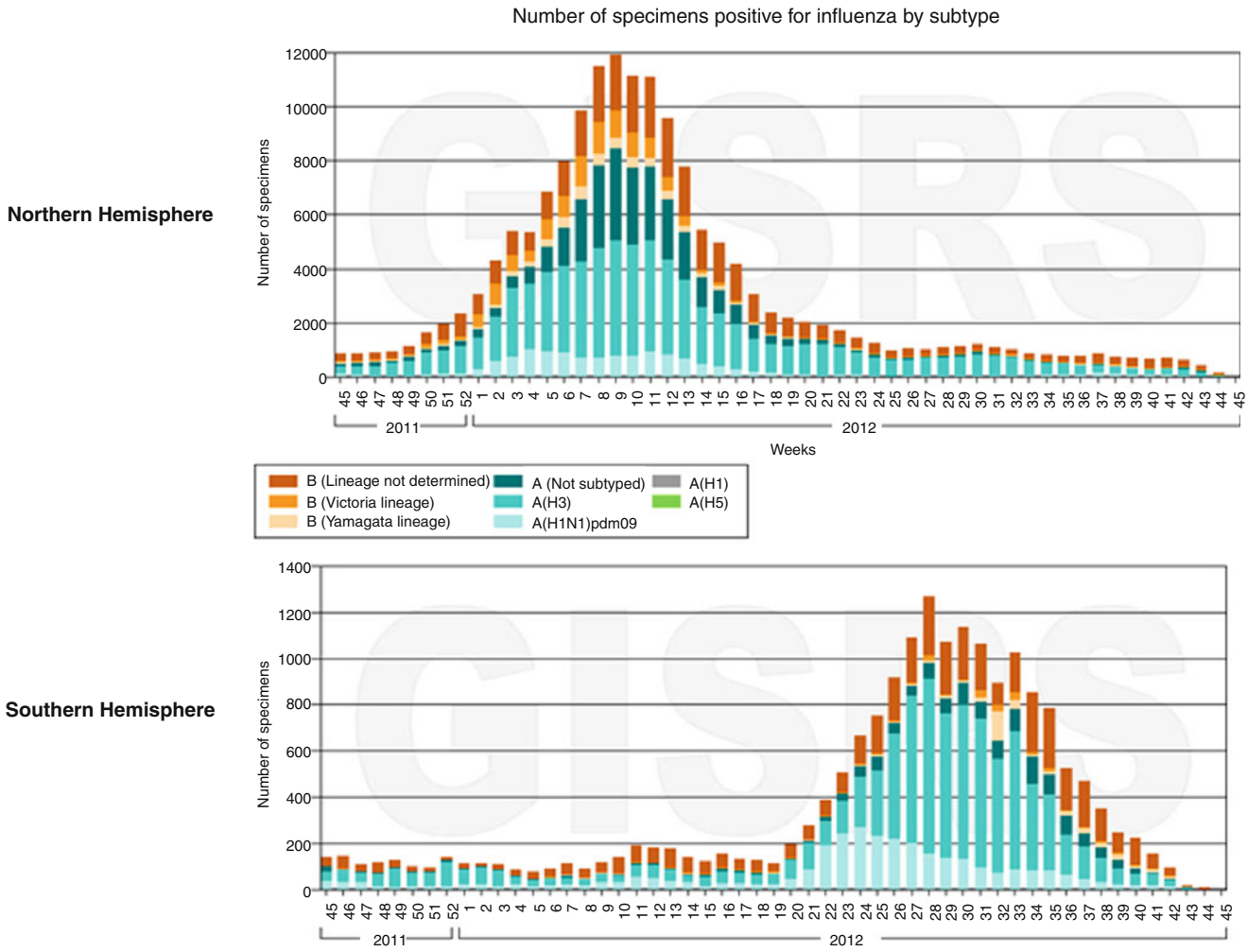

Fig. 19.3 Representative example of the global circulation of influenza in the northern and southern hemisphere. In temperate climates, influenza peak activity occurs in months with cooler, drier climate conditions

recent decades, possibly because of the increasing numbers of older, at-risk members of the population. Recent estimates suggest an average of approximately 8,000 excess P&I-associated deaths annually, but much higher numbers of all-cause mortality associated with influenza, with an average of 36,000 deaths annually with a range as high as 51,000 deaths in the United States [59]. Generally, the level of excess mortality is highest in years when influenza A (H3N2) viruses predominate [60] and lower in years with predominant H1N1 activity, possibly reflecting age-related susceptibility to these two subtypes.

Excess morbidity and mortality are particularly high in those with certain high-risk medical conditions, including adults and children with cardiovascular and pulmonary conditions such as asthma, or those requiring regular medical care because of chronic metabolic disease, renal dysfunction, hemoglobinopathies, or immunodeficiency, and in individuals with neurologic conditions that compromise handling respiratory secretions [61]. Influenza also results in more severe disease and significant mortality in individuals with human immunodeficiency virus (HIV) infection [62, 63].

The increased risk of influenza during pregnancy was dramatically demonstrated during the 2009 pandemic [64]. Previous studies had identified an increased risk of hospitalization associated with influenza epidemics during pregnancy, especially in the second and third trimester and in the immediate postpartum period [65]. Several groups at increased risk for severe influenza-related disease and deaths were recognized during the 2009 H1N1 pandemic. Women in each stage of pregnancy, or in the immediate post partum period, were clearly over represented among those admitted to hospitals and ICUs. This observation was perhaps not surprising as pregnancy has long been recognized as a risk factor for influenza mortality during previous pandemics and to a lesser extent during seasonal influenza as well. The mechanism(s) by which pregnancy enhances the risk of

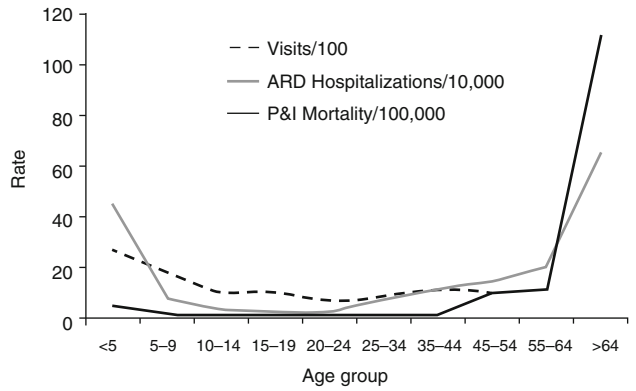

Fig. 19.4 Age-related annual incidence of acute illness visits, hospitalizations, and deaths during the interpandemic era in Houston, Texas (Data from Couch et al. [66])

influenza is not clear but might include the increased cardiovascular demands of pregnancy as well as hormonally mediated changes to the innate and adaptive immune response.

Obesity also emerged as a risk factor for influenza morbidity and mortality that had not been recognized in previous seasonal epidemics or pandemics. While compromise to respiratory mechanics as a direct result of extreme obesity undoubtedly plays a role, there is also evidence to support a detrimental role of adipose tissue in the inflammatory response that might also enhance the influenza disease process. As a result of these observations, obesity is not recognized as an important factor for targeting influenza vaccine.

Influenza is usually associated with a U-shaped epidemic curve (Fig. 19.4). Attack rates are generally highest in the young, whereas mortality is generally highest among older adults [66, 67], in part because the prevalence of high-risk conditions is greater in this group. Influenza is also recognized as an important health problem in young children. Rates of influenza-related hospitalizations are particularly high in healthy children under 2 years of age, where rates approach those of older children with high-risk conditions [68–70]. In addition, a high rate of secondary complications, particularly otitis media and pneumonia, occurs in children with influenza infection [71]. Outpatient clinic visit rates for laboratory-documented influenza have been observed at 50–95 and emergency room visit rates of 6–27 per thousand person years in children under five [72]. While rare, influenza-related deaths occur each year in previously healthy children [61]. Notably, many of these deaths occur in children who were not recognized to have high-risk conditions prior to their illness.

Much of the impact of influenza is related to the malaise and consequent disability that it produces, even in young, healthy individuals. It has been estimated that a typical case of influenza, on average, is associated with 5–6 days of restricted activity, 3–4 days of bed disability, and about 3 days lost from work or school [73]. The average number of medical visits for cases in which medical attention was sought was from 1.1 to 3.6 per year, depending on severity of the outbreak and age of the patient. In children, outpatient visits are 10–250 times more common than hospitalizations [72]. It is worth noting that direct medical costs of illness account for only about 20 % of the total expenses of a case of influenza, with a major proportion (30–50 %) of the economic impact due to loss of productivity [74]. In one study, influenza in schoolchildren resulted in 37 missed school days by children and 20 days of missed work by parents, per 100 children [75]. Influenza is also associated with decreased job performance in working adults [76] and reduced levels of independent functioning in older adults [77]. Data from the Tecumseh Community Health Study have been used to estimate that influenza is responsible for 13.8–16.0 million excess respiratory illnesses per year in the United States among individuals less than 20 years of age and for 4.1–4.5 million excess illnesses in older individuals [78].

5 Mechanisms and Routes of Transmission

Influenza viruses are transmitted from person to person via the respiratory route. Three potential modes of transmission have been suggested [79]. Coughing and sneezing could generate small particle aerosols (<10 um mass diameter) which can remain suspended in air for many hours and could transmit infection to individuals at a substantial difference. Larger particles or droplets will typically fall to the ground within 3 m of the infected person and would be expected to infect individuals in direct contact. Finally, viral particles could land on surfaces, where influenza viruses remain infectious [80, 81] and could infect others through indirect contact. There is substantial evidence for all three modes of transmission in experimental studies and epidemiologic observations, but the relative roles of each mode of transmission are uncertain and remain controversial, with obvious implications for infection control practices and for potential interventions to mitigate pandemics.

Small particle aerosols are generated by infected humans, and influenza genome can be detected in these small particles by polymerase chain reaction techniques [82, 83]. It has not been proven that these aerosols contain significant amounts of infectious virus, but experimental studies in humans have shown that vary small amounts (~5 infectious particles) may be sufficient to infect humans by the aerosol route [84, 85]. Aerosol transmission has also been demonstrated in animal models in which infected and exposed ferrets or guinea pigs are separated by several meters, with transmission occurring in the direction of airflow [56, 86].

Airborne transmission has also been implicated in multiple observations of outbreaks where an airborne route of transmission appears to be the most plausible explanation for the characteristics of the outbreak. The most often cited such

outbreak occurred in a commercial airliner that was delayed for approximately 4½ h with a poorly functioning ventilation system. The risk of transmission of influenza A from the index case to other passengers was related to the amount of time passengers spent on the aircraft and not on their seating proximity to the index case. Since most of the passengers did not have direct contact with the index case, airborne transmission appears to be likely [87]. In a well-investigated hospital outbreak, nosocomial cases occurred significantly less frequently in a hospital ward where the air was treated with UV light than in an otherwise similar ward without UV light treatment [88]. In an outbreak in a long-term care facility, there appeared to be an association between the risk of nosocomial influenza and the air handling systems in several wards [89].

Additional observations consistent with airborne transmission were made during an early study of zanamivir prophylaxis of influenza in families. In this study [90], subjects who received short-term prophylaxis with inhaled zanamivir were protected compared to placebo recipients, but recipients of zanamivir administered by nasal spray were not. In addition, the combination of nasal and inhaled zanamivir was no better than inhaled zanamivir alone.

In most of these outbreaks, there are alternative explanations for the observations that could at least partially explain the epidemic behavior without requiring aerosol transmission [91], and the real role of aerosol transmission remains controversial. If aerosol transmission plays a dominant role in influenza, then health-care workers would need to wear filtering face masks, and patients would require negative pressure isolation, to prevent nosocomial transmission of influenza. This has prompted several studies that have attempted to evaluate the role of facemasks in infection prevention in hospitals. In one large, randomized trial, nursing staff who were randomly assigned to wear N-95 respirators had the same rate of influenza as staff assigned to wear simple surgical masks while caring for patients with influenza [92]. This study suggests that airborne transmission does not play a major role at least in nosocomial influenza, although it has been pointed out that cases in the N-95 group could have been acquired outside the hospital and that compliance with these masks is frequently poor. In contrast, hand hygiene and simple surgical masks were reported to be modestly effective in the prevention of influenza transmission in households [93] suggesting that in this setting, droplet spread was the predominant modality.

6 Pathogenesis and Immunity

6.1 Pathogenesis

Once virus is deposited on the respiratory tract epithelium, it can attach to and penetrate columnar epithelial cells if not prevented from doing so by specific secretory antibody (IgA), by nonspecific mucoproteins to which virus may attach, or by the mechanical action of the mucociliary apparatus. After adsorption, virus replication begins, leading to cell death by inhibiting cellular protein synthesis and disrupting other cellular functions and by inducing apoptosis. Virus release continues for several hours before cell death ensues. Released virus then may initiate infection in adjacent and nearby cells, so within a few replication cycles, a large number of cells in the respiratory tract are releasing virus and dying as a result of the virus replication. The time between the incubation period and the onset of illness and virus shedding varies from 18 to 72 h depending in part on the inoculum dose [94].

Quantitation of virus in respiratory tract specimens from otherwise healthy young adults reveals a characteristic pattern (Fig. 19.5). Virus is first detected just before the onset of illness (within 24 h), rapidly rises to a peak of 3.0–7.0 log10 TCID50/mL, remains elevated for 24–48 h, and then rapidly decreases to low titers [95]. Usually, virus is no longer detectable after 5–10 days of virus shedding. However, because of the relative lack of immunity in the young, more prolonged shedding of higher titers of virus is expected in children.

fections of healthy adults, the severity of illness correlates well with the quantities of virus shed, suggesting that a major mechanism in the production of illness is cell death resulting from viral replication. Although the clinical manifestations of influenza are dominated by systemic symptoms, viral replication is limited to the respiratory tract. Instead, systemic symptoms are probably due to the release of potent cytokines, such as type I interferons, tumor necrosis factor, and interleukins (ILs), by infected cells and responding lymphocytes [95]. In fact it has been suggested that an overly vigorous cytokine response to infection may contribute to the high fatality rate seen with H5N1 influenza [96, 97].

Bronchoscopy of individuals with typical, uncomplicated acute influenza has revealed diffuse inflammation of the larynx, trachea, and bronchi, with mucosal injection and edema [98, 99]. Biopsy in these cases has revealed a range of histologic findings, from vacuolization of columnar cells with cell loss to extensive desquamation of the ciliated columnar epithelium down to the basal layer of cells [99]. Individual cells show shrinkage, pyknotic nuclei, and a loss of cilia. Viral antigen can be demonstrated in epithelial cells [100]. Generally, the tissue response becomes more prominent as one moves distally in the airway [99]. Epithelial damage is accompanied by cellular infiltrates primarily composed of lymphocytes and histiocytes. Histologic findings on autopsy in more severe cases show extensive necrotizing tracheobronchitis, with ulceration and sloughing of the bronchial mucosa [101], extensive hemorrhage, hyaline membrane formation, and a paucity of PMN infiltration. Patients with secondary bacterial pneumonia have the changes characteristic

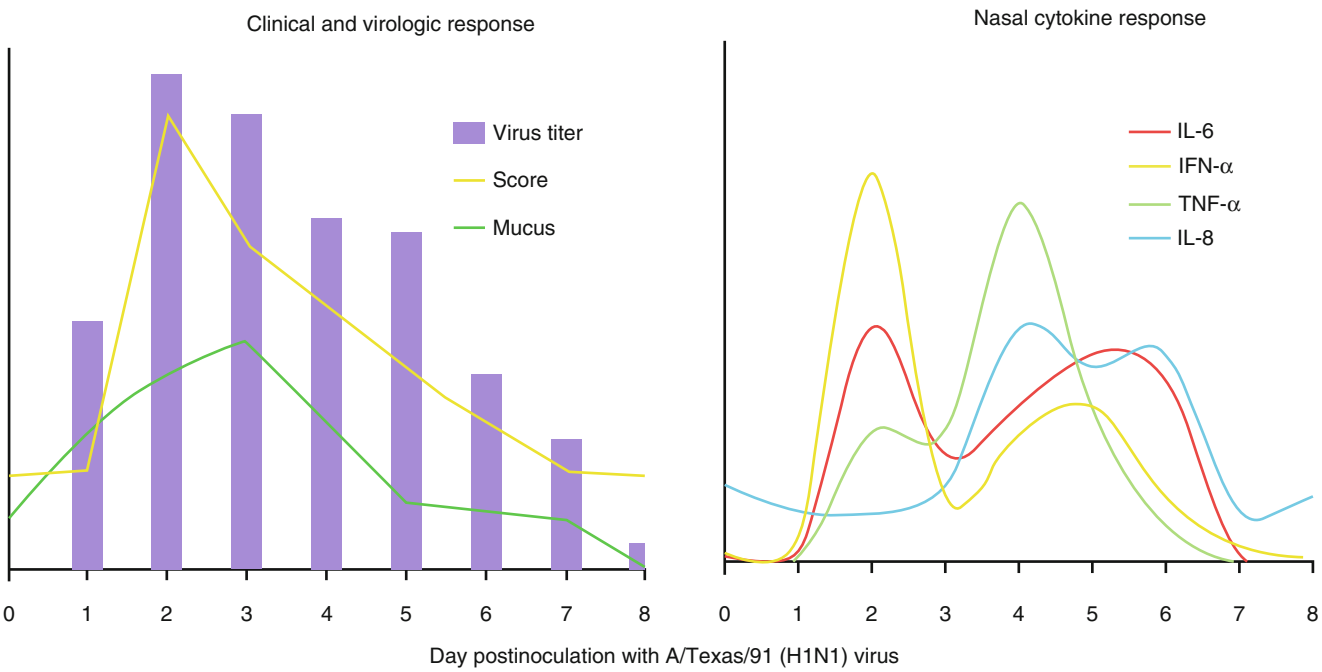

Fig. 19.5 Time course of influenza in healthy adults who were experimentally infected with influenza A/Texas/91. Symptoms are temporally correlated with the peak of virus shedding and production of a variety of cytokines (Data adapted from Hayden et al. [95])

of bacterial pneumonia in addition to the tracheobronchial findings of influenza. Recovery is associated with rapid regeneration of the epithelial cell layer and with pseudometaplasia.

Abnormalities of pulmonary function are frequently demonstrated in otherwise healthy, nonasthmatic young adults with uncomplicated (nonpneumonic) acute influenza. Demonstrated defects include diminished forced flow rates, increased total pulmonary resistance, and decreased density-dependent forced flow rates consistent with generalized increased resistance in airways less than 2 mm in diameter [102, 103], as well as increased responses to bronchoprovocation [102]. In addition, abnormalities of carbon monoxide diffusing capacity [104] and increases in the alveolar-arterial oxygen gradient [105] have been seen. Of note, pulmonary function defects can persist for weeks after clinical recovery. Influenza in asthmatics [106] or in patients with chronic obstructive disease [107] may result in acute declines in forced expiratory vital capacity (FVC) or forced expiratory volume in 1 s (FEV1). Individuals with acute influenza may be more susceptible to bronchoconstriction from air pollutants such as nitrates [108].

Primary viral pneumonia is an uncommon but frequently severe complication of acute influenza. In this situation, virus infection reaches the lung either by contiguous spread from the upper respiratory tract or by inhalation. The trachea and bronchi contain bloody fluid, and the mucosa is hyperemic [109]. Tracheitis, bronchitis, and bronchiolitis are seen, with loss of normal ciliated epithelial cells. Submucosal hyperemia, focal hemorrhage, edema, and cellular infiltrate are present. The alveolar spaces contain varying numbers of neutrophils and mononuclear cells admixed with fibrin and edema fluid. The alveolar capillaries may be markedly hyperemic with intra-alveolar hemorrhage. Acellular hyaline membranes line many of the alveolar ducts and alveoli [109]. Pathologic findings seen by biopsy of lung in nonfatal cases are similar to those described in fatal cases [110].

Bacterial superinfection is a well-recognized complication of influenza that may account for a substantial proportion of morbidity and mortality, especially in adults. For example, the frequency of pneumococcal hospitalizations has been shown to increase in association with influenza epidemics [111]. Consequently, the spectrum of disease and pathophysiology of bacterial superinfection has been studied intensively, and a number of factors have been identified in viral respiratory disease that could play a role in increasing the risk of bacterial infection. Uncomplicated influenza is associated with significant abnormalities in ciliary clearance mechanisms [112]. In addition, increased adherence of bacteria to virus-infected epithelial cells has been demonstrated [113]. The disruption of the normal epithelial cell barrier to infection and loss of mucociliary clearance undoubtedly enhance bacterial pathogenesis. In addition, influenza infection may upregulate certain cell surface receptors involved in bacterial adherence [114]. Alterations in PMNs and mononuclear cells may also contribute to enhanced bacterial infection.

6.2 Immunity

Epidemiologic and experimental observations in humans have shown that infection with influenza virus results in long-lived resistance to reinfection with the homologous virus. In addition, variable degrees of cross-protection within a subtype have been observed [115]. Infection induces both systemic and local antibody, as well as cellular immune responses, each of which plays a role in recovery from infection and resistance to reinfection (Fig. 19.6) [116].

6.2.1 Systemic Antibody Responses

Infection with influenza virus results in the development of antibody to the influenza virus envelope glycoproteins HA and NA, as well as to the structural M and NP proteins. Some individuals may develop antibody to the M2 protein as well [117]. The antibody response is more rapid after reinfection. Peak antibody responses after primary infection are seen at approximately 4–7 weeks and decline slowly thereafter. Antibody titers can sometimes be detected years after exposure, for example, persons born before 1968 frequently have detectable titers of antibody against H2 viruses. The mechanisms that allow such persistent antibody are not known.

As implied by the name, the HA protein of influenza is defined by the ability to hemagglutinate red blood cells, a function which is directly related to binding to cellular receptors. Thus, antibody that can block hemagglutination, or so-called hemagglutination-inhibiting antibody (HAI), has been studied intensively and is generally accepted as a surrogate for virus neutralization and protection against infection. Serum antibody to the HA has been demonstrated to have a protective role against influenza infection and disease in both animal models as well as in experimental infection of humans and in epidemiologic observations. An increased risk of laboratory-documented influenza among those with the lowest titers of preexposure HAI or neutralizing antibody is a consistent finding of most but not all studies. However, there is considerable uncertainty about the actual level of HA antibody that is the best predictor of protection, with estimates ranging from HAI titers of 1:8 to 1:160 or higher [118]. Given the substantial variation from laboratory to laboratory in the estimation of the HAI titer on the same set of samples [119], the inability to use an absolute value for protection is not unexpected. In addition, the amounts of antibody needed to mediate protection could vary by population, degree of exposure, age, and specific influenza type or subtype, although this has not been analyzed comprehensively.

B cells secreting HA-specific antibody that binds to the stem region rather than the head of the HA have been detected in the blood of individuals experiencing infections with novel influenza viruses such as pH1N1 or H5N1 [120, 121].

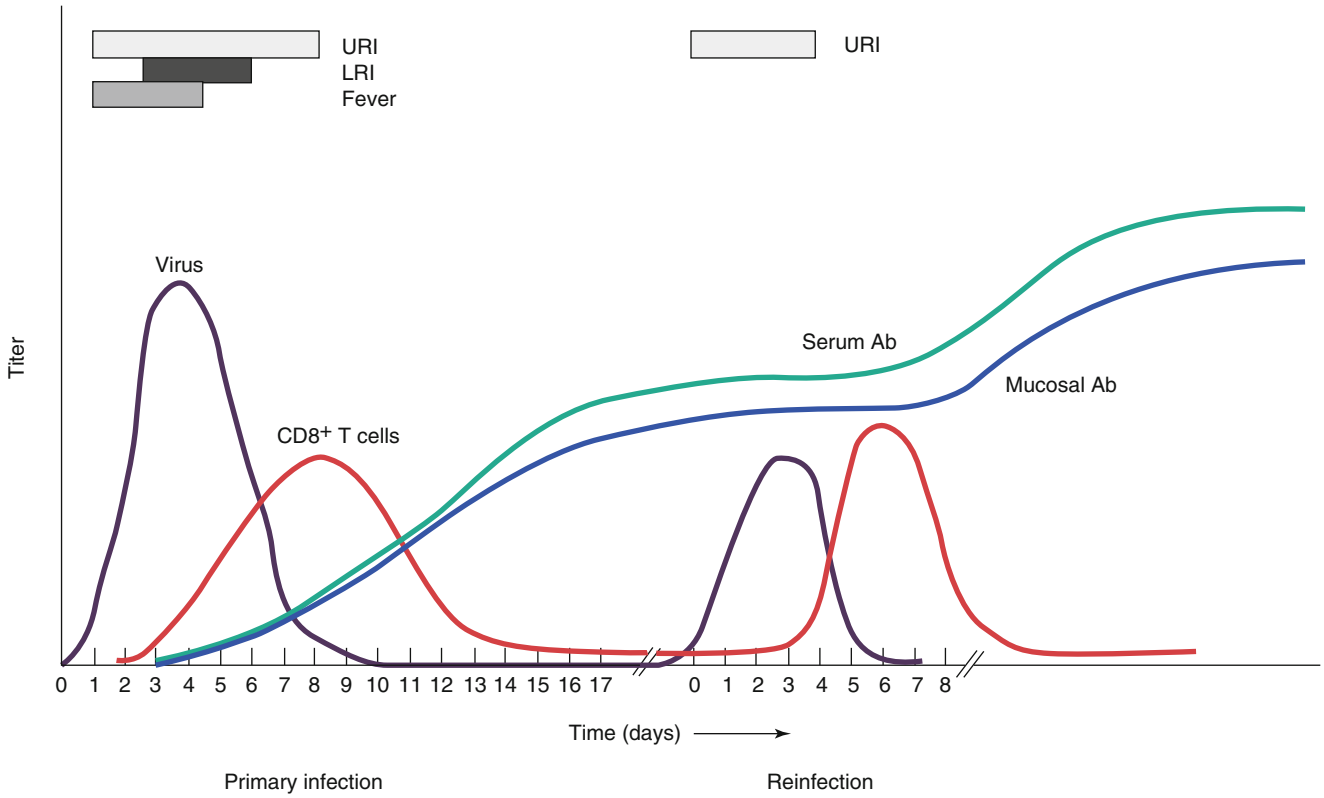

Fig. 19.6 Immune response to influenza infection and reinfection (Adapted from Subbarao et al. [116])

These antibodies exhibit neutralizing activity in some assays but do not inhibit hemagglutination. Stalk-binding antibody can be detected in serum as well [122]. Because the stalk region is well conserved, these antibodies can be cross-neutralizing among HA subtypes and have increased interest in the potential creation of a universal influenza vaccine.

In contrast to anti-HA antibody, anti-NA antibody does not neutralize virus infectivity but instead reduces efficient release of virus from infected cells, resulting in decreased plaque size in in vitro assays [123] and in reductions in the magnitude of virus shedding in infected animals [124, 125]. Observations on the relative protection of those with anti-N2 antibody during the A/Hong Kong/68 (H3N2) pandemic [26, 126], as well as experimental challenge studies in humans [127], have shown that anti-NA antibody can also be protective against disease and results in decreased virus shedding and severity of illness but that it is infection permissive [128].

Antibody to other influenza viral proteins has also been evaluated for potential protection. Antibody to M2 reduces plaque size in vitro, and passive transfer studies in mice have also suggested that antibody to the M2 protein of influenza A viruses may be partially protective if present in large enough amounts [129]. The mechanism of protection in vivo is related to mediation of antibody-dependent cytotoxicity [130]. Antibody to internal viral proteins such as M or NP is also cross-reactive among type A viruses, but they are non-neutralizing. Studies in mice have suggested that such non-neutralizing but cross-reactive antibody may mediate protection under some circumstances [131]. The mechanism by which antibody to viral proteins that are not exposed on the surface can mediate protection is unclear.

6.2.2 Mucosal Antibody Responses

Both natural viral infection and live attenuated vaccines have been found to induce significant mucosal antibody responses. Nasal HA-specific IgG is predominantly IgG_1, and its levels correlate well with serum levels of HA-specific IgG_1, suggesting that nasal IgG originates by passive diffusion from the systemic compartment [132]. Nasal HA-specific IgA is predominantly polymeric and IgA_1, suggesting local synthesis. Studies in mice and ferrets have emphasized the importance of local IgA antibody in resistance to infection, particularly in protection of the upper respiratory tract. Studies in humans have also suggested that the resistance to reinfection induced by virus infection is mediated predominantly by local HA-specific IgA, whereas that induced by parenteral immunization with inactivated virus depends also on systemic IgG [133]. Importantly, either mucosal or systemic antibody alone can be protective if present in high enough concentrations, and optimal protection occurs when both serum and nasal antibodies are present [134, 135].

6.2.3 Cellular Immune Responses

The induction of cellular immune response to influenza virus infection has been studied intensively in murine models, and such studies suggest that B cell, CD4+ T cell, and CD8+ T cell responses all can play a role in protection against disease and recovery from infection. A large number of HLA class I-restricted (CD8+ T cell) and HLA class II-restricted (CD4+ T cell) epitopes have been described, and in situations where those epitopes are on relatively well-conserved influenza proteins such as the polymerase, NP, and M proteins, the cellular responses are cross-reactive between subtypes, but not between types A and B.

Cellular immune responses to influenza vaccination and infection have not been studied as extensively in humans, but B cell (memory B cell and antibody-secreting cell), CD4+ T cell, and CD8+ T cell responses in peripheral blood have been described after infection or vaccination [136]. It can be difficult to capture the peak of the response and detectable increases in antigen-specific cells may only be seen on a few days after exposure. Generally, the peak cellular response occurs somewhere between 5 and 14 days depending on the status of the subject and the nature of the response. As seen in murine models, a major component of the cellular response is directed at conserved peptides to which the subject has already been exposed during previous infections or vaccinations.

Antibody-secreting cells (ASC) appear in blood and tonsils as early as 2 days after vaccination and are detected in the blood of adults and older children more frequently than in young children after immunization [137]. An increase in cytotoxic T lymphocytes, directed primarily at the conserved internal proteins, has been shown in healthy adults with a peak at 14 and 21 days after vaccination and return to baseline at 6 months. An increase in HA-specific CD8+ T cells on day 7 after vaccination has also been detected by tetramer staining in adults receiving inactivated influenza vaccine [138].

An important role of the cellular immune response in recovery from influenza infection in humans is strongly supported by the observation of prolonged illness and viral shedding in individuals who are lymphopenic as a result of disease or chemotherapy. However, it has been difficult to develop specific markers of T cell immunity as correlates of protective immunity. Activated T cells, in the form of granzyme B-positive T cells, have been associated with protection in older subjects [139]. In the human challenge model, early studies identified the early induction of virus-specific cytotoxic T cells as measured by cytochrome release assays as correlated with reductions in the duration and level of virus replication in adults [140]. In a subsequent study done many years later by the same group, prechallenge CD4+ T cells, but not CD8+ T cells, correlated with relatively lower levels of viral shedding and symptoms following experimental

infection [141]. In a large study of the efficacy of live attenuated vaccine in children, it was shown that higher levels of influenza-specific T cells assayed by gamma-interferon ELISPOT was associated with a decreased risk of PCR-documented influenza [142]. The development of more sophisticated markers that can specifically identify reactive cells in peripheral blood will help to define the role of cellular immunity in protection, but the field remains limited by the lack of convenient access to compartments other than peripheral blood in humans.

7 Patterns of Host Response

Typical uncomplicated influenza often begins with an abrupt onset of symptoms after an incubation period of 1–2 days. Many patients can pinpoint the hour of onset. Initially, systemic symptoms predominate, including feverishness, chilliness or frank shaking chills, headaches, myalgia, malaise, and anorexia. Usually, myalgia or headache is the most troublesome symptom, and the severity is related to the height of the fever. Respiratory symptoms, particularly cough and sore throat, are usually also present at the onset of illness. The predominance of systemic symptoms is a major feature distinguishing influenza from other viral upper respiratory infections. Older adults may simply present with high fever, lassitude, and confusion without the characteristic respiratory complaints, which may not occur at all. Generally there is a wide range of symptomatology in healthy adults, ranging from classic influenza to mild illness or asymptomatic infection.

Two manifestations of pneumonia associated with influenza are well recognized: primary influenza viral pneumonia and secondary bacterial infection. The syndrome of primary influenza viral pneumonia was first well documented in the 1957–1958 pandemic, predominantly among persons with cardiovascular disease, especially rheumatic heart disease with mitral stenosis, and to a lesser extent in others with chronic cardiovascular and pulmonary disorders [109]. The illness begins with a typical onset of influenza, followed by a rapid progression of fever, cough, dyspnea, and cyanosis. Secondary bacterial pneumonia classically presents after a brief period of improvement [143, 144], although most patients do not fit this classic pattern. Bacteria frequently associated with influenza include *Streptococcus pneumoniae* or *Haemophilus influenzae* and, notably, an increased frequency of *Staphylococcus aureus*. An increased frequency of community-acquired, methicillin-resistant *S. aureus* has been seen in children and adults following influenza outbreaks [145].

In addition to pneumonia, other pulmonary complications of influenza include croup [146] and exacerbations of chronic bronchitis or asthma [147, 148]. Recognized non-pulmonary complications include myositis and myoglobinuria [149], myocarditis and pericarditis [150, 151], toxic shock syndrome [152, 153], Guillain-Barré syndrome and transverse myelitis [154], and Reye's syndrome particularly in children who have been given aspirin to treat fever.

8 Control and Prevention

8.1 Antiviral Drugs

Two classes of antiviral drugs have been used clinically to treat and prevent influenza. The adamantanes amantadine and rimantadine are related primary symmetrical amines whose mechanism of action involves inhibition of the M2 ion channel activity of susceptible viruses. The function of the M2 ion channel in viral replication is to acidify the interior of the virion, disrupting the interaction between the matrix and nucleoproteins and allowing the ribonucleoproteins to be transported to the nucleus, where replication occurs [155]. Similar ion channels have been described for influenza B and C viruses; however, at clinically achievable levels, these drugs are active against only influenza A.

Amantadine and rimantadine are effective in the therapy of both experimentally induced and naturally occurring influenza A, with more rapid decrease in fever, more rapid improvement in symptoms, and decreased shedding of virus [156, 157]. Rimantadine has also been shown to be effective in the treatment of influenza A in children [158].

Drug resistance has been a factor in limiting the use of these antiviral agents. Resistant viruses emerge frequently in treated individuals, particularly children, in whom subpopulations of resistant virus can be detected following treatment in virtually all cases [159]. Resistance is the result of single point mutations in the membrane-spanning region of the M2 protein, and it confers complete cross-resistance between amantadine and rimantadine [160]. Resistant virus can be transmitted to, and can cause disease in, susceptible contacts. Prolonged shedding of resistant viruses may occur in immunocompromised patients, particularly children, and may continue even after therapy is terminated [161], consistent with the relative fitness of these resistant viruses. While previously rare, a rapid increase in the prevalence of de novo resistance to M2 inhibitors was noted in 2005, and essentially all H3N2 viruses are now resistant to these agents [162, 163]. Although previously circulating seasonal H1N1 viruses remained sensitive to these agents, the emerging pH1N1 viruses are also completely resistant to the M2 inhibitors, which now lack activity against all strains of influenza virus currently circulating.

The neuraminidase inhibitors act by inhibiting the functioning of the influenza virus neuraminidase, which is critical in allowing newly formed viruses to egress from the cell

and spread to other cells [164]. The two licensed inhibitors, zanamivir and oseltamivir, have shown very similar results in clinical trials. Both drugs reduce the duration of symptoms and enhance the return to normal activities when used within the first 36 h of symptoms in otherwise uncomplicated influenza in healthy adults [165–168]. Both drugs have also been shown to be effective in reducing the duration and severity of influenza in children [169, 170].

Antiviral resistance to these agents has also been detected both in treated and untreated individuals. Mutations within the catalytic framework of the NA that abolish binding of the drugs have been described [171, 172]. The specific mutations conferring resistance are dependent on the specific NA, that is, common resistance mutations in N1 (e.g., H274Y) are different than the ones seen in the N2 (e.g., R292K or E119V) or influenza B (e.g., D198N). In addition, depending on the location of the mutation, these viruses may be specifically resistant to only one inhibitor [173]. Resistance mutations in the NA may be associated with altered characteristics of the enzyme with significantly reduced activity [174, 175].

Some resistant viruses appear to have reduced fitness, with reduced levels of replication, attenuation in animals, and reduced ability to be transmitted from animal to animal [176–179]. Drug resistant viruses were also isolated very infrequently from oseltamivir-treated individuals in clinical trials, being seen in less than 2 % of treated adults and detected in 5.6 % of children [169]. However, subsequent studies have demonstrated that resistant viruses can be detected in up to 18 % of treated children when using sensitive PCR techniques to pick up minor subpopulations [180].

Beginning in 2006, spontaneously resistant H1N1 viruses carrying the H274Y mutation began to be detected in viruses from individuals who did not have a history of exposure to oseltamivir [181]. By 2008, all H1N1 viruses isolated in the United States were resistant to oseltamivir. The mechanism that led to the selection of seasonal H1N1 clades resistant to oseltamivir as the predominant circulating H1N1 viruses is unclear. However, the emerging pH1N1 virus has remained largely susceptible to oseltamivir.

8.2 Vaccines

Two types of influenza vaccines are currently licensed and are used in various countries, inactivated influenza vaccines (IIV) and live attenuated influenza vaccines (LAIV). Shortly after these vaccines were introduced, it was recognized that their efficacy might depend on the antigenic match between the strain(s) contained within the vaccine and the circulating viruses [182], and since that time, the vaccines have been continuously reformatted to keep pace with the ongoing evolution of influenza viruses. Since 1977, influenza vaccines have contained a representative A/H3N2, A/H1N1, and B virus, so-called trivalent influenza vaccine. As mentioned above, two antigenically distinct lineages of influenza B viruses have cocirculated in humans since 2004, and quadrivalent formulations of vaccine are currently being considered.

8.2.1 Safety

IIV are chemically inactivated and have been administered either as the so-called "whole-virus" preparations or as detergent-disrupted and partially purified "split product" or "subunit" vaccines. Hundreds of millions of doses of IIV are administered each year, and the safety of these vaccines has been repeatedly confirmed. For example, no increase in clinically important medically attended events has been noted among over 251,000 children <18 years of age who were enrolled in one of the five health maintenance organizations within the Vaccine Safety Datalink, the largest published post-licensure population-based study of vaccine safety [183]. The most common adverse events reported following immunization with IIV are tenderness and/or pain at the injection site. Most injection site reactions are mild and rarely interfere with daily activities. Systemic reactions following immunization of adults with inactivated vaccine are uncommon. In placebo-controlled clinical trials in younger and elderly adults, rates of systemic reactions were similar among groups given inactivated vaccine or placebo [184, 185]. However, whole-virus IIV are associated with fever in children [186] and are not recommended in this age group.

Recently, an increased frequency of fever and febrile seizures was observed among young children given one specific IIV during the 2010 influenza season in Australia [187]. The reasons for this unexpected reactogenicity are unclear, but preliminary studies have suggested that this vaccine preparation stimulated unusually high cytokine responses in in vitro assays. In addition, concomitant immunization of young children with IIV and pneumococcal conjugate vaccine (PCV) was shown to be associated with an increased risk of developing febrile seizures.

Immediate hypersensitivity reactions (hives, wheezing, angioedema, or anaphylactic shock) following inactivated vaccine can also occur, and vaccine is considered contraindicated for persons who experienced a previous anaphylactic reaction following vaccine. Clinical protocols have been proposed to administer inactivated vaccine to persons who are at high risk for severe or complicated influenza who also have a history of immediate hypersensitivity to eggs, if the benefit of immunization is judged to outweigh the risk [188].

Several unusual syndromes have been associated with IIV. The Guillain-Barré syndrome (GBS), an acute inflammatory demyelinating polyneuropathy, was associated with the 1976 swine influenza vaccination campaign, with an increased risk of approximately 1 per 100,000 vaccinees. More recent studies have suggested a statistically significant

but very slight increased relative risk of GBS within 7 weeks of influenza vaccination [189]. The oculorespiratory syndrome (ORS) is a syndrome of red eyes, facial edema, and/or respiratory symptoms such as coughing, wheezing, sore throat, hoarseness, difficulty breathing, or chest tightness that develop within 2–24 h after vaccination, associated with a specific influenza vaccine used in Canada, but not elsewhere [190]. The specific mechanism underlying this phenomenon is unknown.

Although not studied as extensively, LAIV also appear to be quite well tolerated. Nasal symptoms (runny nose, nasal congestion, or coryza) and sore throat have been the most frequently identified adverse symptoms following LAIV. Children under 8 have had slightly increased rates of low-grade fever, runny nose, and abdominal symptoms in the 7 days following vaccination compared to placebo recipients. However, when considering all the pediatric studies in aggregate, no consistent symptom was significantly more common in LAIV compared to placebo recipients. In older children, 11 to <16 years of age, sore throat was observed slightly more frequently in LAIV recipients than IIV recipients.

In larger studies, wheezing has been associated with LAIV in young children, although occurring at low rates. In the largest trial, medically significant wheezing within 42 days of vaccination was reported in 3.8 % of children <2 years old after receipt of LAIV compared to 2.1 % in those who received IIV [191]. Wheezing generally occurs in the youngest, previously unvaccinated children following the first dose of vaccine. Because of this observation, LAIV is currently approved for use in the United States for children ≥2 years old who do not have a history of asthma.

LAIV can be recovered from nasal secretions of about half of adult recipients, although generally shedding of LAIV by adults is of low titer and short duration [192]. Although young children shed much higher levels of vaccine virus, no transmission of LAIV from vaccine recipients to susceptible contacts was detected in studies of young children involved in day-care-like settings where LAIV and placebo recipients played together for up to 8 h a day for 7–10 days after vaccination. In the largest study, 197 children between 8 and 36 months of age were randomized to receive LAIV or placebo, and vaccine virus shedding was assessed for 21 days after vaccination. Although 80 % of LAIV recipients shed at least one vaccine strain, for a mean of 7.6 days, clear evidence of transmission was detected in only one placebo recipient [193].

8.2.2 Efficacy and Effectiveness

The ability of influenza vaccines to prevent influenza has been assessed in numerous clinical studies which vary greatly in design, populations, and endpoints. These studies have included prospective, randomized controlled studies, in which case they are referred to as efficacy studies, as well as a wide variety of nonrandomized cohort and retrospective study designs which assess vaccine effectiveness. Endpoints evaluated in these studies have included both laboratory-confirmed influenza and non-laboratory-confirmed respiratory illnesses. In this regard, it has been recognized that studies that utilize a serologic definition of influenza infection may overestimate the efficacy of influenza vaccine, since it will be harder to demonstrate postvaccination to post-season antibody increases in the vaccinated group [194].

Randomized studies of IIV efficacy against laboratory-confirmed influenza have mostly been conducted in healthy adults. These studies have shown a wide range of efficacy, from approximately 40–80 %, with lower levels of efficacy typically seen in years with apparent antigenic mismatch. A recent meta-analysis of 8 randomized, controlled trials in healthy adults during 2004–2008 estimated the pooled efficacy of IIV against culture-confirmed influenza to be 59 % (95 % CI 51–67) among those aged 18 through 64 years [195]. The role of antigenic mismatch in the efficacy observed in these trials is unclear, and some studies in young adults have demonstrated high levels of efficacy (76 %) despite a degree of antigenic mismatch. Recent studies using virus culture and/or PCR endpoints have demonstrated similar levels of efficacy for both egg-grown and cell culture-grown IIVs [196].

Relatively few placebo-controlled trials of the efficacy of LAIV have been conducted in adults. In the human challenge model, cold-adapted and inactivated influenza vaccines were of approximately equal efficacy in the prevention of experimentally induced influenza A (H1N1), A (H3N2), and B. The combined efficacy in preventing laboratory-documented influenza illness due to the three wild-type influenza strains was 85 % for LAIV [197]. In a randomized, controlled study in healthy persons aged 1 through 64 years, of whom most of the participants were adults, the efficacy of a pre-licensure, bivalent preparation of LAIV for preventing culture-confirmed influenza A illness in adults was 85 % (95 % CI 70–92 %) for H1N1 and 58 % (95 % CI 29–75 %) for H3N2 [198]. LAIV was also evaluated in a large study against clinical endpoints performed in 4,561 healthy working adults [199]. In this study, the effectiveness of LAIV-T in preventing severe febrile respiratory illness of any cause during the influenza season was 29 %.

Relatively few recent prospective trials have assessed IIV efficacy in children. In one randomized, controlled trial in healthy children aged 6 through 23 months, vaccine efficacy was 66 % (95 % CI 34–82) in the first year, but efficacy could not be assessed in the second year due to a very low influenza attack rate (efficacy −7 %: 95 % CI −247–67) [200]. Immunization of asthmatic children has also been shown to reduce the incidence of influenza. More recently, the efficacy of IIV against PCR-confirmed influenza was assessed in a randomized, placebo-controlled trial in healthy children

between the ages of 6 and 72 months [201]. The efficacy of IIV against all influenza strains was 43 % compared with the placebo group.

LAIV was demonstrated to be efficacious in the prevention of influenza in a pivotal 2-year, randomized placebo-controlled trial conducted in 1,314 children aged 15 to <72 months [202]. The efficacy against culture-confirmed influenza illness in the first year of this trial was 95 % (95 % C.I. 88–97 %) for influenza A/H3N2 and 91 % (95 % C.I. 79–96 %) for influenza B. In the second year of the trial, the H3 component of the vaccine (A/Wuhan/93) was not a close match with the predominant H3 virus that season, A/Sydney/95. However, the efficacy of LAIV against this variant was 86 % (95 % C.I. 75–92 %) [203]. Overall, the efficacy of LAIV to prevent any influenza illness during the 2-year period of surveillance in this field study was 92 % (95 % C.I. 88–94 %). The overall efficacy of LAIV against culture-confirmed influenza among children 6 to <36 months who were attending day care was recently shown to be 85 and 89 % in the first and second year of the study, respectively [204]. Significant protection against flu-associated acute otitis media also was demonstrated (>90 % in both years). Studies done in Asia have reached similar conclusions, with an efficacy of LAIV compared to placebo of between 64 and 84 % over multiple seasons, depending on the antigenic match with the vaccine [205].

Although annual vaccination of elders and other high-risk persons has been recommended for many years, there are very few randomized trials demonstrating efficacy in these groups, in part because the existing vaccine recommendations make it difficult to do studies using a placebo group. In the most commonly referenced study, TIV was 52 % (95 % CI 29–67) efficacious in preventing serologically documented influenza illness in a population of adults 60 years of age and older [184]. When the groups were further stratified by age, efficacy estimates against serologically documented influenza illness were 57 % (95 % CI 33–72 %) in those 60 through 69 years old but only 23 % (95 % CI −51–61 %) in those ≥70 years old.

In a recently reported randomized, double-blind, placebo-controlled clinical trial of LAIV among community-dwelling ambulatory adults ≥65 years old, the overall efficacy of LAIV against viruses that were antigenically similar to the vaccine was 42 % [206], similar to the protection seen with inactivated vaccine. However, LAIV is not currently licensed for use in individuals over 49 years of age.

Monitoring influenza vaccine efficacy on an annual basis by conducting randomized placebo-controlled studies would clearly be a very difficult undertaking and is probably not possible in children, elders, and other high-risk groups. Therefore, a number of observational study designs have been used for this purpose. Many recent studies have utilized a test-negative, case–control design, in which individuals meeting a particular case definition are tested for influenza using a highly sensitive and specific diagnostic test, and the vaccination exposure of test-positive cases and test-negative controls is determined [207]. Large surveillance networks for this purpose have been established in Canada, the United States, Europe, and Australia for purposes of making interim and end-of-season estimates of vaccine effectiveness.

Studies using this design have shown variable results with estimates generally ranging from as low as 20 %, or in some cases, no effectiveness, to as high as 70 % [195]. While the various networks vary in their study design and the specific selection criteria for subject inclusion, a few overall generalizations can be stated. Failure to detect VE has typically occurred in studies with very low prevalence of influenza in the study population, or in years with substantial antigenic mismatch between the vaccine and circulating strains, most often involving influenza B lineage mismatch. The relationship of antigenic mismatch with vaccine effectiveness for influenza A/H1N1 and A/H3N2 viruses is not as consistent, but even in situations of antigenically matched viruses, VE remains in the 50–60 % range [208]. In some cases, viruses recovered from subjects in studies with low vaccine effectiveness have been shown to have substantial changes on a HA sequence level despite appearing well matched by traditional HAI tests [209].

Most studies have not enrolled enough subjects in a single season to make age-specific estimates of VE. However, there is a trend towards decreased VE in elderly, not surprising given their diminished immune response to vaccination. After accumulating cases over several seasons, it was recently possible to use the same test-negative case–control design to demonstrate VE of approximately 60 % against influenza-related hospitalization in a population of community-dwelling older adults [210].

While the use of a study design in which testing is performed without knowledge of vaccination status may eliminate some biases related to health-care access and health-seeking behavior, the results are influenced by the accuracy of the diagnostic testing, since errors in assignment to the case or control group will bias VE towards nil. Recently, in a study done in children, it was demonstrated that using the test-negative case–control approach, estimates of VE were substantially higher when children with documented infections with viruses other than influenza were used as a control group, rather than using all children who were test negative for influenza [211].

A larger body of data exists from nonrandomized or observational studies of vaccine effectiveness. These studies have suggested that influenza vaccination can reduce pneumonia and influenza (P&I) hospitalizations and death among the elderly regardless of whether they have other conditions that place them at high risk for complications following influenza [212]. While post-licensure observational studies are

important tools for monitoring vaccine effectiveness, such studies relating to the elderly are particularly challenging to perform and interpret. Frailty selection bias (a higher baseline risk of hospitalization and death among unvaccinated vs. vaccinated subjects) and nonspecific endpoints may overestimate vaccine effectiveness in cohort studies [213].

Infants less than 6 months of age are at substantial risk for influenza-related morbidity but are too young to receive influenza vaccine. One strategy to protect vulnerable infants is maternal immunization, with protection mediated by both transfer of maternal antibody and reduced potential for contact with an influenza-infected mother. In a randomized study of maternal immunization, infants born to mothers immunized with influenza vaccine had substantially lower rates of laboratory-documented influenza in the first 6 months of life than did infants born to mothers immunized with pneumococcal vaccine [214]. Similarly, in a retrospective case–control study, the frequency of influenza immunization was substantially lower in the mothers of infants hospitalized with PCR-confirmed influenza than in mothers infants hospitalized who were PCR negative, with an estimated protective effect of 92 % [215].

There has been considerable interest in potential strategies to protect such individuals indirectly by preventing illness and viral transmission within highly susceptible populations that probably play a role on community transmission, such as schoolchildren. Several studies have suggested that this may be possible. During the 1968 pandemic, it was observed that the incidence of respiratory illness during the period of influenza A circulation was among unvaccinated adults was substantially lower in a community in which schoolchildren were immunized than in a comparison community with no school immunization. Influenza B was not contained in the vaccine, and during a subsequent influenza B epidemic, there was no difference in adjusted respiratory illness rates in adults in the two communities [216]. In a recent study, closed agricultural communities of Hutterites were randomized to vaccination of schoolchildren with influenza vaccine or with hepatitis A vaccine as a control. In the subsequent influenza A epidemic, the rate of laboratory-documented influenza A in unvaccinated adults residing in school-vaccinated communities was reduced by 61 % (95 % CI 8–83 %) compared to adults in unvaccinated communities [217]. Observations in Japan, where it appeared that substantial fluctuations in overall influenza-related mortality (occurring mostly in the elderly) were directly related to the rate of school-aged influenza vaccination, also support a potential role for school vaccination in protection of elders [218].

8.2.3 Adjuvants

For many years, licensed inactivated influenza vaccines have been administered without adjuvants. However, it is clear that the immune response to vaccination and the protective effects of vaccine are suboptimal, particularly in some groups at high risk for influenza-related complications including young children and elders. Thus, there has been significant interest in the potential use of adjuvants to enhance the protective effects of vaccination. Although influenza vaccines adsorb well to aluminum salts, these compounds have not been effective adjuvants for influenza, for unknown reasons. Early studies suggested that water in oil emulsions using mineral oil could very substantially improve antibody responses in military recruits allowing the use of lower doses [219]. However, the use of these adjuvants was restricted by substantial local side effects including the development of sterile abscesses at the site of administration; [220] the use of peanut oil appeared to reduce the observed toxicity [221].

Subsequently, oil-in-water emulsions based on the metabolizable oil squalene have been shown to substantially improve immune responses with an excellent safety profile. While there is relatively little effect on the immune response to seasonal vaccines in healthy adults [222], the oil-in-water emulsion MF59 results in an approximately 50 % increase in antibody titers in older adults [223], and MF59 adjuvanted seasonal IIV has been licensed for use in elders in Italy for several years. Recently, a large randomized trial in young children compared MF59-adjuvanted IIV with unadjuvanted IIV over two seasons. Absolute efficacy against all influenza strains was 86 % in the group given IIV-MF59 and 43 % in the group given unadjuvanted IIVs when compared with the placebo group [201].

Oil-in-water emulsions have demonstrated especially striking improvements in the antibody responses to candidate H5 pandemic vaccines. These studies have shown higher titers of antibody against the vaccine virus, as well as against antigenic variants, the development of B cells that recognize a larger variety of HA epitopes, and broadened and more vigorous CD4 T cell responses [224, 225].

8.2.4 Comparisons of Inactivated and Live Influenza Vaccines

While relatively few randomized direct comparisons of the efficacy of live and inactivated vaccines have been performed, the available studies are consistent with the observed effects of age and prior influenza experience on immunogenicity. When these vaccines have been compared in young children 12 months through 59 months of age, LAIV has shown consistently superior protection, with an approximately 50 % greater protective efficacy than inactivated vaccine [191, 226]. Studies conducted in healthy young children have generally concluded that LAIV may be more efficacious than IIV, including both against viruses which are well matched antigenically to the vaccine virus and those which are antigenically drifted [191].

In contrast to young children, studies that have directly compared the vaccines in adults have suggested that the

vaccines have similar efficacy or that IIV vaccine is slightly more efficacious than live vaccine. In one 3-armed study, the efficacy of LAIV compared to placebo for prevention of laboratory-confirmed influenza in healthy adults was 57 %, while the efficacy of the IIV was 77 %, but the difference between the two vaccines was not statistically significant [227]. In a subsequent season in the same population, the absolute efficacies of IIV and LAIV were 68 and 36 %, respectively [228]. Similar results have been reported from a retrospective study evaluating the effectiveness of IIV and LAIV against medical visits for pneumonia- and influenza-related diagnoses in the US military [229]. Generally, rates of such visits were lower in recipients of IIV, except for personnel who had not been vaccinated in previous years, in which there was not apparent difference in the effectiveness of the two types of vaccines. In a recent effectiveness study that included both LAIV and IIV recipients, there was no clear-cut difference in effectiveness between the two vaccines [208].

9 Unresolved Problems

Despite many years of research and vast energies devoted to uncovering its mysteries, influenza remains an enigma and effective strategies for its control are elusive. Influenza vaccines, including unadjuvanted inactivated vaccines of various formulations and live attenuated vaccines based on cold-adapted master donor viruses, have clearly been demonstrated to reduce the risk of influenza in selected populations. However, these vaccines have never been shown to have any substantial impact on the overall disease burden of influenza. In part, this is a problem of inconsistent supply and limited uptake, compounded by the need for annual reformulation and administration, but in part this also reflects our growing recognition of the limited effectiveness of these vaccines and an incomplete understanding of the nature of protective immunity.

Development of more effective vaccines, perhaps to include broadly cross-protective vaccines, is an obvious goal. The specific types of immune responses that could contribute to more effective vaccination are unclear, and the best strategies for generating these responses remain to be determined. Many exciting new discoveries have recently been revealed, and multiple innovative approaches for new vaccines and adjuvant systems are underway. Each of these pathways will need to carefully balance safety and efficacy in the context of the existing very safe but modestly effective options.

Antiviral agents are another strategy to mitigate the impact of seasonal and pandemic influenza, but their real utility in this disease has also been quite limited. The rapid emergence of resistance is a major obstacle facing effective use of antiviral strategies, but the basic pathogenesis of influenza creates an extremely narrow time window for antiviral intervention in most cases, further complicating antiviral therapy. Novel approaches that focus on modifying the host or the host innate immune response may offer one alternative that might overcome some of these problems.

Although it should be recognized that the cumulative disease burden of seasonal influenza is generally larger than that of pandemic influenza, the threat of a severe influenza pandemic represents another unresolved issue in the field. Extensive efforts have been devoted to comprehensive surveillance for novel influenza viruses and potential pandemic threats in a wide variety of domestic and wild animal populations, and our understanding of the ecology of influenza is expanding rapidly. However, our ability to actually predict the emergence of any specific pandemic remains extremely limited.

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Noroviruses, Sapoviruses, and Astroviruses

20

Ben A. Lopman, Jan Vinjé, and Roger I. Glass

1 Introduction

Acute gastroenteritis (AGE) is among the most common diseases of humankind affecting people of all ages and particularly those at the extremes of life: children and the elderly. In developing countries, AGE remains one of the most important causes of death in children [1] and has been associated with a cycle of malnutrition and problems of impaired neurocognitive development [2]. In developed countries, the disease is an important cause of doctor visits and hospitalization of children, and a less frequent but persistent problem for adults and the elderly, all at appreciable medical cost [3, 4]. Prior to 1970, an etiology could be specified for fewer than 15 % of episodes of AGE, but since that time, more than 50 different infectious agents and toxins have been identified to cause the disease. This wealth of new information has challenged investigators and public health professionals to understand the relative importance of each agent, assess its contribution to human health, and consider prospects for prevention and control. Control has focused on understanding routes of transmission of each pathogen and determining whether public health interventions could interrupt their spread or investigating whether natural immunity to repeat infection might provide an opportunity to consider prevention with vaccines. Many of the infectious agents of AGE have been discovered in epidemics where a common illness has led to the identification of a single point source of infection. However, patients with these infections also present individually as "sporadic cases" in clinics and hospitals, where despite careful questioning, their illnesses cannot be linked to an outbreak or to other common cases or exposures.

Noroviruses and sapoviruses belong to two separate genera of the family *Caliciviridae* [5]. These single-stranded RNA nonenveloped viruses are highly infectious agents transmitted through a variety of routes including person-to-person; contact with fecally contaminated food, water, or environmental surfaces; or possibly via airborne droplets. In the United States, noroviruses are now recognized to be the most common cause of outbreaks of AGE, the most common cause of sporadic AGE across all ages, and the most common cause of foodborne disease [6]. They are also an important cause of healthcare-associated infections in both long-term and acute care settings. In addition to accounting for the high burden of AGE, noroviruses are often detected in people without the occurrence of symptoms, particularly among people in resource-poor settings [7]. Immunity is complex and incompletely understood but generally regarded to be short-lived [8]. The sapoviruses, which initially were thought to cause AGE in children, are now recognized to cause outbreaks in people of all age groups including the elderly [9]. Astroviruses are non-enveloped, single-stranded RNA viruses in the family *Astroviridae*. Although astroviruses have been detected in all age groups, most infections are in children <2 years of age and tend to be relatively mild, rarely requiring hospitalization [10].

Over the past two decades, this new appreciation of the major burden of norovirus and sapovirus AGE has been brought about by laboratory advances in the molecular detection of these viruses and their genetic characterization. In the 1990s, the prototype Norwalk virus and its close relative, Southampton virus, were cloned and sequenced [11, 12],

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paving the way for the development of new molecular diagnostic tests based upon RT-PCR detection and sequencing of virus directly from fecal specimens [13–15]. Furthermore, expression of the capsid proteins meant that serologic assays could be performed with antigens that could be replenished in the laboratory [16] rather than relying on immunoassays that required fecal specimens for antigen and paired sera collected from human volunteers. Genetic factors associated with resistance to infections and acquired immunity both play a role in susceptibility to infection and disease. With this in mind, and because of the great difficulty in controlling most outbreaks through routine public health interventions, vaccines for norovirus are currently a priority for development.

Unfortunately, there are currently few specific interventions to prevent the spread of these viruses. Management of outbreaks is based on identifying, where possible, the means of spread, be it from person to person or from fecally contaminated food and water, and then intervening with control measures to interrupt that spread. Treatment is supportive and based on oral rehydration solution (ORS) or intravenous fluid replacement in cases of severe dehydration. Recent advances have led not only to major changes in our recognition of the importance of these viruses in human health but also novel approaches to their control through public health measures and the potential future use of vaccines.

2 Historical Background

The history of noroviruses and our understanding of the viruses that cause AGE track directly with the development of modern diagnostic methods to detect and characterize these viruses. Each advance in diagnostics has furthered our understanding of the epidemiology of the virus and expanded our appreciation of the role that these viruses play in human health. Consequently, over the past 40 years, noroviruses have emerged from being an obscure and rarely detected viral cause of outbreaks of AGE to become the most common cause of these outbreaks and of sporadic episodes of AGE in developed countries and a common pathogen in developing countries. In the 1940s, investigators suspected viruses to be possible causes of AGE because volunteers administered bacteria-free stool filtrates from patients suffering from AGE developed the disease [17]. Unfortunately, they were unable to isolate a pathogen. Most episodes and outbreaks of AGE, one of the most common illnesses of humans, went without an etiologic diagnosis and were defined with descriptive terms – diarrhea of weaning, travelers' diarrhea, winter vomiting disease, or idiopathic diarrhea. The first major breakthrough occurred in 1972 when Kapikian discovered a virus-like particle by immune electron microscopy (IEM) in the fecal specimen of victims linked to an outbreak of AGE at a school in Norwalk, Ohio, which had been investigated by CDC epidemiologists 4 years earlier [18]. The discovery of the Norwalk virus opened a golden era for electron microscopists to discover new viral pathogens in fecal specimens and use IEM to characterize the patient's immune response in acute and convalescent sera. A stream of other enteric viruses were then discovered – rotaviruses, astroviruses, "classic caliciviruses," as well as antigenically distinct variants of Norwalk virus, named according to the location where first identified (e.g., the Hawaii agent, the Snow Mountain agent, the Sapporo virus, the Taunton agent) [19–21]. Other viruses were seen in fecal specimens in the absence of a corresponding serologic response, a *sine qua non* of a true infection – parvoviruses, reoviruses, enteroviruses, and picobirnaviruses. Thus began the journey to discover the relevance of each of these viruses to cause disease and to public health. Viruses found in stool were first classified by their morphologic appearance by EM. Noroviruses were first classified simply as "small round-structured viruses" (SRSVs) to distinguish them from small round viruses (SRVs) without a distinct surface structure such as parvovirus and enterovirus. Differentiating between variant SRSVs required IEM using human paired sera specimens, but few research groups had the human reagents or skills needed to distinguish one SRSV from another.

Following their discovery, the Norwalk-like viruses began to be linked to the majority of outbreaks of AGE where another bacterial or parasitic pathogen could not be found [22]. The frequency of detection depended initially upon the skill of the microscopist. Because no animal model for norovirus infection was available, a long series of human volunteer challenge studies was conducted to assess immunity to infection [23, 24] and cross-protection between strains [25] and to develop novel immunoassays using fecal specimens and paired acute and convalescent sera from human volunteers. Virus was often not seen in fecal specimens from patients directly implicated in outbreaks. Therefore, serologic assays, both radioimmunoassay (RIA) and an enzyme immunoassay (EIA), were developed using human reagents (stool for antigen and paired sera) to monitor the immune responses of those involved in outbreaks [26, 27]. At CDC, for more than a decade, these assays became the diagnostic tests of choice [28]. The application of serology greatly increased the number of outbreaks of AGE that were attributed to the Norwalk virus. Of note, however, was that some people with documented exposure to the contaminated food or water did not become ill or seroconvert, suggesting a role for innate or acquired immunity. Also, some people involved in outbreaks of norovirus did not seroconvert to stool filtrates from Norwalk volunteers; however, they did respond to human reagents developed from volunteers challenged with an inoculum from patients infected with antigenically distinct Norwalk-like viruses such as the Snow Mountain agent

or the Hawaii agent suggesting serotype differences in susceptibility and immunity to disease [25]. A laboratory diagnosis of norovirus was rarely sought in patients routinely hospitalized for diarrhea because no simple diagnostic test was commercially available, and few had a history of exposure in an outbreak. Noroviruses remained almost exclusively agents of outbreaks of AGE and were not associated with sporadic illness leading to hospitalization [28].

All this changed in the early 1990s when the Norwalk and Southampton viruses were cloned and sequenced [11, 12, 29], an advance that revolutionized the field. These discoveries led immediately to the development of molecular assays that were more sensitive, specific, and reproducible than electron microscopy and that used chemical reagents that could be produced or purchased rather than biological reagents obtained from human volunteers. Molecular characterization immediately placed the Norwalk virus and the Norwalk "family of viruses," i.e., the other SRSVs, into a separate genus, *Norovirus*, in the family *Caliciviridae*. The Sapporo virus and related viruses with typical calicivirus morphology were grouped in their own genus *Sapovirus* [5]. Knowledge of the sequence meant that individual strains could be identified and traced within and between outbreaks allowing for the identification of common source exposures [30, 31]. Furthermore, strains previously characterized simply by the location where they had been found, or studied for antigenic differences by IEM, could now be sequenced and classified into genogroups and genotypic clusters [32]. When reverse transcription polymerase chain reaction (RT-PCR) was applied to examine fecal specimens from a large collection of outbreaks of AGE in the United States, Europe, Australia, and Japan, which had gone without an etiologic diagnosis, more than 80 % could be attributed to a norovirus, making it the most common cause of outbreaks of AGE [28, 33, 34]. Knowledge of sequence allowed new studies of the molecular epidemiology of the virus and facilitated tracing transmission of a single strain to a common source, such as fecally contaminated shellfish or foods prepared by a single sick food handler [30, 35, 36]. Similarly, outbreaks caused by foods irrigated with sewage (e.g., raspberries transported in a worldwide distribution chain) had a mixture of viruses representing multiple sources of contamination [37, 38].

The cloning of a number of HuCVs cleared the way for their single capsid protein to be expressed in different vectors [11, 12, 29]. When the Norwalk virus capsid gene was introduced into a baculovirus expression system, it formed viruslike particles (VLPs) that were indistinguishable by EM from natural virus [11]. VLPs could be used as antigens in immunoassays to monitor the immune response to specific infections, to differentiate between strains of virus, and to consider as potential vaccines [39]. They also provided targets to study the structure of related viruses and consider novel targets for drugs. Noroviruses can genetically be grouped into at least six different genogroups of which viruses from GI and GII viruses being the most common [6, 40]. GII viruses have caused the majority of all norovirus outbreaks over the past decade worldwide [41].

Traditionally, public health laboratories studied noroviruses almost exclusively in the context of outbreaks reported to them. However, AGE is responsible for both mild disease leading to doctor and clinic visits and severe disease leading to hospitalizations and some deaths [3, 42, 43]. That few of these patients are directly linked to an outbreak suggested a role for noroviruses as a cause of sporadic AGE. A national cohort study in England was the first to widely apply RT-PCR for noroviruses to study patients with AGE in the community [44–46]. Norovirus was identified as the most common cause of disease among adults and second only to rotavirus as the causative agent among children. A study of the etiology of AGE in adults hospitalized or treated in the emergency room in three sites in the United States yielded similar results: noroviruses were the most common cause of diarrhea in US adults [47]. With the advent and widespread use of rotavirus vaccine in the United States, norovirus has become the most common cause of medically attended gastroenteritis [48]. Application of molecular diagnostics have now demonstrated noroviruses and sapoviruses to be common in children and adults as well and a major cause of AGE in low- and middle-income countries [49].

In a parallel trajectory, astroviruses were first discovered in 1975 through electron microscopic examination of stools from an outbreak of pediatric diarrhea [50, 51]. The modern standard for laboratory diagnosis is real-time RT-PCR [52, 53]. In the pediatric population, astroviruses are consistently associated with acute diarrhea, though at substantially lower levels than the noroviruses [54].

3 Epidemiology

Noroviruses are now recognized to be one of the most common pathogens causing AGE in a wide range of settings, ages, and risk groups. In the United States, they are the most common cause of AGE in the community, the most common cause of outbreaks of AGE, and the most common cause of foodborne disease outbreaks [3, 55, 56]. Following the introduction of routine rotavirus immunization in the U.S., noroviruses are now the most common cause of pediatric gastroenteritis requiring medical care [48]. They also cause the great majority of outbreaks of AGE in institutional settings such as nursing homes, hospitals, and chronic care facilities in industrialized countries [57]. Sapoviruses are a less common cause of AGE in all age groups and in a wide variety of settings as well. For norovirus, sapovirus, and astrovirus, our more complete appreciation of the burden of disease has developed over the last decade, largely due to the availability of more sensitive and specific diagnostic tests.

3.1 Methods for Epidemiological Analysis

3.1.1 Epidemics and Outbreak Investigation

Data from the United States and Europe have demonstrated that norovirus is responsible for approximately 50 % of all reported AGE outbreaks (range 36–59 %) [58]. These generally occur throughout the year, although there is a seasonal pattern of increased activity during the winter months (Fig. 20.1) [58]. Outbreaks occur in various settings; they are propagated by nosocomial transmission in hospitals and nursing homes, foodborne spread in restaurants and aboard cruise ships, and waterborne outbreaks, all affecting people of all ages. Although initial reviews of norovirus outbreaks in the United States implicated contaminated food as the main vehicle of infection [59], newer reports suggest that the majority involve person-to-person transmission [55, 60–62]. Moreover, given the high infectivity and environmental stability of noroviruses, transmission during outbreaks may involve multiple routes [63], and contaminated fomites, surfaces, and droplets may also act to perpetuate outbreaks [64–66].

HuCV infections often come to the attention of public health authorities in the form of outbreaks, and these events present an opportunity to learn about transmission of the virus. In the United States, thousands of outbreaks have been identified and investigated over the past four decades, and a few have provided critical clues to understand transmission. The original Norwalk virus outbreak occurred in an elementary school and provided important initial observations. It was clear that the virus was highly infectious with primary and secondary attack rates of 50 and 30 %, respectively [18, 67]. The incubation period was determined to be short at <48 h, and occurrence in both students and teachers implied a lack of protective immunity with age. Other outbreak investigations from the 1980s demonstrated that norovirus could be transmitted by drinking water [10], as well as recreational water use [68]. Transmission by contaminated oysters was demonstrated to be an important route of transmission, as were a range of other foodborne exposures (e.g., to leafy greens, raspberries, and foods prepared by ill food handlers) [69–72]. The role of norovirus in healthcare facilities, including nursing homes, also becomes apparent from these early investigations [73]. Outbreak investigation led to the observation that norovirus can also be transmitted directly by apparent airborne or droplet spread [74–77], not only directly from person to person. More recent investigations have provided compelling evidence of environmentally mediated transmission [78, 79] and even by unusual fomites such as reusable shopping bags [80, 81]. An other outbreak started from a contaminated food product, subsequently spread by person to person among players on opposing football teams, and went on to attack the roommates of the players several days later; it demonstrated the potential for multiple transmission routes in a single outbreak [82]. While astroviruses primarily cause sporadic disease, outbreaks have been reported in a range of settings and may be a fairly common cause of nosocomial gastroenteritis in children's hospitals [83].

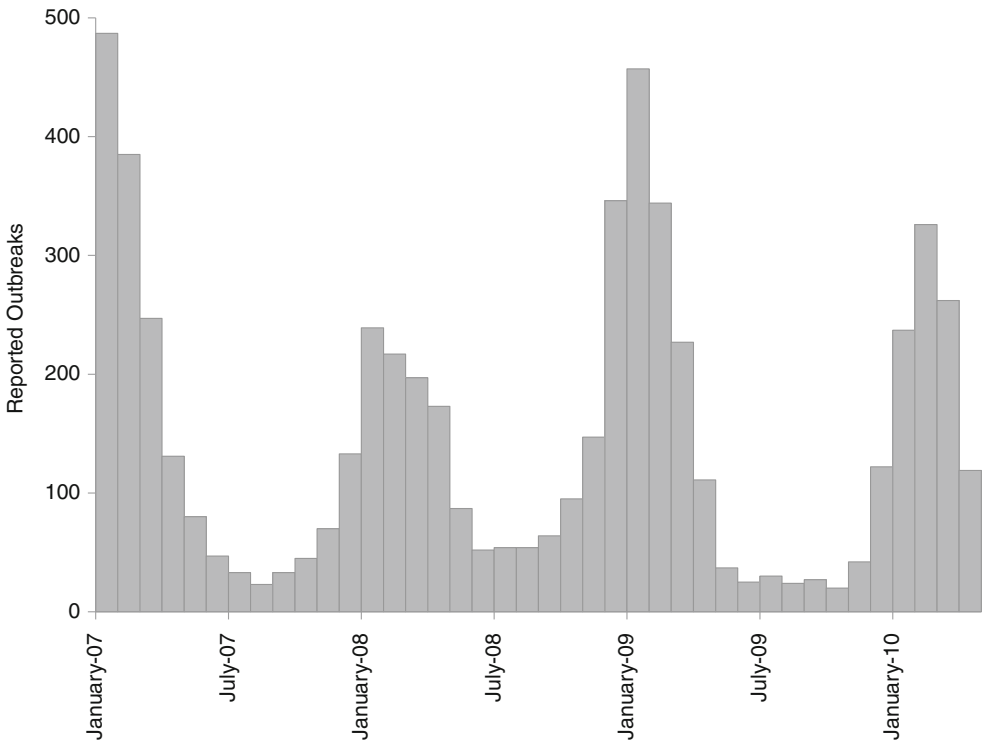

Fig. 20.1 Suspected and confirmed norovirus outbreaks reported by 30 US states, January 2007–April 2010 (Adapted from Yen et al. [55])

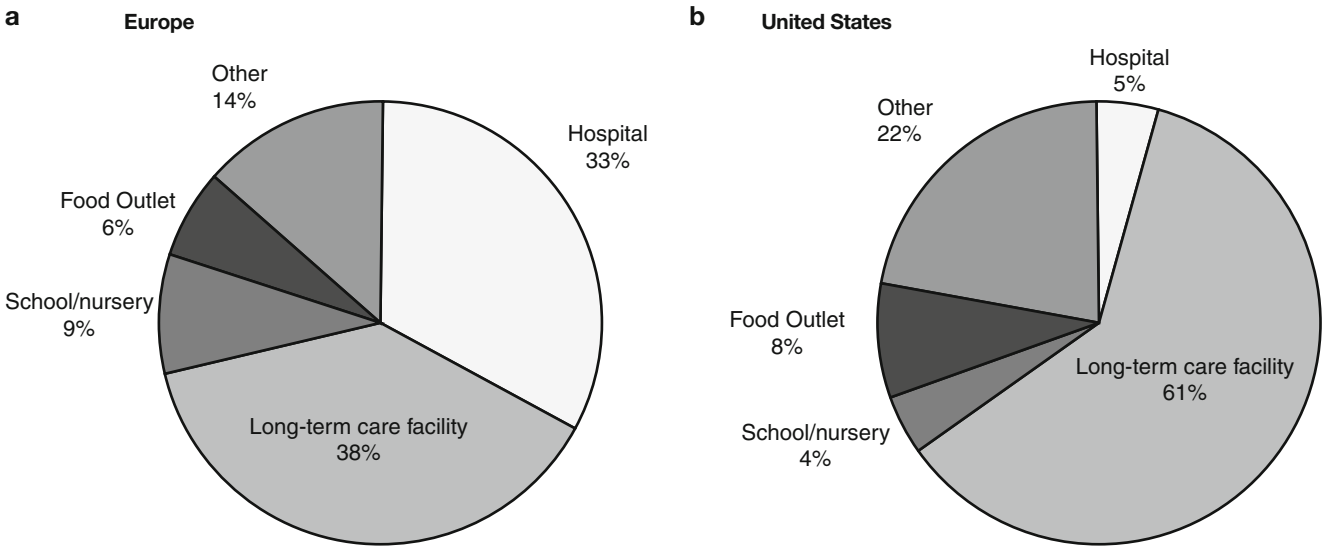

Fig. 20.2 Setting of reported outbreaks of norovirus AGE in (**a**) Europe, 2004 [33], and the (**b**) United States, 2006–2008 [89]. In both settings, the majority of outbreaks occur in healthcare settings; in Europe many more outbreaks are reported from acute care hospitals

Periodically, the number of norovirus outbreaks has increased at the same time that new genogroup II genotype 4 (GII.4) strains have emerged, likely because these new variants escape immunity in the population [84, 85]. These emergent GII.4 strains rapidly replace circulating strains and can sometimes cause unusually severe seasons, as occurred in 2002/2003 and 2006/2007 [41, 86, 87]. GII.4 variants predominate across all settings, though the role of GI and other GII genotypes appears to be greater in settings that involve foodborne or waterborne transmission compared to the GII.4 viruses [88].

A major difference in the reported epidemiology of norovirus between the United States and most other high-income countries lies in the frequency of outbreaks in acute care (hospital) settings. In Europe approximately one-third of reported outbreaks occur in hospitals compared with 4 % in the United States (Fig. 20.2) [90]. It is not known whether this difference in reported outbreaks represents a real difference in the epidemiology of outbreaks, an underreporting of hospital outbreaks in the United States, or differences in infection control practices in hospitals.

Recently, more sophisticated statistical and modeling studies have used data from traditional outbreak investigations to examine issues of virus transmission. Transmission models have been fit to demonstrate the important role that vomiting plays in the spread of norovirus [91]. By using methods that reconstruct the most likely transmission trees [92], Sukhrie and colleagues found that in healthcare settings, people with symptoms are far more likely to transmit infection than those who remain asymptomatic, and patients are more important to virus transmission than staff [93, 94].

Outbreaks of sapovirus-associated AGE have been reported in settings similar to those of noroviruses – schools [95], child care facilities, [96] hospitals [97], long-term care facilities, and occasional foodborne outbreaks [98, 99]. The broad age range of individuals affected in these outbreaks demonstrates that sapovirus infection is not restricted to young children as was previously thought. Although the molecular epidemiology of sapovirus is less well studied than norovirus, strains belonging to four different sapovirus genogroups (GI, GII, GIV, GV) have been observed to infect humans. Two of these viruses, GI.2 and GIV, have been most commonly associated with outbreaks in Europe, Asia, and North America primarily affecting older adults [9, 100, 101].

Similar to the norovirus and sapovirus, astrovirus outbreaks may occur in a range of settings including long-term and acute care facilities, schools, and child care centers [102]. However, such outbreaks appear to be much less common than those caused by norovirus.

Few studies have quantified the direct healthcare or societal costs due to the noroviruses, but given how common these infections are, the direct healthcare costs and the indirect costs to society are likely to be substantial. Most studies to date have only quantified the cost of outbreaks, as opposed to endemic disease, for which the cost must be much greater. For example, an outbreak in a single 946-bed US hospital cost an estimated $650,000 [103]. During the 2002–2003 season, the cost to the English National Health Service of nosocomial AGE outbreaks was estimated at $184 million [57]. Norovirus foodborne disease in the United States costs an estimated $2 billion annually [4].

3.2 Surveillance

There are some major limitations to what can be learned from individual outbreak investigations since published reports likely represent a biased sample of events in terms of disease severity [104, 105] and provide limited insight into the efficacy of control measures and the full burden of disease [106]. In most countries, AGE from norovirus is not a notifiable cause of disease; surveillance has focused on the recognition of outbreaks, many of which would not be reported in the peer-reviewed literature. What constitutes an outbreak can be hard to define; as a result, the data collected by any given surveillance system may in part reflect the definitions used, the size of the outbreaks reported, and the focus of the surveillance effort. For example, in the United States, outbreaks linked to food historically were prioritized for reporting, whereas in England and Wales, surveillance targeted outbreaks occurring in National Health Service hospitals, thereby emphasizing nosocomial spread [107]. When more broad-based assessments have been conducted, the profile of outbreaks in the United States has been consistent with those from other industrialized countries where a majority occur in healthcare settings and are spread by person-to-person transmission [55]. In response to this surveillance gap, CDC has recently developed the National Outbreak Reporting System (NORS) as an integrated national surveillance system for all enteric disease outbreaks [108]. Launched in February 2009, NORS now provides the framework through which all outbreaks of enteric disease, regardless of transmission mode, may be reported from state and local health departments to the CDC. CDC also coordinates a norovirus outbreak surveillance network known as CaliciNet, also launched in 2009 [109]. State and local public health and food regulatory agency laboratories upload sequences of norovirus outbreaks to allow rapid comparison to potentially link outbreaks with a common (e.g., food) source as well as to identify emerging norovirus strains (e.g., GII.4 Sydney in late 2012).

Large reviews of surveillance datasets have been published from a number of countries including England and Wales, [61, 110] some European countries (as part of the Foodborne Viruses in Europe group) [33, 90], Australia, and New Zealand [111, 112]. These broad-based surveillance systems have consistently demonstrated that the majority of reported outbreaks occur in healthcare settings (including nursing homes or hospitals) and are predominantly spread from person to person while at the same time identifying an important role for food in disease transmission. Systems dedicated to the surveillance of outbreaks in healthcare settings have shown a large burden of disease, in both acute and long-term care settings, as well as a high degree of severe disease and economic burden [112, 113]. Regional or state-wide surveillance reports have also been useful for understanding patterns of disease when national surveillance systems are incomplete or do not exist [114, 115].

3.2.1 Etiologic Studies and Endemic Disease

Globally, norovirus is estimated to account for 12 % (95 % CI 9–15 %) of community- or clinic-based AGE cases and 11 % (95 % CI 8–14 %) of emergency department- or hospital-based cases [49]. These proportions are similar in developing and developed country populations [49]. In the United States, norovirus causes an estimated 21 million cases of AGE [3], 1.7 million outpatient visits [116], 400,000 emergency care visits, 70,000 hospitalizations [42], and 800 deaths annually across all age groups (Fig. 20.3) [43]. The burden of hospitalizations and death surges by approximately 50 % in those years when novel GII.4 variant strains emerge [86, 87, 117]. Although symptomatic norovirus infections are usually mild and self-limiting in otherwise healthy adults, they may be fatal among the elderly [118] and immunocompromised persons [119]. Excess mortality associated with norovirus has been documented in a number of countries as well [120, 121].

Etiologic studies of norovirus can be grouped into two categories: (1) community-based cohort/case–control studies or (2) healthcare facility-based studies, with the former being much less common. Community-based cohort studies are expensive and logistically complicated to conduct, but when conducted, have established norovirus to be the most common cause of AGE in the community [46, 54, 122]. In England and the Netherlands, these studies have estimated the incidence of norovirus in the general population to be 4.1 and 4.6 cases per 100 person-years [46, 54, 122], with regional studies providing generally consistent results [116, 123]. This means that with a life expectancy of ~80 years, a person will experience an average of three to five episodes in their lifetime. Incidence is approximately five times higher in children under the age of 5 years [46]. The incidence of norovirus at the general practitioner (primary care) level has been estimated at 0.49 per 100 person-years in England and 0.62 in northwest Germany, suggesting about one in ten who are ill with AGE seeks medical care [46, 54, 123].

Community-based studies of the incidence of sapoviruses have been less common. In England and Wales, the incidence of sapovirus-associated illness in the community was estimated at 2.6 cases per 100 person-years [54]. Estimates from a study of a Healthcare Management Organization population in the state of Georgia yielded similar results, 9 and 1 cases per 1,000 population, respectively [116]. A prospective study of Finnish children <2 years of age reported sapoviruses in 9 % of sporadic gastrointestinal illness compared with rotavirus in 29 % and norovirus in 20 % [124].

As noted earlier, astroviruses are also comparatively less common than norovirus. In the England and Wales study, incidence across the age range (at 0.5 per 100 person-years)

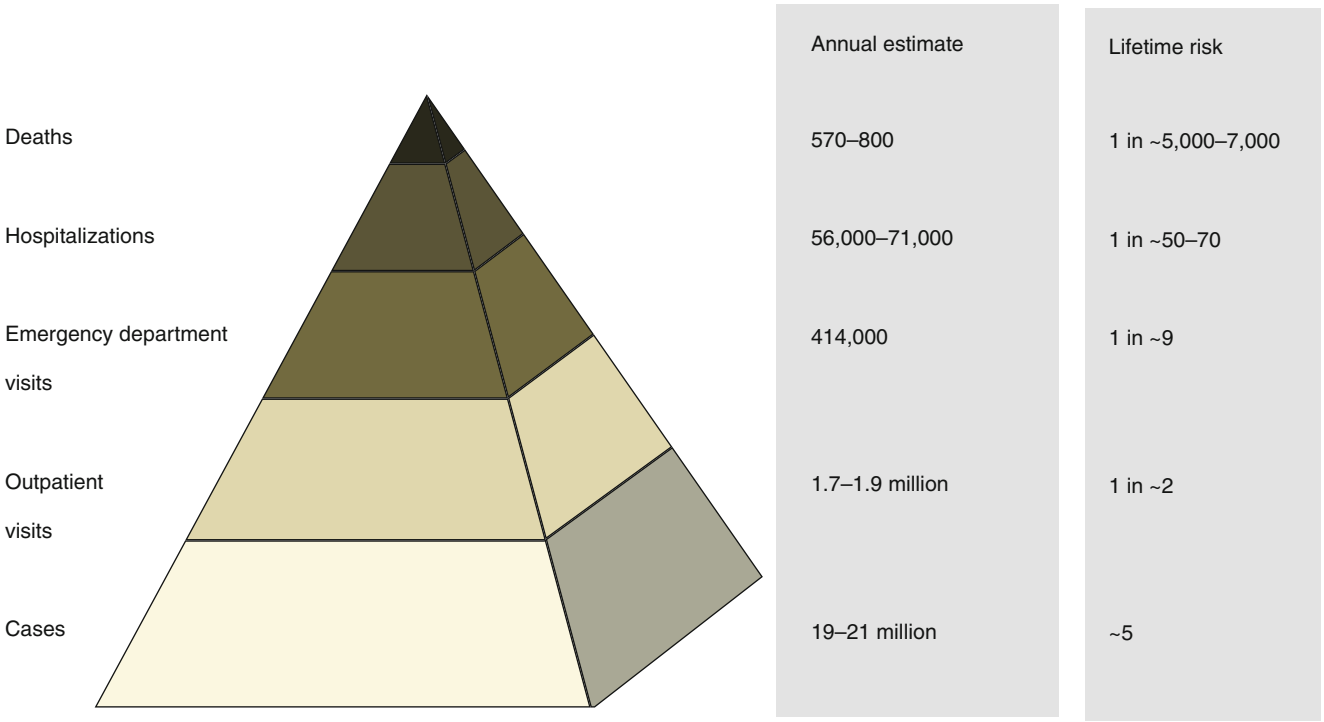

Fig. 20.3 Annual cases, outpatient visits, emergency room consultations, hospitalizations, and deaths from norovirus annually in the United States. Lifetime risk estimate based on a life expectancy of 80 years and US population of 300 million [125]

was about one-tenth and one-fifth of the incidence of norovirus and sapovirus, respectively, in 2008/2009, while in a recent study in the United States, the classical human astroviruses caused about one-fourth of the norovirus cases [54, 126]. Astroviruses are usually detected in <10 % of young children treated for gastroenteritis in outpatient clinics or in hospitals [127, 128].

The only nationally comprehensive estimate of the burden of disease due to human caliciviruses and the associated disability-adjusted life years (DALYs) exists for the Netherlands. This assessment included the incidence of cases in the community that did not seek healthcare, those visiting a general practitioner, hospitalizations and deaths that were derived from cohort studies, and surveillance data and literature. In total, these yielded an estimate of 1,622/100,000 (95 % CI 966–2,650) disability-adjusted life years in a population of 16.5 million [129]. This burden is similar to that of disease due to *Salmonella spp*. in the same population. While endemic disease-related costs have not been comprehensively assessed, norovirus-associated hospitalizations specifically have been estimated at nearly $500 million annually in the United States [42].

3.2.2 Severe Disease Outcomes

Although norovirus AGE is typically a mild self-resolving illness in healthy adults, it can lead to severe dehydration, hospitalization, and, in rare cases, death. These severe outcomes are more common in vulnerable populations such as young children and the elderly residing in long-term care facilities (LTCFs) or hospitals [105, 107]. In nearly all instances of norovirus-associated death reported in the literature, the decedents had other serious underlying heath conditions, such as immunosuppression [130–133]. Data from passive surveillance systems and outbreak investigations provide estimates of norovirus case hospitalization in the range of 0.1–5 hospitalizations per 1,000 cases [61, 107, 110, 112, 134]. A large systematic review of published outbreak reports estimated norovirus-associated hospitalization and mortality rates at 7 and 0.7 per 1,000 cases, respectively [105]. However, severity may be overestimated when based on published outbreak reports since the larger and more severe outbreaks are more likely to be investigated and reported.

It has been challenging to isolate the risk factors leading to hospitalization and death because the mode of transmission, population affected, and genotype causing the outbreaks are highly correlated. Specifically, GII.4 outbreaks are predominant in healthcare facility outbreaks, which affect vulnerable individuals (i.e., the elderly) and are typically spread from person to person [135, 136]. A review of over 800 outbreaks has highlighted that hospitalizations and deaths were much more likely to occur in healthcare outbreaks and, somewhat surprisingly, in GII.4 virus-associated outbreaks, independent of those factors that could be analyzed. This suggests that in addition to increased vulnerability of certain population groups, there is increased severity

associated with GII.4 viruses. GII strains are shed at higher levels [137], may be more likely to induce vomiting [138], and cause more severe disease in children; [139] this genogroup thus appears to demonstrate a consistent pattern of higher virulence. The relatively high hospitalization rates in long-term care facilities and mortality in all healthcare settings underscore the vulnerability of populations affected by outbreaks in these settings.

3.2.3 Risk Factors

Consistently, the strongest risk factors for community disease are proxies for contact with an infectious person. For both young children and older children/adults, reporting a symptomatic household member, especially a child, is a strong predictor of disease [122, 123, 140, 141]. It appears that young children frequently bring infection into the household, and older children/adults acquire many of their infections within the household [141, 142]. Foreign travel is also a risk factor; [141, 143] the increased risk may be attributable to changes in behavior while traveling or exposure to a different spectrum of norovirus strains. Although outbreak investigations frequently attribute norovirus AGE to contamination of food during preparation by a range of mechanisms and in a range of settings, food-related risk factors have not shown consistent associations with disease in community-based studies [122, 141]. In fact, consumption of raw fruits and vegetables, often considered potential vehicles for transmission, is generally associated with a protective effect [141]. Other potential factors such as recreational water exposure and animal contact have also been associated with reduced risk [122, 123, 141]. Taken together, the unexpected relationships observed in these studies suggest that these putative risk factors are correlated with other lifestyle factors that may actually be protective against norovirus or that frequent exposure results in immunity. The foods consistently associated with disease are oyster and other shellfish harvested from areas where the seabeds are contaminated with sewage [141, 144]. However, in most populations, consumption of these products is not common, and this exposure likely accounts for only a small fraction of disease.

4 Mechanisms and Routes of Transmission

Norovirus is extremely contagious, with an estimated infectious dose as low as 18 viral particles [145]. In contrast, approximately five billion infectious doses are contained in each gram of feces during peak shedding. Humans are the only known reservoir for human norovirus infections. Transmission occurs via fecal-oral and vomit-oral pathways by four general routes: direct person-to-person, foodborne, waterborne, or through environmental fomites (Fig. 20.4). Because humans are the only known reservoir for human norovirus, sapoviruses, or astroviruses, in a sense, all transmission is ultimately person to person. From that perspective, foodborne, waterborne, and environmental transmission are "special cases" of person-to-person spread.

4.1 Direct Person-to-Person Spread

Direct person-to-person transmission is believed to be the primary mode of spread in most outbreaks [55, 61] and in sporadic disease [141, 146]. The proportion of outbreak spread primarily by person-to-person transmission is highest in settings with close contacts. Direct person-to-person transmission is reported as the primary route in >90 % of norovirus outbreaks in hospitals, long-term care facilities, and schools [61]. GII and, specifically, GII.4 viruses are more commonly associated with person-to-person transmission [62] or found in settings where person-to-person transmission is common [135, 136]. Furthermore, there is a strong wintertime seasonality of person-to-person outbreaks [55, 147–149], a pattern not clearly seen for norovirus spread by other routes of transmission [61]. However, when new strains of GII.4 emerge by escaping population immunity, aberrant seasonal patterns may occur, [41, 87, 150, 151] highlighting the importance of host factors (i.e., population immunity) in transmission [152]. The most consistently identified risk factors for transmission are all related to the exposure of a symptomatic contact (see Sect. 3.2.3). Although infection in asymptomatic individuals is common, the importance of these shedders in person-to-person spread is unclear. However, based on current evidence, it appears that disease symptoms, especially vomiting, are fundamental to disease transmission [91, 93, 94, 153].

4.2 Foodborne Disease

Norovirus is the most common cause of foodborne disease outbreaks in the United States [154, 155], accounting for >50 % of foodborne disease outbreaks with known causes reported to CDC during 2006–2008 [154, 155]. Foodborne transmission most frequently occurs by contamination from infected food handlers during preparation and service. Only a small dose of virus is needed to cause infection, and thus infected food handlers can contaminate large quantities of product, especially when they put their hands into large-volume liquids (e.g., salad dressing), which allow for mixing. In one example, an estimated 3,000 cases of AGE were traced to icing prepared by an ill baker and put on a variety of bakery products [71]. Unlike with direct person-to-person transmission, the role of both pre- and post-symptomatic shedding has been clearly linked with onward transmission

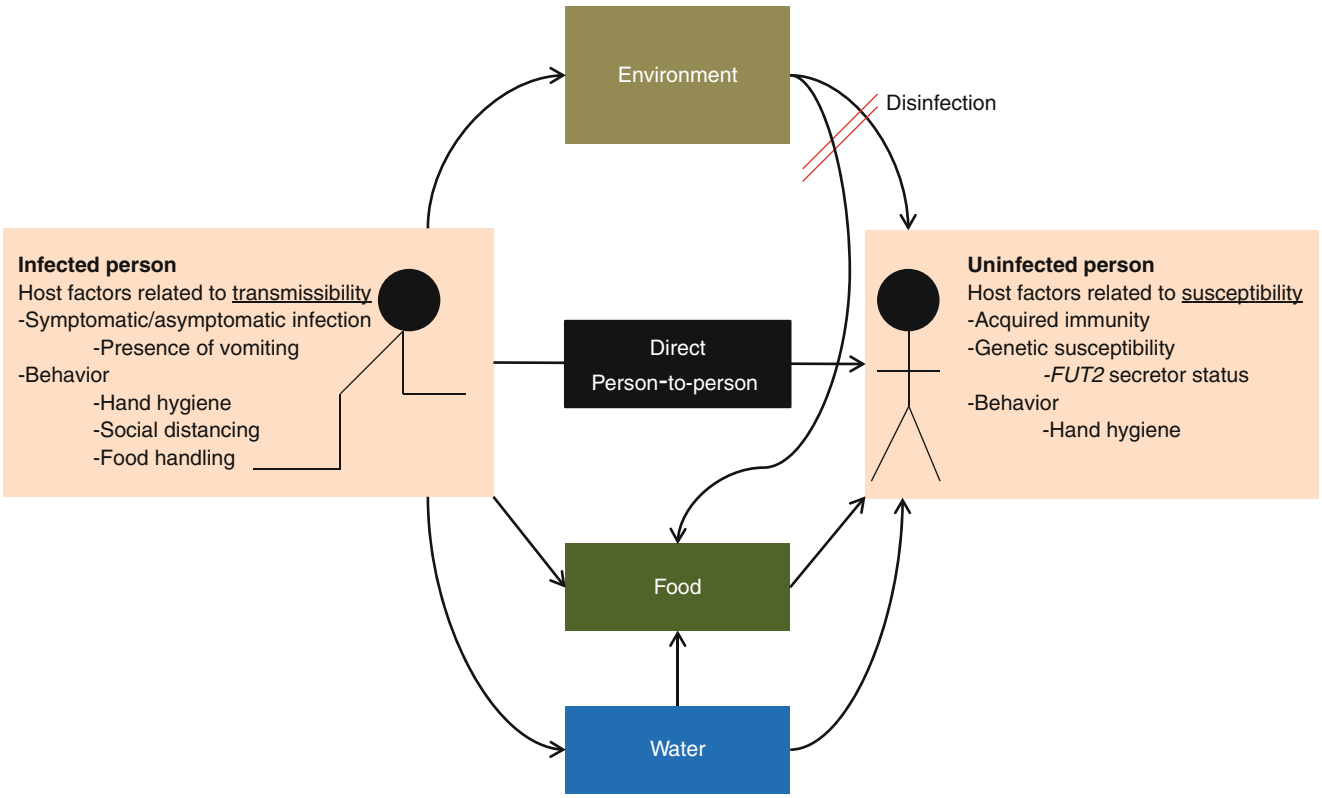

Fig. 20.4 Routes of transmission of norovirus from infected to uninfected people (Reproduced with permission from Current Opinion in Virology [7]). Norovirus transmission can occur via a range of transmission routes. Characteristics and behaviors of the infected host and potential susceptible individuals may mitigate the risk of transmission. This simple schematic is not meant to depict all the intricacies of each pathway but rather to highlight the interaction of the various routes and to illustrate that all pathways require shedding of virus from infectious hosts. Different control measures may be targeted at each *arrow*; here, the role of environmental disinfection is highlighted. Certain practices (such as hand hygiene) may reduce transmission through all pathways, while targeted interventions (such as exclusion of ill food handlers from work) may reduce transmission through specific pathways

[156–158]. Foodborne astrovirus outbreaks have been reported, [159] though this route of transmission, relative to person-to-person spread, is not well defined.

Food can become contaminated with norovirus at any point along the farm-to-fork continuum, including production, processing, and preparation. Thus, a variety of products have been implicated in outbreak investigations, especially those foods irrigated with or grown in fecally contaminated water and eaten raw, such as leafy vegetables, fruits, raspberries, and shellfish [31, 108, 154, 160–163]. Because gross sewage contamination will contain a collection of viruses circulating in the community, multiple norovirus genotypes are often detected in such outbreaks.

4.3 Environmental Transmission

Many factors may facilitate environmental transmission of norovirus. They include a large human reservoir, [3, 46, 116, 122] high levels of shedding [164, 165] (which can be asymptomatic and prolonged [164, 166, 167]), small infectious dose, [145] and widespread contamination by vomitus [65, 77, 91, 164, 165, 167–169]. The viruses are relatively stable in the environment, [149, 167, 170] resistant to disinfection, [171, 172] and can contaminate a range of fomites [65, 165, 168, 173, 174]. The most convincing evidence of environmental transmission comes from outbreaks where groups in a common setting with no known direct contact have been sequentially affected [175]. Such transmission has occurred aboard airplanes and cruise ships, [78] [63] in a concert hall, [176] and from the use of a reusable grocery bag [80]. In all these examples, the environment or fomites likely became contaminated by airborne transmission following an episode of vomiting. Widespread contamination of environments during outbreaks has been documented, particularly in hospital settings where virus has been detected on surfaces of many different objects – switches, televisions, cellular phones, public phones, water taps, toilet light switches, microwave ovens, keyboards, bed frames, and chairs [165, 168]. The role of this contamination is nevertheless

unclear because noroviruses are hardy: outbreak strains have been detected on environmental surfaces during non-outbreak periods, and the converse has also been observed [173, 177]. Although the highest levels of contamination probably occur on surfaces directly contaminated by vomitus or feces, virus has been detected on mantle pieces and light fittings, located above 1.5 m in a hotel affected by an outbreak [65].

4.4 Waterborne Transmission

Sewage-contaminated water is also a recognized route of transmission [178–180]. Norwalk virus can remain infectious in groundwater for at least 2 months and can be detected for over 3 years [170, 181]. Drinking water or ice may become contaminated with norovirus and result in outbreaks in food service settings. The same contaminated water can cause disease directly through drinking and when used in food preparation [182]. Recreational and drinking water can become contaminated from septic tank leakage and sewage or from breakdowns in municipal treatment plants, resulting in large community outbreaks [183–185] [186]. However, outbreaks have even been reported from wells built in compliance with regulations when groundwater becomes contaminated by septic systems or percolation of sewage through unusual geologic formations [187]. For reasons that are not clear, most waterborne outbreaks are associated with GI noroviruses [88, 188]. However, in contrast to these epidemiological observations, some laboratory data suggest that GII viruses are more stable in water as well as on surfaces [181, 189]. Waterborne transmission of astrovirus has also been documented [190]. Because they often result from gross contamination, waterborne outbreaks are also more commonly associated with mixed infections with multiple noroviruses or even multiple pathogens [88].

5 Biological Characteristics

Noroviruses are a group of nonenveloped, single-stranded RNA viruses with an icosahedral symmetry classified into the genus *Norovirus* of the family *Caliciviridae*. Other genera within this virus family include *Sapovirus*, which also causes AGE (AGE) in humans, as well as *Lagovirus*, *Vesivirus*, and *Nebovirus*, which are not pathogenic for humans [5]. Upon approval by the calicivirus study group of International Committee on the Taxonomy of Viruses (ICTV), novel caliciviruses detected in rhesus monkeys and swine may be accepted as additional genera [191, 192]. By structural analysis, the Norwalk VLP capsid is formed with 180 capsid molecules organized into 90 dimers with a T =3 icosahedral symmetry (where T is triangulation number), [193] using two distinct dimer types to form the higher-order structure [194]. Noroviruses cannot be classified by serotypes, since it cannot be grown in cell culture and neutralized with antisera, but can be divided into at least five genogroups (G), designated GI–GV, based on amino acid identity in the major structural protein (VP1) [195]. Viruses from at least one additional genogroup have been recognized in dogs [40, 196]. The strains that infect humans are found in genogroups GI, GII, and GIV, whereas the strains infecting cows and mice are found in GIII and GV, respectively (Fig. 20.5a). Although interspecies transmission of noroviruses has not been documented, strains that infect pigs are found in GII [197], and a GIV norovirus was discovered recently as a cause of diarrhea in dogs [196], suggesting the potential for zoonotic transmission. On the basis of phylogenetic analysis of the complete VP1, noroviruses can be further classified into genotypes, with at least nine genotypes belonging to GI and 21 genotypes belonging to GII (Fig. 20.5a). At least since 2001, GII.4 viruses have caused the majority of viral AGE outbreaks worldwide [41, 198]. Recent studies have demonstrated that these viruses evolve over time through serial changes in the VP1 sequence, which allow evasion of immunity in the human population [117]. Sapoviruses are divided into at least seven different phylogenetic clusters, four (GI, GII, GIV, and GV) of which include viruses that infect humans, while GIII, GVI, and GVII have only been found in swine [199]. Detection of additional sapovirus genogroups in mink and recently in bats illustrates the enormous genetic variability and host range of viruses within the *Sapovirus* genus (Fig. 20.5b).

Astroviruses, first discovered in 1975 [200, 201], are nonenveloped, single-stranded RNA viruses in the family Astroviridae. Astroviruses are 28 nm in diameter with a smooth edge and may have a characteristic 5- or 6-pointed starlike appearance in the center (Greek, *astron* = star). Since then eight serotypes of human astrovirus (classical human astroviruses) have been identified and characterized. Serotype 1 is the most common, though multiple serotypes can co-circulate during the same season. Greater serotype diversity may be found in developing countries [202, 203]. In addition, viruses belonging to two other phylogenetic clades (MLB and VA) have been detected in human stools, some of which has been directly linked to AGE in children [204, 205].

6 Pathogenesis

The primary replication site for noro- and sapoviruses has not been established. It is likely that these viruses replicate in the upper intestinal tract because volunteers who develop

gastrointestinal illness following oral administration of virus have histopathologic lesions on biopsies from the jejunum [19, 206]. Pigs infected with porcine sapovirus demonstrate blunting and shortening of the villi of the proximal small intestine [207]. Interestingly, the same characteristic jejunal lesion has also been observed in volunteers who were fed Norwalk or Hawaii virus but did not become ill [208–210]. Increased mononuclear infiltrates in the lamina propria and villous blunting in intestinal biopsies of pediatric patients compared with uninfected controls have been reported [211]. Levels of small intestinal brush border enzymes (trehalase and alkaline phosphatase) are significantly decreased compared with baseline and convalescent-phase values [206]. It has been proposed that abnormal gastric motor function is responsible for the nausea and vomiting associated with noroviruses [208], but the precise mechanism responsible for illness is not known. Astrovirus infection appears to be restricted to villous enterocytes and the exposed epithelium. Histological damage and inflammation is generally mild [212], and the specific mechanism by which astrovirus causes diarrhea is not known [102, 213].

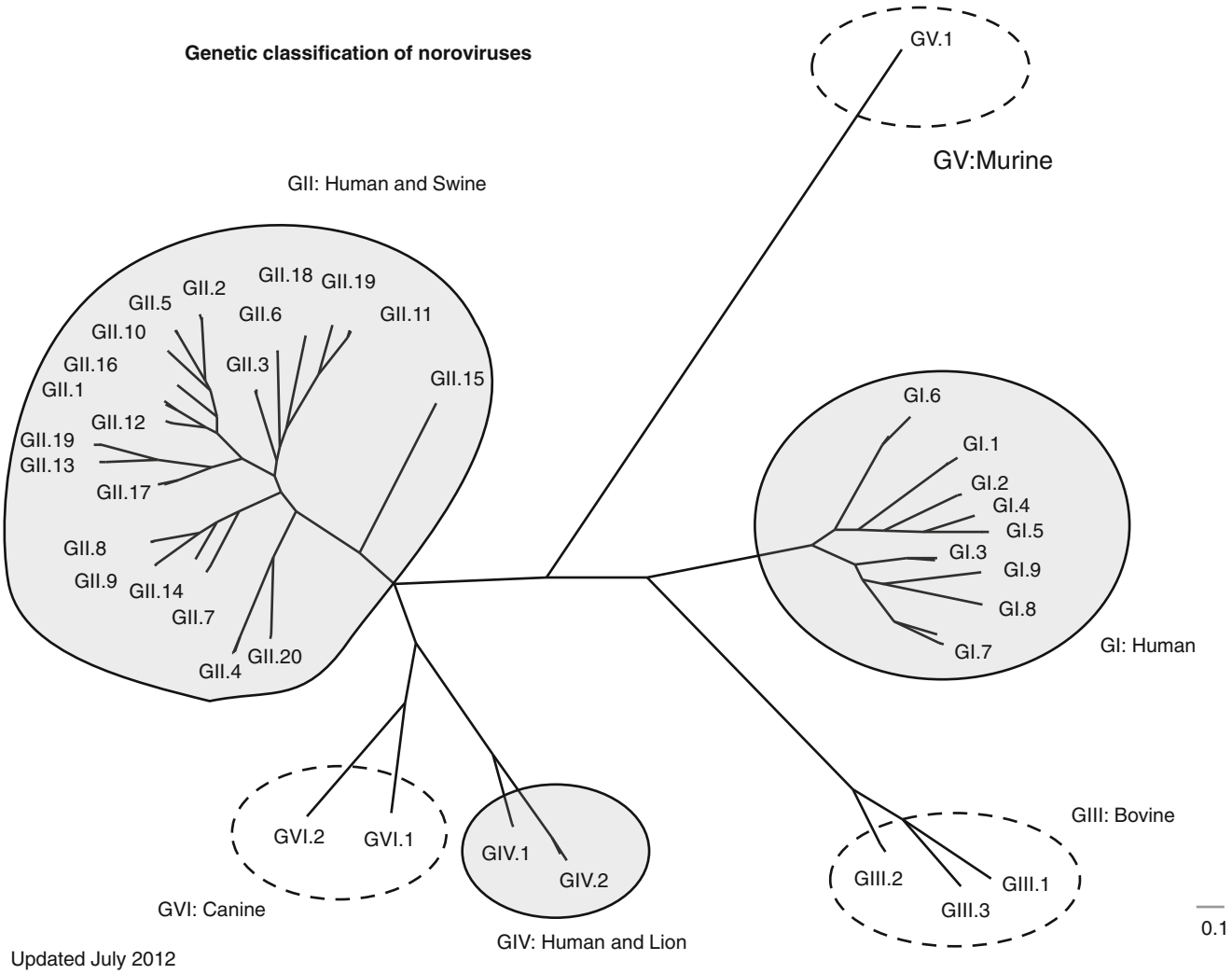

Fig. 20.5 Genetic classification of (**a**) noroviruses and (**b**) sapoviruses based on phylogenetic analysis of sequences of the major capsid protein VP1. Noroviruses can be grouped into six different genogroups (GI–VI) of which *GI* (9 genotypes), *GII* (18 of the 21 genotypes), and *GIV* (1 of the 2 genotypes) can infect humans. Strains belonging to *GIII*, *GV*, and *GVI* infect bovine, murine, and canine species. (**b**) Sapoviruses can be grouped into at least seven different genogroups (GI–VII) with several additional viruses (mink sapovirus and bat sapovirus) that are tentative new genogroups. Sapoviruses in *GI* (5 genotypes and 1 unassigned), *GII* (3 genotypes and 2 unassigned), *GIV* (1 genotype), and *GV* (1 genotype) can infect humans. The scale bar of 0.1 reflects the number of amino substitutions per site (Data analysis and graphs were developed by Everardo Vega, CDC)

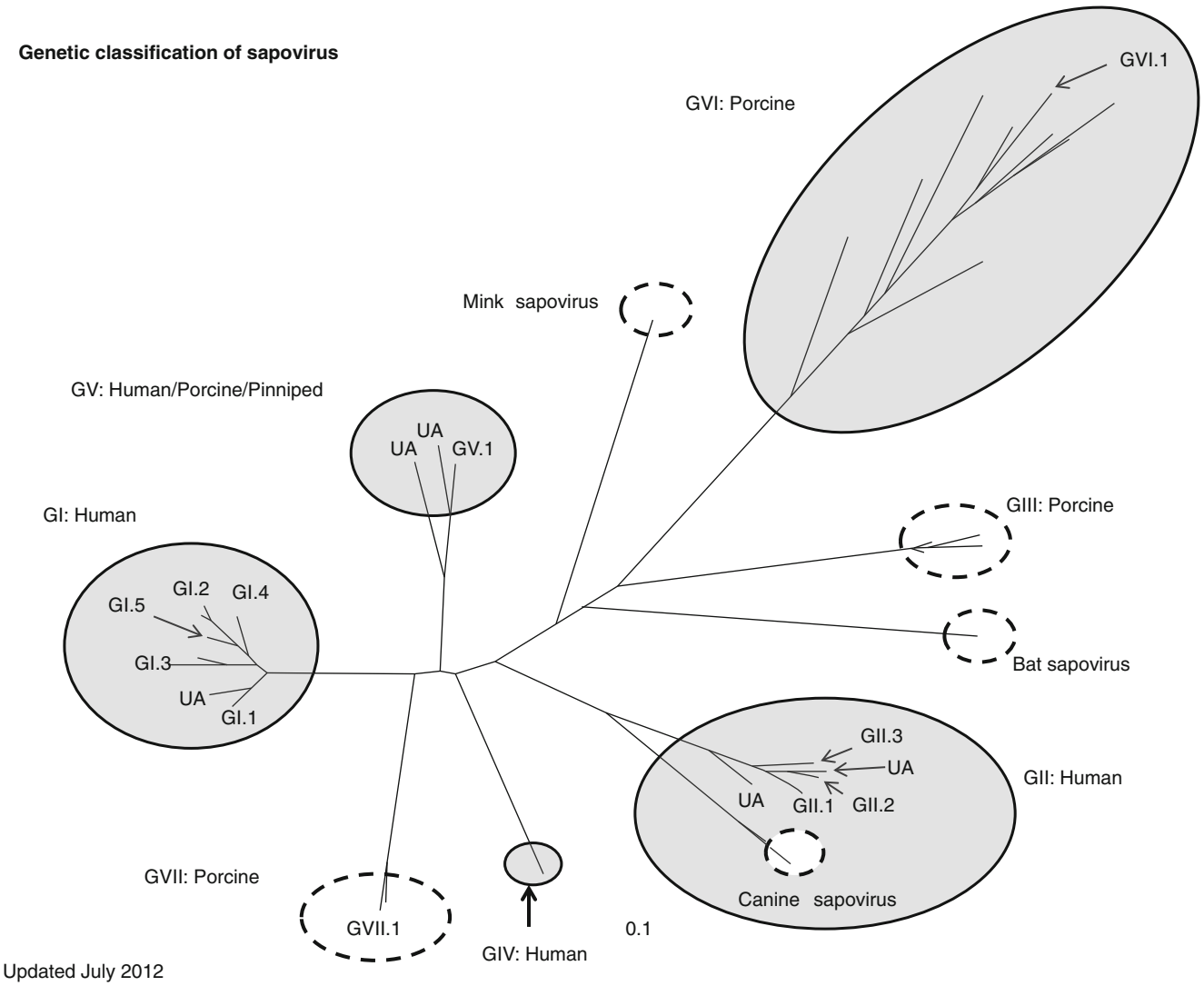

Fig. 20.5 (continued)

7 Patterns of Host Response

Protective immunity to norovirus appears to be different from other enteric viral pathogens and is incompletely understood. Although seroepidemiological studies have shown an antibody prevalence to norovirus of >80 % by age 20, adults consistently demonstrate a high degree of susceptibility to both naturally occurring and experimentally administered noroviruses. In a classic study from the 1970s, 50 % of adult volunteers infected with Norwalk virus consistently developed illness following challenge [25, 214]. In human challenge studies, infected volunteers were susceptible to reinfection with the same strain as well as to infection with heterologous strains [23, 25, 215, 216]. In addition, individuals with preexisting antibodies were not protected from infection unless repeated exposure to the same strain occurred within a short period. Two of these studies demonstrated that homologous antibody protection might last anywhere from 8 weeks to 6 months [23, 216]. Because preexisting antibodies among challenged volunteers did not confer immunity in all individuals and because some persons remained uninfected despite significant exposure, both innate host factors and acquired immunity have been hypothesized to contribute to the susceptibility to infection [23]. However, the infectious dose of virus given to volunteers in these challenge studies was several thousand-fold greater than the small dose of virus capable of causing human illness, and thus immunity to a lower, more natural challenge dose might be greater and more cross-protective. A mathematical modeling study suggests the duration of protective immunity may be on the order of 4 to 8 years [217].

Histo-blood group antigens (HBGAs), including H type, ABO blood group, and Lewis antigens, are a diverse family of carbohydrates expressed on mucosal surfaces. Several

studies indicate that they act as putative receptors or co-receptors for noroviruses. Although there is no evidence that binding to HBGA is mediating viral entry, there is a strong correlation between polymorphic expression of HBGA and human susceptibility to norovirus infection [8]. Genetic resistance to norovirus infections has been associated with mutations in the *FUT2* (or "secretor") gene, which encodes an α1,2-fucosyltransferase; in the homozygous state, null mutant alleles (FUT2–/–) lead to the absence of the α1,2-linked fucose residue on the H-type carbohydrate structures, which characterize the so-called non-secretor phenotype [218]. The most frequent null allele (se428) is characterized by the 428G > A nonsense mutation, and homozygous non-secretors (FUT2–/–) represent up to 20 % of the European population [219]. Results from two landmark studies of human volunteers who were challenged with Norwalk virus demonstrated that non-secretors were not infected by the virus and none of the volunteers showed an increase in anti-Norwalk virus antibodies or had detectable RNA in feces, while secretors (FUT2+/+ or FUT2+/–) excreted the virus and developed a strong antibody response [8, 220]. In addition, both volunteer studies demonstrated that FUT2-positive individuals of the B blood group type were less likely to be infected than A or O individuals but, if infected, more likely to remain asymptomatic. Thus, alleles at the *FUT2* locus determine sensitivity or resistance to the Norwalk virus strain, and the polymorphism at the ABO locus modulates sensitivity within the secretor-positive group. HBGAs are differentially expressed in humans, and several conserved amino acids of the P2 domain of VP1 are important for HBGA binding [221]. The expression of HBGAs has been shown to be associated with strain-specific susceptibility to norovirus infection [8, 222–224]. However, specific binding profiles are not genotype or genogroup exclusive [225] suggesting a host-pathogen coevolution driven by carbohydrate-protein interactions. For GII.4 viruses, single amino acid replacements seem to drastically alter the binding capacity of the VLP [226]. Overall no single norovirus strain seems to be able to cover the whole spectrum of human HBGAs diversity, although collectively, they are likely to be able to infect nearly everyone. The only notable exception is the subgroup of individuals who have a rare genotype, either FUT2–/– or FUT3–/– [218], who may be completely resistant.

Recent studies have reported that innate immunity plays an important role in the control of murine norovirus infection, but little is known about cell-mediated immune responses against noroviruses [227, 228]. A study using oral immunization of human volunteers with Norwalk viruslike particles showed an increase in interferon-γ (IFN) in the absence of IL-4 production, suggesting a dominant Th1 pattern of cytokine production [229]. This dominant Th1 response was confirmed in a study of 15 volunteers infected with Snow Mountain virus, who experienced significant increases in serum IFN-γ and IL-2, but not IL-6 or IL-10, on day two after challenge [224]. Interestingly, in an in vitro study using a Norwalk virus replicon-bearing cells, IFN-α efficiently cleared the NV replicon in a dose-dependent manner at comparable levels to hepatitis C virus, indicating a potential therapeutic application of IFN to norovirus infection [230].

Because diarrheal disease caused by astrovirus is largely restricted to children, immunity is believed to be long lasting; however, little is known regarding the specific immune responses that result in immunity to astroviruses. $CD4^+$ T cells may be involved in the anti-astrovirus response [231] and animal models point to a possible role for the innate immune system [232].

8 Control and Prevention

Efforts to prevent norovirus and sapovirus disease are directed at interrupting the person-to-person transmission cycle, even in the case where contaminated food or water can be identified. Most gastroenteritis viruses are transmitted via the ingestion of infectious fecal (or, less commonly, vomitus) material. Therefore, standard sanitation and hygienic precautions are key. These include frequent hand hygiene, environmental disinfection, proper disposal of fecal or vomit-soiled materials, and limited contact with ill persons. Even when these precautions are put firmly in place, our ability to control outbreaks remains limited [233].

8.1 Hand Hygiene

The single most important method to prevent norovirus infection and control transmission is appropriate hand hygiene [234, 235]. Washing with soap and water is the preferred method to prevent norovirus transmission, with alcohol-based hand sanitizers useful only as an adjunct when hands are not visibly soiled [236]. Plain soap and water reduces the number of microbes on hands via mechanical removal of loosely adherent microorganism [234]. In finger pad studies, soap and water used for 20 s have been shown to reduce norovirus by 0.67–1.20 $\log_{10}$ by RT-PCR assay [235]. The use of alcohol-based hand sanitizers remains controversial, due to both inconclusive in vitro finger pad studies [171, 235, 237] and epidemiological studies where higher rates of infection have been detected during outbreaks in long-term care facilities that use alcohol-based hand sanitizers [238], though the reasons for association in this one study are debated [239].

Surrogate viruses such as murine norovirus (MNV) or feline calicivirus (FCV) and porcine sapovirus are typically used since norovirus cannot be cultured, so its infectivity cannot be directly assessed. Additionally, detection and quantification of viral RNA is not necessarily a reliable

means of estimating the effectiveness of hand sanitizers against human norovirus [171]. Studies on disinfectant and hand sanitizers using MNV and FCV have given contradictory results [237, 240]. The sensitivity of FCV to low pH and the relatively high susceptibility of MNV to alcohols suggest that disinfectants that are effective against both surrogate viruses may be more likely to be effective against human norovirus [171].

8.2 Exclusion and Isolation

Considering the highly infectious nature of norovirus, exclusion and isolation of infected individuals are often the most practical means of interrupting transmission of virus and limiting contamination of the environment. This is true in settings where people reside or congregate such as long-term care facilities, acute care hospitals, cruise ships, and college dormitories as well as in the case of infected food handlers.

Unfortunately, empirical evidence for the effectiveness of exclusion and isolation strategies is limited; [241] these strategies are based on general infection control principles rather than direct evidence. The principle underpinning isolation is to minimize contact with persons during the most infectious periods of their illness. This includes the acute phase of illness, a period following recovery while the person is still shedding virus at high levels, and, in some situations in healthcare facilities, exclusion of exposed and potentially incubating individuals. Isolation of well persons (i.e., quarantine) may be useful during outbreaks in long-term care facilities and hospitals to help break the cycle of transmission and prevent additional cases.

In healthcare facilities, ill patients may be cohorted together in an isolatable unit, with the same dedicated nursing staff providing care only for infected individuals [242]. Ill patients should not generally be transferred to unaffected units in the facility – except in the case of medical necessity and after consultation with infection control staff. Analogously, passengers with AGE on cruise ships may be asked to remain isolated in their cabins during their illness and for a period of 24–48 h after recovery. To minimize the risk of spread from incubating or asymptomatically infected patients and staff in healthcare facilities, such individuals should not be transferred to or work in unaffected areas, typically for 48 h after exposure. In certain situations, units in a healthcare facility may be closed to new admissions to prevent the introduction of new susceptible patients, though guidelines differ on this point [236, 243–245]. Ill staff members in healthcare facilities as well as infected food handlers should be excluded during their illness and for 24–48 h following resolution of symptoms [108].

8.3 Food Handling

Food may also be potentially contaminated with enteric viruses during production if growing or irrigation waters are contaminated with human feces; thus, shellfish should be adequately cooked and fresh produce washed thoroughly before consumption [108].

8.4 Environmental Decontamination

Chemical disinfection is a central approach inactivate norovirus [246, 247]. The US Environmental Protection Agency maintains a list of approved products for norovirus disinfection (http://www.epa.gov/oppad001/list_g_norovirus.pdf) based on their efficacy against FCV. Notably, FCV exhibits different physiochemical properties than human norovirus and therefore might not reflect a similar disinfection efficacy profile. Largely due to the uncertainty from in vitro studies, CDC recommends chlorine bleach solution at a concentration of 1,000–5,000 ppm (5–25 tablespoons household bleach [5.25 %] per gallon of water) for disinfection of hard, nonporous, environmental surfaces whenever feasible [108, 172]. In healthcare settings, cleaning products and disinfectants used should be EPA registered and have label claims for use in healthcare settings [108]. Hand hygiene (discussed above) is also a key part of the environmental transmission cycle since contaminated hands can transfer virus to touched surfaces, and hands may be a vehicle for transferring virus from contaminated surfaces back to humans [175].

8.5 Vaccination and Treatment

No specific treatment exists for most AGE viruses, so treatment is supportive and includes therapy for dehydration and electrolyte imbalances. First-line treatment should be oral rehydration solutions, while severe dehydration or shock may warrant intravenous fluid therapy. Antiemetics, antimotility agents, and antibiotics are generally not recommended [248]. Certain compounds with antiviral properties have shown promise in laboratory studies, but their value in the clinic remains uncertain given the short and acute infection caused by caliciviruses.

No vaccines for noroviruses are currently available, but a number of norovirus vaccines are at various stages of development. The product furthest developed is based on recombinant virus-like particles (VLPs) produced by the expression and spontaneous self-assembly of the major capsid protein VP1. An intranasally delivered formulation was shown to be safe and immunogenic in phase 1 and 2 trials [249]. In a challenge study, where participant were vaccinated and

subsequently exposed to homotypic Norwalk virus, the vaccine was shown to be effective against disease and, to a lesser extent, infection [250]. A number of remaining questions and challenges include whether efficacy in the community against commonly circulating viruses can be achieved, whether more vulnerable groups (children and the elderly) will be protected, and whether duration of protection will be long enough to be clinically useful [251].

9 Unresolved Problems

A number of issues remain unresolved, hamper our understanding of norovirus, and preclude a solid basis to develop effective prevention and control strategies. Most fundamental is our inability to grow norovirus in a cell-culture system [252]. This limitation has restricted development of infectivity assays and our ability to differentiate infectious virus from inactivated particles. That capability could ultimately lead to a better understanding of the biology of noroviruses. Second, a perplexing result from many studies is the high level of asymptomatic infection, mainly in children, but occasionally in older age groups [44]. These findings raise questions about the interpretation of diagnostic results as well as the role of asymptomatic infection in transmission. For example, considering the long duration of asymptomatic shedding, we do not know how long infected food handlers or healthcare workers should be excluded from work. Finally, and most importantly, the burden of the noroviruses among economically poor populations in developing countries remains to be fully understood.

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Orthopoxviruses: Variola, Vaccinia, Cowpox, and Monkeypox

21

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1 Introduction

1.1 Biological Characteristics

Poxviruses are a family of large, complex DNA viruses characterized by their unique ability to replicate entirely within the cytoplasm of infected cells [1]. The *Poxviridae* family is divided into the subfamilies *Chordopoxvirinae* and *Entomopoxvirinae* which infect vertebrate and insect hosts, respectively. Orthopoxviruses are members of the *Chordopoxvirinae* subfamily along with seven other genera: *Parapoxvirus, Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscopoxvirus,* and *Yatapoxvirus*. Four species of orthopoxviruses are known to infect humans: variola (smallpox), vaccinia (smallpox vaccine), cowpox, and monkeypox.

Due to their large size compared to other animal viruses, poxviruses were the first viruses to be seen with a microscope [1, 2]. With the increased resolution of electron microscopy, orthopoxvirus virions appear brick shaped with slightly rounded edges [1, 3]. The mature virion (MV) is the most basic infectious form of poxvirus and consists of a dumbbell-shaped core surrounded by a single lipid membrane bilayer [1, 4] (Fig. 21.1).

The orthopoxvirus genome is located in the viral core within a nucleoprotein complex (nucleosome) [4]. It consists of a single, linear, covalently closed double-stranded DNA molecule with inverted terminal repetitions (ITRs) at each of the two ends. The genomes of most orthopoxviruses are ~200 kbp in length and contain a guanine plus cytosine content of ~36 % [1]. The complete genomic DNA sequences of many orthopoxviruses, including representative strains all of

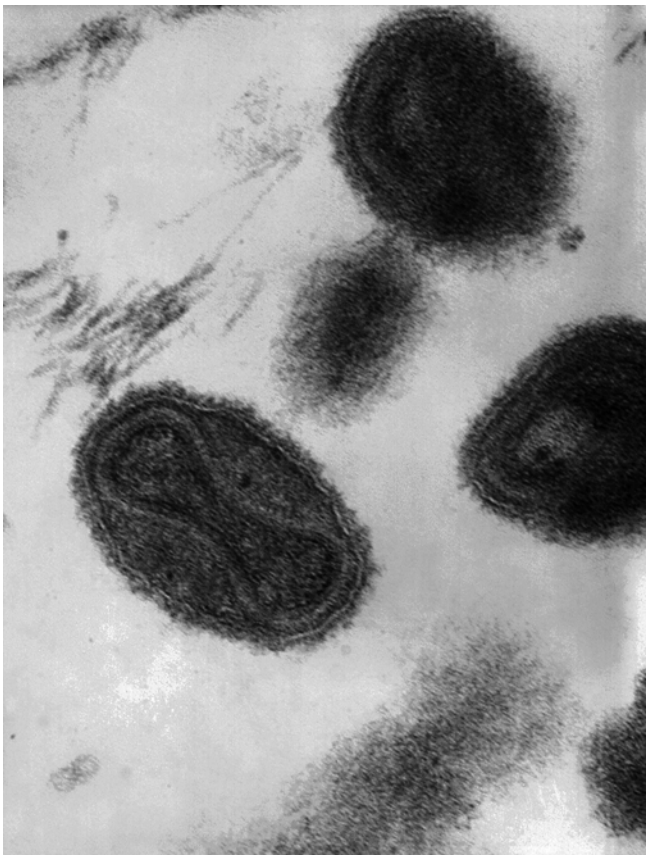

Fig. 21.1 Electron micrograph of smallpox virus virions depicting the dumbbell-shaped viral core (From the US CDC Public Health Image Library [PHIL] Image ID 1849. Credit: CDC/Dr. Fred Murphy; Sylvia Whitfield. This image is in the public domain and thus free of any copyright restrictions)

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the viruses described in this chapter, have been determined and described previously [5–8].

During virion morphogenesis, a minority of MV undergo further processing with the addition of a second outer lipid membrane bilayer to form an extracellular enveloped virion (EV). Both MV and EV are infectious particles but are thought to have different mechanisms of binding and entering cells. MV binding to cell glycosaminoglycans or laminin is mediated by four viral proteins associated with the single MV membrane [9]. While the binding protein(s) of EV has yet to be identified, it is believed that the second outer membrane is disrupted to expose the MV membrane prior to entry [9, 10]. Subsequently, membrane fusion is initiated by 11–12 transmembrane proteins on the MV surface, a process that can occur directly with the cell plasma membrane or with the membrane of an endocytic vesicle [9, 10]. When fusion occurs at the plasma membrane, the viral entry and fusion proteins now embedded in the cell membrane also mediate the formation of syncytia with adjacent cells and cell to cell spread of virus [10]. Once inside the cell, viral replication commences and occurs completely within the cytoplasm. For this reason, poxvirus virions contain a nearly complete RNA polymerase system that enables primary transcription of viral genes without the use of the cell's nuclear machinery [1]. This early gene expression provides nonstructural proteins needed for DNA replication as well as gene expression of intermediate and late genes. The intermediate and late genes encode structural proteins required for virion assembly in addition to early transcription factors that are packed together with RNA polymerase in assembling virions [1]. In total, orthopoxvirus genomes contain approximately 200 genes of which half encode proteins that are ultimately packaged into virions [4].

1.2 Patterns of Host Response

At present, there is an incomplete understanding of the patterns of host response that occur in human orthopoxvirus infections. Of the four orthopoxviruses that infect humans, vaccinia virus has been the best studied as a model for understanding poxvirus biology. Vaccinia virus has also been exploited as a vaccine due to the ability of orthopoxviruses to induce cross-reactive antibodies that protect against infection from other orthopoxvirus species. Neutralizing antibodies to orthopoxviruses generally appear within the first week of illness and can remain present beyond 20 years [11, 12]. In contrast, hemagglutination-inhibition and complement-fixation antibodies become detectable 16–18 days after infection with levels decreasing after 1 year [2, 13]. The development of such antibodies has been associated with protection in studies of both animals and humans [14–17]. However, in addition to humoral responses, cell-mediated and T-cell responses also appear to be important in the development of immunity against orthopoxviruses [18]. To thwart these host responses, poxviruses have developed numerous mechanisms to evade the immune system [19, 20]. Many of these disrupt the innate immune system by targeting mediators of inflammation such as interferons, tumor necrosis factors, interleukins, complement, and chemokines [19, 21–27]. Further studies using DNA microarrays to evaluate gene expression profiles in response to infection with variola, vaccinia, and monkeypox viruses confirmed the absence of interferon and tumor necrosis factor responses and other factors with critical roles in the activation of the innate immune response [28, 29]. This complex interplay between the host response and viral immune evasion mechanisms has made it difficult to establish a direct correlate of immunity despite technological advances [4]. Historically, the presence of a "take," i.e., demonstrable lesion or scar at the administration site of smallpox vaccine, was used as evidence of successful vaccination against smallpox and protection against disease. However, this practice can mistake a bacterial superinfection of the inoculation site or vaccination with bacillus Calmette-Guérin (BCG) vaccine with protection [4]. Furthermore, an alternate correlate of protection would be useful for more recently developed highly attenuated smallpox vaccines that do not produce visible lesions. Continued research is needed to better elucidate the patterns of host response and correlates of immunity for orthopoxvirus infections.

2 Smallpox

2.1 Historical Background

Smallpox is one of the oldest described human diseases. Examinations of several mummies from ancient Egypt, including that of the pharaoh Ramses V, suggest that smallpox may have occurred in humans over 3,000 years ago [2]. Early written records containing detailed descriptions of smallpox also exist including those of Ko Hung from China in 340 AD, Ahrun from Alexandria in 622 AD, and the Persian scholar Al-Razi in 910 AD [2]. Periodic epidemics of smallpox likely plagued primitive civilizations until their populations became sufficiently dense to establish endemicity [2]. By the mid-eighteenth century, smallpox had become endemic on all continents with the exception of Australia [2]. Throughout the centuries, smallpox caused untold human suffering and loss of life. Even as late as the 1950s, over 150 years after Edward Jenner introduced vaccination, smallpox was estimated to produce 50 million cases worldwide each year [30]. Subsequently, a global eradication campaign was initiated by the World Health Organization (WHO) in 1959 to rid the world of this ancient scourge. The success of these efforts culminated in the identification of the last known case of naturally occurring smallpox in Somalia in 1977.

In 1980 the WHO's General Assembly officially declared smallpox eradicated, and recommendations for routine vaccination against smallpox were discontinued. All known stockpiles of variola virus were collected and consolidated and are currently stored in two locations at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, United States, and the State Research Center of Virology and Biotechnology VECTOR in Novosibirsk, Russia. Following eradication, smallpox was allegedly developed as a biological weapon by the Soviet Union and produced in large quantities [31]. This revelation, along with other intelligence concerns, has led to the belief that unauthorized stocks of smallpox may exist and could be used as a biological weapon by terrorists or rogue nations. Though the likelihood of this happening is believed to be low, efforts to be prepared, utilizing a smallpox response research and development program, for the possible reemergence of smallpox into human circulation are ongoing [32, 33].

2.2 Methods for Epidemiologic Analysis

Given the eradication of the disease, all epidemiologic data on smallpox is historical in nature. However, the widespread nature of the disease prior to eradication provided numerous cases for study. Among the largest and most systematic clinical and epidemiologic analyses of smallpox in hospitalized patients were those done by Thomas F. Ricketts, A. Ramachandra Rao, James P. Marsden, and Cyril W. Dixon [2, 34]. The work of Ricketts, Rao, and Dixon was based on personal examination of hundreds of patients with smallpox hospitalized in London, England; Madras, India; and Tripolitania (modern-day Libya), respectively [35–37]. Similarly, Marsden's experience in the London smallpox hospitals provided insights into the epidemiology of the milder form of smallpox variola minor [2, 38]. Further studies evaluating the epidemiology of smallpox in nonhospital settings were also undertaken during the global eradication campaign [39, 40]. Currently, smallpox surveillance is focused on strengthening and maintaining the capacity to identify the disease in the event that human infection reoccurs. Smallpox is a nationally notifiable disease in the United States, and the CDC has developed a clinical algorithm to evaluate the risk of smallpox in patients with acute vesicular or pustular rash illnesses, to assist in developing a differential diagnosis, and to help inform the need for diagnostic testing [41, 42]. To increase capacity for diagnostic testing, the Laboratory Response Network (LRN) was established by the CDC with collaboration from multiple partners including the Association of Public Health Laboratories and the Federal Bureau of Investigation [43, 44]. The LRN provides laboratory diagnostic testing for smallpox in addition to other potential biological warfare agents.

2.3 Descriptive Epidemiology

The host range of variola virus was restricted to humans, and there are no known animal or insect reservoirs [2]. However, recent genetic analyses suggest that the disease may have emerged from a rodent-borne variola-like ancestor in Africa [45, 46]. Among humans, smallpox cases occurred in all ages, genders, races, and ethnicities. Indeed, smallpox has been described as a disease of "princes and peasants" due to its indiscriminate nature in inflicting disease upon all people [47]. Two principal forms of disease were distinguished epidemiologically: variola major and the less severe variola minor (including a biologically distinct virus type alastrim). Outbreaks of variola major produced case fatality rates up to 30 %, while those for variola minor were generally less than 1 % [2, 4]. Population studies including both vaccinated and unvaccinated subjects revealed that case fatality rates were highest in the very young and older age groups [40, 48]. Pregnant women also had extraordinarily high case fatality rates from smallpox (34.3 % overall and nearly 70 % for unvaccinated pregnant women) and were particularly susceptible to hemorrhagic smallpox [2, 49–51]. Secondary attack rates of 30–80 % among unvaccinated contacts within households were similar for both variola major and minor [4]. The age distribution during outbreaks of smallpox depended largely on the immunization or disease-convalescent status of the population affected. Children generally had the highest attack rates in smallpox outbreaks in most areas including the United States, Europe, and Asia as most adults had acquired immunity through either vaccination or previous smallpox infection [52]. In contrast, the age distribution of cases was generally proportional to the age distribution of the population in rural areas where vaccination was less common and adults were more likely to be vaccine naïve and susceptible to disease [40, 52]. Smallpox outbreaks exhibited a seasonal pattern with the highest incidence of cases occurring during the winter and early spring [40, 53]. This observation was consistent with the findings that other orthopoxviruses (i.e., vaccinia) survived better under conditions of low temperature and humidity [54].

2.4 Mechanisms and Routes of Transmission

The most common route of transmission of smallpox was from person to person primarily via respiratory-salivary droplets expelled from the oropharynx of infected individuals. Transmission generally occurred following direct face-to-face contact, though rare occurrences involving airborne dissemination over longer distances have been documented [55]. Common features linked to these occurrences included primary cases with extensive rash and cough, low humidity, and hospital ventilation favoring the formation of air currents

[55]. Transmission of smallpox also occurred through direct contact with active rash lesions or other sloughed exudative materials, though this route of transmission was uncommon [2]. Smallpox was also rarely transmitted via contaminated fomites, e.g., soiled clothing or bed linens [2, 34]. Lastly, smallpox infection by direct inoculation occurred among nursing mothers, postmortem workers, and fabric workers (particularly those working with lace) or more commonly through variolation – a technique for immunizing patients by purposefully infecting them with material from the pustules of smallpox patients with mild smallpox disease [2]. Variolation was practiced widely before the advent of vaccination.

Peak viral shedding is seen during the rash phase of illness; consequently, almost all transmission occurred during the time when rash is present [56, 57]. As such, the majority of smallpox transmission occurred within households, hospitals, and other health-care settings. Large outbreaks in schools were uncommon, and transmission on trains, planes, or buses was also rare [58, 59]. Despite containing significant amounts of virus, scabs were not found to be particularly infectious presumably due to the encasement of viral particles within the protein-fibrin matrix [2].

2.5 Pathogenesis and Immunity

Infection with smallpox began with entry of the virus through the respiratory mucosa or alveoli. The clinical course of ordinary smallpox then began with an asymptomatic incubation period generally lasting 10–14 days (range of 7–17 days). Illness onset generally occurred suddenly with fever and malaise. Temperatures rose to 38.5–40.5 °C and were variably accompanied by headache, backache, abdominal pain, vomiting, and pharyngitis [2]. These prodromal symptoms lasted approximately 2–3 days before the first appearance of lesions [2]. The rash developed in a centrifugal pattern with lesions appearing first and in highest numbers on the oropharynx, face, and extremities before spreading to the trunk. Those on the oropharynx quickly ulcerated as a result of the lack of stratum corneum, releasing high titers of virus into the saliva [58, 60]. The presence of lesions on the palms and soles of the hands and feet was also characteristic (Fig. 21.2). Lesions progressed from macules to papules to vesicles over the course of 4–5 days. Vesicles were often umbilicated and evolved to pustules within a day or two (Fig. 21.3).

Pustules were described as round, tense, firm to the touch, and deep seated within the dermis. At any given time, lesions exhibited the same stage of development in any one area of the body [4]. Lesions typically began to crust on the eighth or ninth day of rash with scab formation and sloughing around day 14 post rash onset. Resolving skin lesions left pitted scars which were particularly evident on the face due to the destruction of the sebaceous glands, shrinking granulation tissue, and fibrosis [2]. In fatal cases, death usually

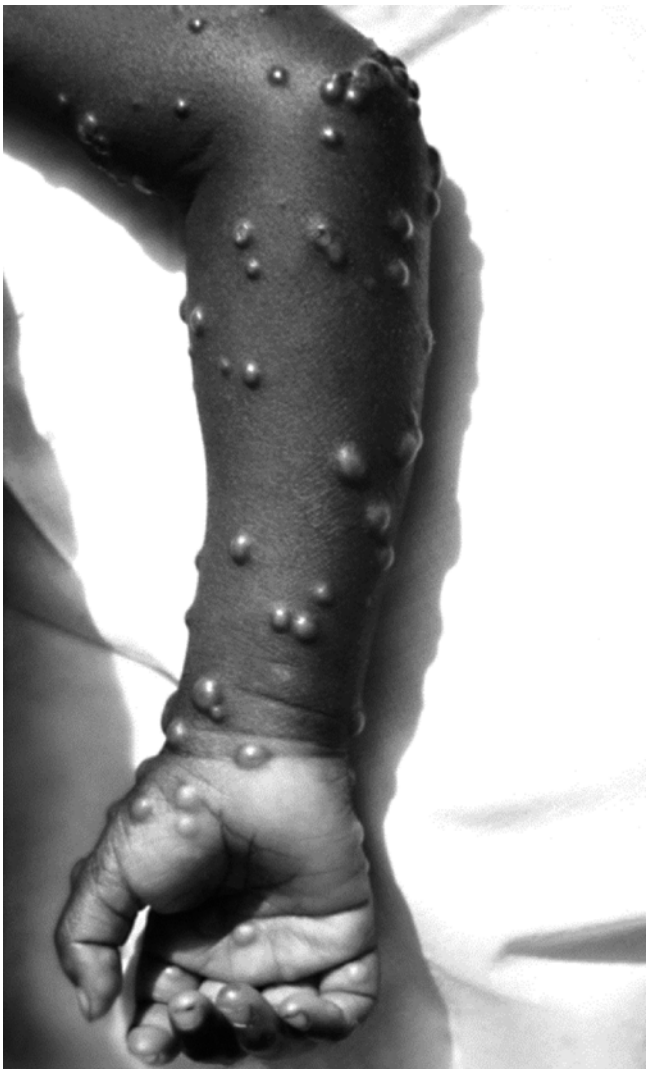

Fig. 21.2 Smallpox lesions on the arm and palm (From the US CDC PHIL Image ID 2007. Credit: World Health Organization; Diagnosis of Smallpox Slide Series. This image is in the public domain and thus free of any copyright restrictions)

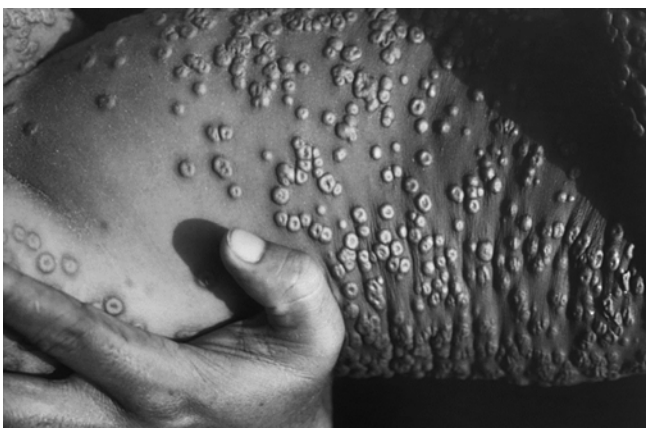

Fig. 21.3 Umbilicated smallpox lesions on the trunk (From the US CDC PHIL Image ID 284. Credit: CDC/James Hicks. This image is in the public domain and thus free of any copyright restrictions)

occurred during the second week of illness. The exact cause of death in smallpox remains unclear, and the pathogenesis may have differed depending on whether the clinical manifestations were hemorrhagic, flat, or "malignant" as opposed to the ordinary type [34]. In some cases reviewed retrospectively, mortality was attributed to some combination of renal failure, shock secondary to volume depletion, and respiratory compromise induced by cytopathic effects of the virus [61]. Toxemia associated with viral antigens in plasma and immune complexes of antigen and antibody have also been proposed to be contributing factors [2, 58].

During the eradication of smallpox, the WHO established four main clinical types of disease based on disease presentation and rash burden: ordinary, modified, flat, and hemorrhagic [62]. The most common type was ordinary smallpox which accounted for approximately 85 % of cases in outbreaks of variola major. The clinical presentation of ordinary smallpox followed the description above and, as classified by WHO for epidemiologic description, was further subdivided based on the density of rash lesions on the face and body. In ordinary confluent, the rash was confluent on the face and arms, whereas ordinary semiconfluent demonstrated a confluent rash on the face only. Ordinary discrete was characterized by areas of normal skin between pustules on the face and body. These subdivisions had prognostic value as higher rash burden portended a higher likelihood of death; confluent, semiconfluent, and discrete ordinary smallpox had case fatality rates of 62, 37, and 9 %, respectively, in unvaccinated individuals [2, 36]. Modified smallpox was similar to ordinary smallpox apart from being "modified" by previous vaccination to produce disease with a milder prodrome, fewer lesions, and an accelerated clinical course. This type accounted for 5–7 % of cases and was not fatal [2, 62]. In contrast, flat smallpox occurred at a similar frequency but was usually fatal in both vaccinated and unvaccinated individuals [2, 36]. The lesions of flat smallpox were slow to mature and generally remained as soft, velvety vesicles without pustulation [30]. Most cases of flat smallpox were seen in children and may have been associated with deficient cellular immune responses though no studies were undertaken to confirm this [2]. Lastly, hemorrhagic smallpox was a rare (<1 % of cases) but deadly form involving extensive bleeding into the skin and mucous membranes that almost invariably led to death within a week of disease onset [4]. This type occurred mostly in adults (particularly pregnant women) with equal frequencies in vaccinated and unvaccinated individuals [2]. Variola infection without the development of rash was thought to occur rarely in previously vaccinated individuals exposed to smallpox cases and was referred to as variola sine eruptione. Patients typically presented with sudden onset of fever, headache, and sometimes backache that resolved within 48 h and showed evidence of acute infection by high or rising antibody titers and occasionally viral isolation [2].

A similar system for subdividing smallpox into categories with prognostic value was described by Dixon [34, 37]. He defined a total of nine types of smallpox, each with associated mortality estimates. Fulminating smallpox (purpura variolosa) was the first and most deadly type with a mortality rate of nearly ~100 %. This type of smallpox was characterized by a hyperacute course leading to death within 4–5 days after onset of symptoms. Illness began with fever, prostration, and anxiety accompanied by early hemorrhages, particularly of the mucous membranes. The absence of any vesicular eruption was a distinct clinical feature making differentiation from other acute hemorrhagic catastrophes difficult. The second and third types of smallpox were termed malignant confluent and malignant semiconfluent. Malignant smallpox typically began with a high initial fever during the first 3–4 days of illness that defervesced before reappearing during the fourth to twelfth days of illness. The rash of malignant smallpox was soft, velvety, hot, and tender with a slow evolution. Vesicular lesions were rare though hemorrhaging could occur late in the skin as well as the mucosa. The prognosis depended largely on the distribution of the rash with 70 % mortality in patients with confluent rash on the face and arms (malignant confluent) as opposed to 25 % mortality in patients with confluent rash on the face only (malignant semiconfluent). Benign confluent and benign semiconfluent were the fourth and fifth types of smallpox described by Dixon. These types were distinguished primarily by the distinctive rash of ordinary smallpox that followed a centrifugal distribution and progressed through the macular, papular, vesicular, and pustular stages. As with malignant smallpox, the rash of benign semiconfluent extended to the face only compared to the face and arms in patients with benign confluent and conferred a mortality benefit (10 vs. 20 %, respectively). Lastly, Dixon described four types of less severe smallpox that differed primarily in the number of lesions; the discrete type exhibited over 100 (but no areas of confluence), 20–100 in the mild type, less than 20 in the abortive type (often without pustulation), and no lesions in variola sine eruptione. Death was uncommonly encountered in these types of smallpox with only discrete smallpox manifesting a 2 % mortality rate.

Variola minor infections produced disease that was clinically identical to the ordinary or modified types of smallpox with typical prodrome and rash [38]. Prior to eradication, variola major and minor could only be distinguished in outbreak settings by the difference in case fatality rates, whereas virologic differentiation is now possible for certain biologically discrete viruses causing minor disease (i.e., alastrim) [2, 58].

Survivors of smallpox were thought to be protected for life [2, 13]. In contrast, complete protection is not lifelong following smallpox vaccination. Vaccine-induced immunity is most effective in the first 1–3 years following vaccination

when preexposure vaccine efficacy may approach 100 %, and substantial protection may endure for up to 15–20 years [4, 63, 64]. Postexposure vaccination was also effective in preventing and/or ameliorating disease when given to contacts of patients with smallpox. Such postexposure prophylaxis appeared to be most effective when given as soon as possible following exposure and particularly in previously vaccinated individuals; vaccination beyond 3 days after exposure appeared to be less effective based on interpretations of data from the eradication era [65–68].

3 Vaccinia and Cowpox

3.1 Historical Background

The disease known as "cowpox" was so named because of the pustular lesions it produces on the teats of milking cows. During the 1700 s, rural Europeans observed that milkmaids (and others exposed to cows) were rarely afflicted with the scars of smallpox, and it was hypothesized that exposure to cowpox induced resistance to infection with smallpox. In 1798, Edward Jenner published the first scientific evidence that cowpox could be used prophylactically to produce immunity against challenge with smallpox. This concept of preventing disease through inoculation was later termed "vaccination" by Louis Pasteur using the Latin word for cow (vacca) to honor the work of Jenner. Initially, poxviruses taken from or grown on a wide range of species including cows, horses, goats, sheep, pigs, and buffaloes were used [2]. However, smallpox vaccination quickly gained acceptance, and by the twentieth century, almost all smallpox vaccines contained vaccinia virus. Recent genotypic analysis suggests that vaccine strains of vaccinia virus are most closely related to cowpox viruses from continental Europe [69]. Regardless of its precise origin, the use of vaccinia virus in combination with surveillance and monitoring led to the eradication of smallpox in 1980.

3.2 Methods for Epidemiologic Analysis

Early interest in the epidemiology of vaccinia stemmed primarily from its use as a routine childhood vaccine for the prevention of smallpox. In particular, considerable attention has been paid to describing the adverse events associated with vaccinia when used as a smallpox vaccine. Two studies conducted by the Centers for Disease Control in 1968 were among the most comprehensive to evaluate the incidence of adverse events following childhood smallpox vaccination with vaccinia virus. The first study relied on passive reporting of patients with suspected complications of smallpox vaccination to seven separate national surveillance systems [70]. The second study actively surveyed physicians in ten states to solicit reports of complications of smallpox vaccination [71]. Not surprisingly, the adverse event rates calculated using the active surveillance from the ten statewide surveys were higher than those calculated from the passive national surveillance [70, 71]. More recently during 2002–2004, adverse events were monitored, while the United States Department of Defense (DOD) and Department of Health and Human Services (DHHS) increased efforts to vaccinate service members and civilians to enhance preparedness against the possibility of a bioterrorism event involving smallpox. In contrast to those persons vaccinated during the 1968 studies, these populations involved adults that were highly screened for predisposing conditions associated with higher rates of adverse events (e.g., eczema, immunodeficiency, or therapeutic immunosuppression). As such, overall rates of adverse events were much lower in both the DOD and DHHS programs when compared to historical rates [72–74]. One exception was the identification of myopericarditis and cardiac ischemic events at higher rates than anticipated based on historical data [73, 75].

With respect to cowpox, the most detailed epidemiologic analysis is based on a series of 54 human cases investigated between 1969 and 1993 [76]. Limited epidemiologic information has also been gleaned from outbreaks and case reports of naturally acquired as well as laboratory-associated cowpox infections [77–85].

3.3 Descriptive Epidemiology

The vast majority of cases of vaccinia virus infection and its complications are associated with vaccination. In the United States, select military service members still receive routine vaccination against smallpox using vaccinia virus [72]. Serious infections with vaccinia virus have been seen among vaccinees as well as contacts of vaccinees [74, 86, 87]. Vaccination with vaccinia virus is also recommended for laboratory workers who work directly with non-highly attenuated strains of vaccinia virus or other orthopoxviruses that infect humans [88]. Vaccinia virus is commonly used in laboratory research, and multiple exposures and infections have been documented in laboratory workers [89].

In addition to its use in laboratory research, vaccinia viruses can also be genetically altered to produce recombinant vaccines. One such vaccine is a recombinant vaccinia virus containing a rabies virus glycoprotein that is distributed in baits to control rabies in wildlife. Two cases of human vaccinia virus infection have been reported in dog owners following exposures to baits retrieved by their dogs [90, 91]. Surveillance for further human exposure to oral rabies vaccine is ongoing and provides additional data for epidemiologic analysis [92].

Natural infection with vaccinia virus is rare but does occur. Evidence suggests that a vaccinia virus related to a Brazilian smallpox vaccine strain has become established in cattle in Brazil and shows signs of spread within the country [93–95]. Human vaccinia virus infections among farmworkers exposed to cattle in Brazil have been reported [94, 96]. In addition, outbreaks of buffalopox virus, a subspecies of vaccinia virus found in milking buffalo and cattle, have been reported to cause vaccinia-like lesions on animals' teats and milkers' hands in India, Egypt, Bangladesh, and Indonesia [97, 98].

Human cowpox virus infection is classically described as a zoonotic infection associated with occupational exposure to cattle. However, multiple other species have been implicated as sources of human infection including rats, pet cats, and zoo and circus elephants [76]. Two cases of laboratory-acquired human infection with cowpox virus have also been documented [77, 78].

3.4 Mechanisms and Routes of Transmission

Both vaccinia virus and cowpox virus are most commonly transmitted to humans through contact of skin with purified virus, active lesions, or materials from lesion exudates. Intentional infection with vaccinia virus for the purpose of immunization is achieved by inoculating virus through scarification of the skin using a bifurcated needle. Inadvertent inoculation (to others or to a distant site of the same patient) also occurs as vaccinia virus and cowpox virus lesions have the capacity to produce infectious material until a scab has formed and detached to reveal an underlying intact layer of skin.

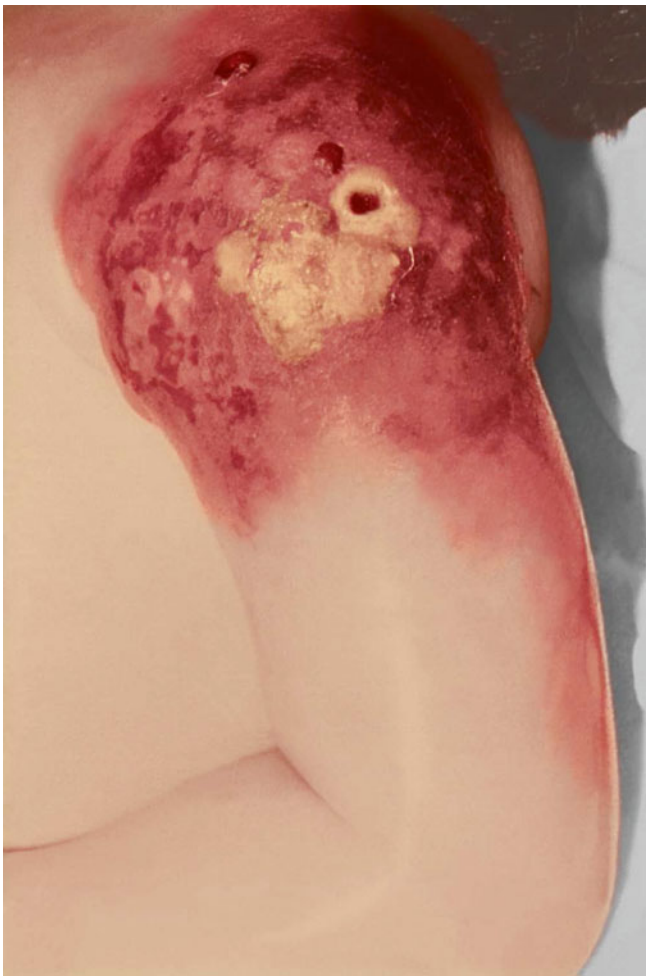

Fig. 21.4 Progressive vaccinia in a 7-year-old male with microcephaly and cerebral palsy after receiving smallpox vaccine (From the US CDC PHIL Image ID 14250. Credit: CDC/Dr. Cocke. This image is in the public domain and thus free of any copyright restrictions)

3.5 Pathogenesis and Immunity

Human infections with vaccinia and cowpox virus produce similar clinical syndromes. Both produce an ulcerative skin lesion that progresses through a well-characterized sequence of events. The site of inoculation develops a papule which becomes vesicular after 3–5 days. Subsequently, the vesicles become pustular and lesions reach their maximum size at day 8–10. The lesions then dry forming a hard black crust which typically separates at day 14–21 leaving a permanent scar. During the vesicular and pustular stages, lesions are often painful with surrounding erythema and edema. Infection is usually also associated with systemic symptoms including fever, lymphadenitis, fatigue, and malaise commonly described as influenza-like. Vaccination with vaccinia virus is typically performed on the upper deltoid; however, secondary autoinoculation or contact transmission can lead to multiple lesions in other anatomical locations. Cowpox typically occurs as a single lesion on the hand, finger, or face but can present with multiple lesions secondary to multiple primary inoculations and autoinoculation and rarely through lymphatic or hematogenous spread of virus [76]. Most vaccinia and cowpox infections are self-limiting and resolve within 6–8 weeks though recovery can take up to 12 weeks in some cases. However, severe systemic illness can be seen in individuals with immunosuppression or immunodeficiency [70, 71, 79, 86].

A number of rare and sometimes fatal complications can result from vaccinia virus infection following vaccination or naturally acquired infections. Progressive vaccinia, also previously referred to as vaccinia necrosum or vaccinia gangrenosum, can occur in persons with severe cell-mediated immunodeficiency [99]. The disease is characterized by uncontrolled spread of infection from the site of inoculation resulting in persistent enlargement of the primary lesion often associated with necrosis (Fig. 21.4).

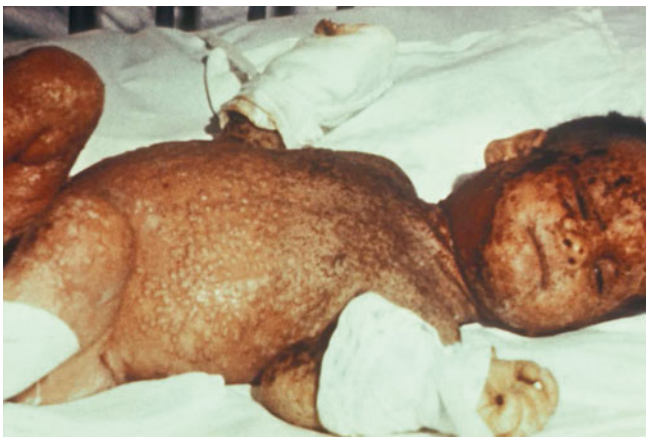

Fig. 21.5 Eczema vaccinatum in an 8-month-old male infected with vaccinia by a sibling recently vaccinated against smallpox (From the US CDC PHIL Image ID 3311. Credit: CDC/Arthur E. Kaye. This image is in the public domain and thus free of any copyright restrictions)

Subsequent dissemination to other parts of the body can sometimes follow. A vaccination site that continues to show progression without signs of healing 15 days after vaccination should prompt consideration of the diagnosis [100]. Progressive vaccinia is generally lethal in those with a complete lack of cellular immunity (e.g., infants with primary immunodeficiencies), though survival has been documented in adults with acquired immunodeficiencies [86, 99]. The incidence of progressive vaccinia was approximately one case per million recipients of routine smallpox vaccination in the United States during 1968 [70, 71].

Eczema vaccinatum is a potential complication of vaccinia virus infection that can occur in individuals with atopic dermatitis (eczema) regardless of the current status of disease activity. Patients typically present with firm, deep-seated vesicles or pustules 3–14 days following exposure to vaccinia virus [101]. The lesions can be locally or widely distributed, often at the site of a previous eczematous lesions, and generally demonstrate the same stage of development (Fig. 21.5).

Lesions may be accompanied by systemic symptoms including fever, malaise, and lymphadenopathy. Bacterial superinfections and supraglottic edema leading to airway compromise are potentially fatal complications [101]. Underlying defects in the immune system of atopic individual's skin render them particularly vulnerable to infection with vaccinia virus and subsequent local spread or wider dissemination [102, 103]. This susceptibility persists even in the absence of active disease as one case series found that two thirds of eczema vaccinatum patients had no active or obvious eczema at the time of vaccination [104]. Eczema vaccinatum occurred at a rate of 10–39 cases per million primary smallpox vaccinations and 1–3 cases per million smallpox revaccinations in the United States during 1968 [70, 71].

Postvaccinial encephalomyelitis (PVEM) is a rare but important complication that occurs following primary vaccination with vaccinia virus. Symptoms of encephalitis or myelitis including fever, headache, vomiting, and malaise typically develop abruptly 10–13 days following vaccination [2]. Decreased consciousness, seizures, and coma frequently ensue [105]. One case with a similar clinical syndrome has also been reported following infection with cowpox virus [106]. Unlike progressive vaccinia and eczema vaccinatum, no known predisposing factors for PVEM are known [2]. The pathophysiology of PVEM is poorly understood with evidence of direct viral infection in some cases, while others display a demyelinating process more closely resembling an acute disseminated encephalomyelitis (ADEM) [105, 107, 108]. Reported rates of PVEM can vary widely based at least partially on differences between vaccine virus strains. The New York City Board of Health (NYCBOH) strain, a strain with lower incidence of PVEM compared to those used in other countries, produced a rate of 3–12 cases per million primary vaccinees in the United States during 1968 [2, 70, 71]. The fatality rate of PVEM is estimated to be 25 % [109].

A syndrome similar to PVEM is also rarely seen in infants less than 2 years of age. Termed postvaccinial encephalopathy (PVE), cases develop the same symptoms as PVEM but typically present earlier following vaccination (usually within 6–10 days) [110]. The development of hemiplegia and aphasia can also be distinguishing features of PVE [110].

Generalized vaccinia is a vesicular rash that develops after vaccination due to dissemination of virus from the site of inoculation. Lesions typically appear 6–9 days after vaccination and follow a similar progression as the primary lesion only more rapid [2]. The rash can be profuse and cover the entire body but does not follow a centrifugal pattern of distribution as seen with smallpox [2]. Generalized vaccinia is typically self-limiting, and serious cases are only seen when underlying immunodeficiency is present [100]. While generalized vaccinia is thought to result from viremic spread of the virus, isolation of virus from lesions is uncommon [111, 112]. During 1968, generalized vaccinia was reported in 23–242 per million primary smallpox vaccinations performed in the United States [70, 71].

Infections with vaccinia virus can pose a risk to the unborn fetus. Although an analysis of pregnancy outcomes among 376 women vaccinated against smallpox did not reveal higher-than-expected rates of pregnancy loss, preterm birth, or birth defects, approximately 50 cases of fetal vaccinia have been documented previously [113, 114]. This complication often results in death of the fetus or neonate and occurs at an estimated rate of 1/10,000 to 1/100,000 in vaccinated pregnant women [114]. For this reason, vaccination with vaccinia virus is generally avoided in pregnant women.

Cardiac complications have been associated with smallpox vaccination, particularly among civilian and military personnel vaccinated in the United States since 2002 [73, 74]. The observed rate of myopericarditis among this vaccinated population has been higher than the calculated background incidence [75, 115, 116]. Clinical studies suggest that myopericarditis may occur in 1 in 175 adults who receive primary vaccination against smallpox [117]. However, most cases appear to be mild and self-limited with few documented cases of dilated cardiomyopathy [115, 118]. The pathophysiology of myopericarditis associated with smallpox vaccine remains unclear. However, the absence of direct viral invasion seen by histopathologic examination of myocardial tissue from vaccinees with myocarditis suggests this may be an immune-mediated phenomenon [115, 119]. While temporally associated cardiac ischemia and myocardial infarction have been observed among smallpox vaccinees, the incidence of these complications does not exceed the expected background rates, and there is currently no evidence to suggest a causal association with vaccination [115, 119].

4 Monkeypox

4.1 Historical Background

Monkeypox was so named due to its identification in 1958 during an outbreak of a pox-like illness in captive monkeys in Denmark [2, 120]. Initially, this gave rise to concerns that smallpox eradication would not be possible if monkeys served as a reservoir for variola virus. However, subsequent laboratory analyses revealed the monkeypox virus to be a distinct species within the *Orthopoxvirus* genus. The first case of human monkeypox was identified in the Democratic Republic of the Congo (DRC) in 1970 [121, 122]. Further human cases were confirmed when nine samples from Zaire, Nigeria, Cote d'Ivoire, Sierra Leone, and Liberia tested positive for monkeypox virus as part of the efforts of the Commission to Certify Smallpox Eradication in West Africa and the Congo Basin sponsored by the WHO [123]. Since that time, sporadic epizootics continue to occur primarily in Central Africa with evidence suggesting the incidence has increased since the cessation of smallpox vaccination [124]. The emergence of human monkeypox cases in the United States in 2003 and in South Sudan in 2005 further demonstrates the potential for outbreaks in locales discrete and/or distant from the historic geographic range of the virus.

4.2 Methods for Epidemiologic Analysis

In the decade following the discovery of human monkeypox infection, 54 cases were identified and investigated in Central and West Africa providing the first opportunities for epidemiologic analyses [125, 126]. Following the eradication of smallpox, the WHO sponsored enhanced surveillance efforts for monkeypox in the DRC during 1980–1986 which detected 350 cases with the assistance of a monetary reward for reporting [126]. Between 1987 and 1995, surveillance efforts were greatly reduced and few cases were reported. A large outbreak in Zaire in 1996 led to further studies over the ensuing 2 years that were partially hampered by civil unrest [126]. The first appearance of human monkeypox outside of the African continent occurred in the United States in 2003 and provided new insights into the epidemiology of the disease outside of the geographic locations and populations typically affected. Human monkeypox continues to occur in the DRC, and efforts to improve prevention and control through enhanced surveillance remain ongoing.

4.3 Descriptive Epidemiology

With the exception of isolated outbreaks in the United States and Sudan, human monkeypox is limited geographically to Central and West Africa. Males and females are affected equally. Younger age groups are disproportionately affected; 90 % of cases identified in the DRC during 1980–1985 were less than 15 years old [127]. As with smallpox, the vaccination status of an affected population may influence the age distribution of monkeypox cases in an outbreak. However, more recent investigations of outbreaks occurring 20–30 years after the cessation of smallpox vaccination have found that the incidence and attack rates for monkeypox remain highest among those 14 years and younger [124, 128]. Furthermore, recent data suggest that the incidence of monkeypox is increasing in rural Africa due to the growing proportion of unvaccinated individuals in the population, waning immunity in vaccinated individuals, decreasing numbers of smallpox survivors, and human encroachment into areas inhabited by animal reservoirs [124, 128–130]. Most reported deaths occur in unvaccinated children under the age of 14 years. While the overall case fatality rate in Africa is typically ~10 % (range 3.7–11.3 %), rates as high as 14.9 % have been seen among the youngest age groups (0–4 years) [2, 127, 128]. In contrast, no deaths were observed in 37 confirmed monkeypox cases in North America [131, 132]. Differences in the level of medical care administered, route of infection, and underlying host factors are all potential contributors to this disparity in lethality. In addition, genetically distinct isolates of monkeypox virus have been identified from West Africa and the Congo Basin [130, 133–135]. The West African clade has shown decreased virulence compared to the Congo Basin clade in animal models of monkeys, mice, and prairie dogs; the West African monkeypox virus identified in North America may also have contributed to the

absence of fatalities and relatively mild disease observed in the 2003 outbreak [134, 136–138]. Serologic surveys of persons without vaccination scars were performed in the 1980s in Central and Western Africa to estimate the prevalence of monkeypox. These studies found that 1,583/10,300 (15.4 %) sera tested demonstrated orthopoxvirus antibodies by hemagglutination inhibition or immunofluorescence assays [125]. Of these positive sera samples, 420 were tested using a more monkeypox-specific serologic assay (the radioimmunoassay adsorption test), 73 of which gave positive results [125]. Prevalence rates did not vary significantly between the African regions surveyed, providing further epidemiologic evidence for a difference in virulence between West African and Congo Basin monkeypox virus clades when one considers that fewer than 10 % of monkeypox cases and no fatalities occurred outside of the Congo Basin [125, 134]. Initial studies through 1979 evaluating seasonality reported that most cases occurred during the dry season (December, January, and February), whereas surveillance involving the use of monetary incentives to report cases during 1981–1986 showed peak incidence in June, July, and August [125, 139].

The complete host range of monkeypox remains uncertain. Humans and monkeys appear to be incidental hosts, and growing evidence suggests that one or more species of squirrels or rodents serve as the animal reservoir for monkeypox virus [140–142]. However, monkeypox virus has only been isolated from one animal infected in the wild – a squirrel (*Funisciurus anerythrus*) with skin eruptions captured in 1985 near a village where human monkeypox was previously identified [140]. More recently, prairie dogs (*Cynomys* species) housed with rodents from West Africa were implicated as vectors in the North America outbreak in 2003 [132, 143]. This discovery has led to the establishment of prairie dogs as an animal model for monkeypox infection [138].

4.4 Mechanisms and Routes of Transmission

As a primarily zoonotic infection, monkeypox virus is most commonly transmitted to humans through direct contact with the blood, bodily fluids, or lesion exudates of infected animals. The source and types of animal exposures reported by primary contacts prior to rash onset are varied. Case control studies have implicated hunting, skinning, trapping, eating, cooking, or playing with carcasses of nonhuman primates, terrestrial rodents, antelopes, gazelles, and tree squirrels [123, 125]. These studies, however, were confounded by multiple animal exposures in both controls and cases.

Secondary human-to-human transmission occurs though inhalation of virus-containing respiratory droplets or direct contact with lesion exudates from an infected person. Additionally, vertical transmission has been reported in one case of congenital monkeypox [144]. Despite the ability to spread from person to person, monkeypox does not exhibit sustained transmission within human populations. The secondary attack rate of 9.3 % among unvaccinated household contacts documented in African outbreaks is far lower than that observed for smallpox [123, 145]. Moreover, the longest documented chain of uninterrupted human-to-human transmission lasted six generations [146]. Secondary transmission may also be influenced by the clade of virus involved; the longest chain of interhuman transmission occurred in the Congo Basin, whereas no human-to-human transmission of West African monkeypox virus has been documented [139, 147].

4.5 Pathogenesis and Immunity

The pathogenesis and clinical presentation of monkeypox resembles that of ordinary smallpox in many respects. Following exposure, patients enter an asymptomatic incubation period lasting approximately 12 days (range 7–21 days) [2, 123, 125, 128]. The subsequent prodromal phase is usually heralded by fever and often accompanied by headache, prostration, back pain, myalgia, and malaise. In contrast to smallpox, up to 90 % of patients with monkeypox also display prominent lymph node enlargement during this phase [2]. The lymphadenopathy is most often generalized but can sometimes be localized to the cervical or inguinal regions and present as 1–4 cm firm, tender, and occasionally painful lymph nodes [2, 125]. As with smallpox, the rash of monkeypox progresses from macules to papules to vesicles to pustules before umbilicating, crusting, and separating to leave a pitted scar. The rash lesions are typically found in the same stage of development and are distributed peripherally including the palms and soles [2, 125] (Fig. 21.6).

In total, the illness generally lasts 2–4 weeks. Potential complications include secondary bacterial infections of the skin and respiratory tract, severe dehydration secondary to vomiting and diarrhea, and keratitis and corneal lesions which can lead to blindness [123, 125]. The severity of disease is correlated not only with the source of infection (Congo Basin vs. West Africa) but also with the mechanism of transmission. Monkeypox cases reporting a bite or scratch exposure were more likely to experience systemic signs or symptoms of disease and undergo hospitalization than those with only noninvasive exposures [148].

Recent vaccination against smallpox (within 3–19 years) is estimated to provide 85 % protection against monkeypox based on comparisons of attack rates between vaccinated and unvaccinated contacts of index cases in Africa [2, 123, 145]. The monkeypox outbreak in North America in 2003 provided some evidence that vaccine-induced immunity may be maintained for decades and confer a reduced risk of infection [63, 124]. However, remote vaccination (>30 years) did not provide complete protection against monkeypox [131, 143, 149].

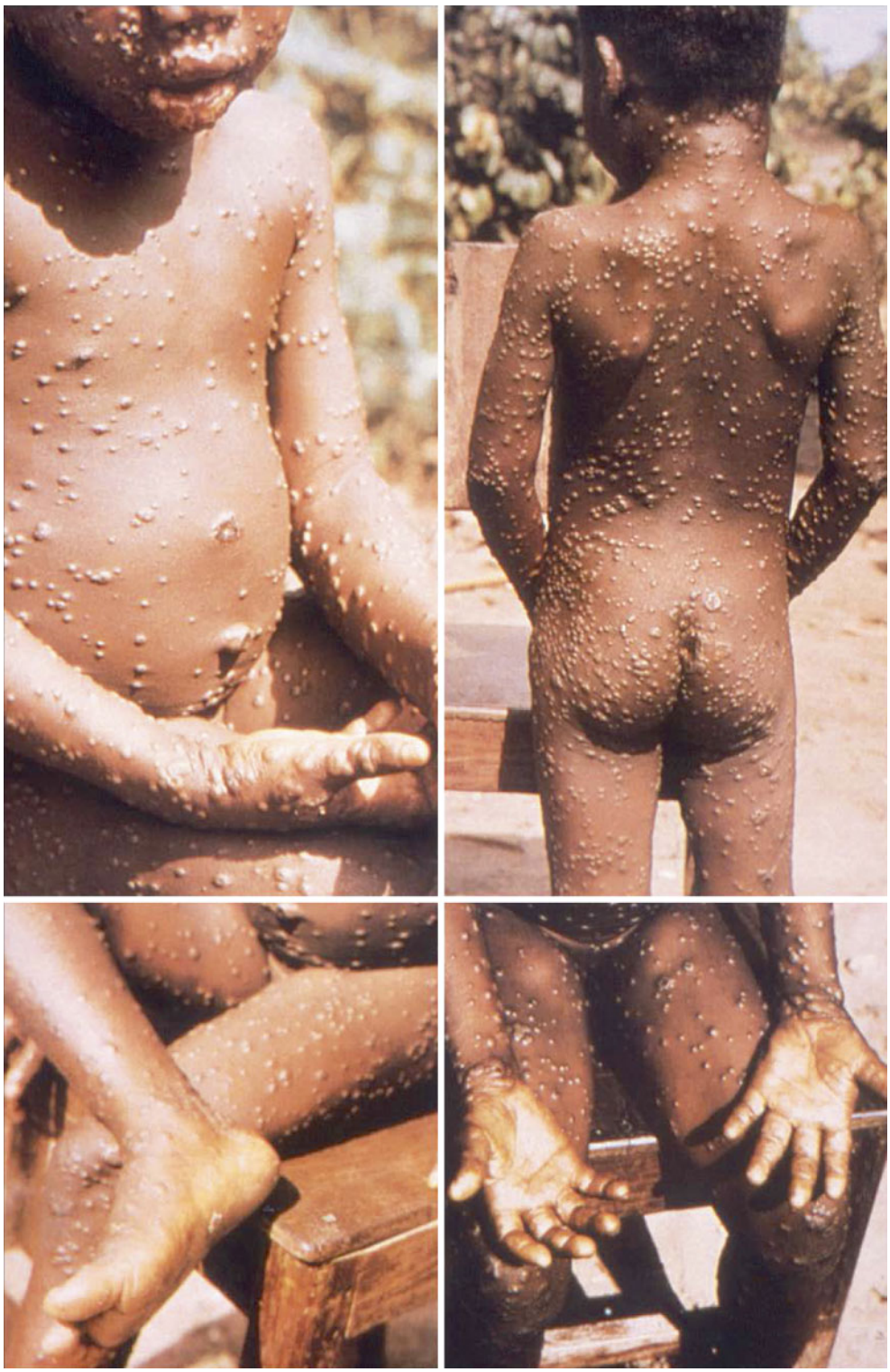

Fig. 21.6 Images of the vesiculo pustular rash of monkeypox. A centrifugal distribution, and lesions on the palms and soles, are characteristic of the generalized rash

5 Prevention, Control, and Treatment

The cross-protective immunity provided by smallpox vaccine makes vaccination an effective method of prevention for all orthopoxvirus infections. Vaccine technology has advanced significantly from the first-generation smallpox vaccines used during the eradication campaign such as Dryvax that were typically propagated in the skin of livestock animals. Second-generation smallpox vaccines are manufactured using modern cell culture techniques but are fully replicative live virus vaccines believed to have essentially equivalent efficacy and safety profiles to the first-generation vaccines. ACAM2000®,

a clonal derivative of the NYCBOH/Dryvax smallpox vaccine, is the prototypical second-generation smallpox vaccine that is currently the principal vaccine stockpiled by the United States for use in an emergency [117]. Third-generation smallpox vaccines are live virus vaccines that have been attenuated to improve the safety profile and decrease the risk of adverse events in high-risk populations. IMVAMUNE® is one such vaccine attenuated through multiple passages in chicken embryo fibroblasts such that it is unable to complete a full replication cycle in mammalian cells [150]. Lastly, fourth-generation vaccines remain in development and include protein subunit, DNA, and recombinant vaccines [151].

The global eradication campaign implemented a strategy of ring vaccination in combination with rapid case identification and isolation to successfully achieve its goals. These concepts have been incorporated into modern plans for responding to the use of smallpox as an agent of bioterrorism [152]. A similar approach would be unlikely to succeed in eradicating monkeypox, however, owing to the ability of the virus to propagate within an animal reservoir. Furthermore, implementing a vaccination program to control monkeypox would be challenging due to the relatively low incidence in remote areas that are difficult to access, risks of adverse events, and associated costs [153]. As such, most programs to prevent human infection with monkeypox currently focus on reducing the risk of exposure to animals that can transmit the virus, controlling the trade of animals to prevent the spread of virus outside of Africa, and avoiding close physical contact to limit human-to-human transmission after an outbreak has already occurred. Similarly, vaccinia and cowpox infections can be prevented by avoiding exposures to the animal vectors. Following smallpox vaccination, proper care of the vaccination site is also critical in preventing inadvertent transmission of vaccinia virus.

Limited options are available for treating orthopoxvirus infections after the onset of clinical signs and symptoms. Vaccinia immunoglobulin is the only product currently licensed by the US Food and Drug Administration (FDA) for the treatment of an orthopoxvirus infection (i.e., vaccinia). The licensed indications include eczema vaccinatum, progressive vaccinia, severe generalized vaccinia, vaccinia infections in persons with skin conditions (e.g., eczema, burns, impetigo, varicella zoster, or poison ivy), and aberrant vaccinia infections in areas that would constitute a special hazard such as the eyes or mouth [110].

Though no FDA-approved antivirals are currently licensed for treatment of orthopoxvirus infections, several drugs with antiviral activity against these viruses have been identified and are in clinical development [154, 155]. One such drug is cidofovir, a nucleotide analog with broad antiviral activity against a number of DNA viruses. It is thought to inhibit viral DNA polymerases as well as the proofreading activity of the poxvirus exonuclease [154, 156]. Cidofovir has demonstrated anti-orthopoxvirus activity in vitro and protection against mortality in mouse models of vaccinia and cowpox [154, 156, 157]. However, cidofovir has low oral bioavailability and must be administered intravenously. The development of CMX001, a lipid conjugate of cidofovir, provides several potential advantages over cidofovir including increased oral bioavailability allowing oral administration, decreased toxicity, and enhanced antiviral activity due to greater cellular uptake [158]. Initial safety and pharmacokinetic studies in humans have been successful in achieving predicted therapeutic plasma concentrations with no dose-limiting adverse events reported [158, 159].

ST-246 is another orally bioavailable drug with antiviral activity specific for poxviruses. It was discovered using a high throughput screening approach that assessed compounds for their ability to inhibit virus-induced cytopathic effects [158, 160]. ST-246 acts as an inhibitor of extracellular virus formation through by targeting a major envelope protein critical to this process [160]. Animal studies in mice, rabbits, and nonhuman primates have shown ST-246 to be efficacious in preventing death following challenge with lethal doses of vaccinia virus, rabbitpox virus, monkeypox virus, and variola virus [160–162]. This protective effect was seen even when treatment was begun up to 3 days after the viral challenge was administered in most cases [158]. Clinical evaluation of ST-246 in humans has been limited to safety and pharmacokinetic studies and rare instances of investigational and compassionate use in treating orthopoxvirus infections [86, 87, 90, 163, 164]. Notably, the development of resistance to ST-246 during the course of treatment of one case of progressive vaccinia has been documented [164]. The clinical significance of this finding remains unclear, however, as the patient did ultimately recover. ST-246 has recently entered the US Strategic National Stockpile and is intended to be used in the treatment of smallpox disease in the event of an outbreak. This would likely occur under an investigational new drug and/or emergency use authorization regulatory mechanism given its current status as an unlicensed product.

New options for the treatment of orthopoxvirus infections continue to be sought. For example, a nineteenth-century therapy for smallpox involving a botanical preparation of the carnivorous plant *Sarracenia purpurea* has recently demonstrated antipoxvirus activity against vaccinia virus, monkeypox virus, and variola virus [165]. However, establishing the efficacy of antiviral therapy in treating human orthopoxvirus infections remains difficult due to the low incidence of cases requiring treatment. In particular, evaluation of the potential synergistic effects of combination antiviral therapy suggested by animal studies is not possible with the limited human data available [166]. While the FDA has the ability to approve drugs or license biological products on the basis

of animal studies when human efficacy studies are not ethical or feasible, only two drugs (neither targeting smallpox) have been approved under this so-called animal rule [167]. Further research is needed to help bridge the gap between human and animal efficacy studies and inform the optimal utilization of antivirals for the treatment of orthopoxvirus infections.

6 Unresolved Problems

The orthopoxviruses continue to present many unanswered questions and unresolved problems with direct relevance to human health. As the 2003 monkeypox outbreak in the United States and increasing incidence of monkeypox in Africa demonstrate, the threat of smallpox being used as a weapon of bioterrorism is not the only threat orthopoxviruses pose to human health. Orthopoxviruses circulate widely among various animal species throughout the world. However, neither the prevalence of these viruses in nature nor the risk of zoonotic transmission to humans is well understood [168–170]. Furthermore, there exists the possibility that an orthopoxvirus could mutate into a more highly virulent human pathogen as very likely occurred with smallpox initially many millennia ago [171]. As such, continued efforts to develop antivirals, improved vaccines, and diagnostic assays specific to orthopoxviruses are justified.

The role of research using live variola virus in these efforts and the timing of the destruction of the remaining stores of variola virus are both topics that remain under active debate. Arguments have been made both for and against the immediate destruction of the remaining stockpiles [51, 171]. Most recently, in 2011 the World Health Assembly (WHA) postponed making a decision on setting a date for destruction until 2014. Regardless of the WHA decision, advances in biotechnology and increasing laboratory capabilities and sophistication worldwide may make synthesizing variola virus de novo or engineering more virulent orthopoxviruses more easily achievable [171, 172]. Similar technologies have been applied to the development of vaccinia as a viral vector for recombinant vaccines as well as immunotherapeutic and oncolytic cancer therapies [151]. In fact, over 30 active clinical trials involving vaccinia viruses are currently registered with the US National Institutes of Health at www.clinicaltrials.gov. The widespread implementation of a recombinant vaccinia vector could result in inducing cross-protective immunity against orthopoxviruses in a significant proportion of the population. This would substantially alter the susceptibility of humans to orthopoxvirus infections and directly impact response activities in the event of an outbreak of smallpox. At present, however, continued research and preparedness planning to address the threat of smallpox and other orthopoxviruses remains a prudent investment.

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Paramyxoviruses: Henipaviruses

22

Stephen P. Luby and Christopher C. Broder

1 Introduction

The henipaviruses are species of the genus *Henipavirus*, family *Paramyxoviridae,* and subfamily *Paramyxovirnae*. The natural reservoir of the three known henipaviruses, Hendra virus, Nipah virus and Cedar virus, are old world fruit bats of the genus *Pteropus* (order *Chiroptera*, suborder, *Megachiroptera*, family *Pteropodidae*). Human infection with either Hendra or Nipah virus causes a widespread vasculitis that commonly progresses to a fatal encephalitis or pneumonia. People who recover from acute infection with either of these henipaviruses are at risk of recrudescent infection.

2 Historical Background

Hendra virus, previously referred to as equine morbillivirus, was first identified in an outbreak in September 1994 in Hendra, a suburb of Brisbane, Queensland, Australia [1, 2]. Nineteen horses residing in or immediately next to the outbreak stable developed illness; 14 horses died. At autopsy the lungs were heavy and edematous with hemorrhage and frothy secretions in the airways. Histopathological investigations identified interstitial pneumonia with focal necrotizing alveolitis and syncytial giant cells within the vascular endothelium [3].

Two employees at the stable, a 40-year-old male stable hand and a 49-year-old male horse trainer, had particularly close contact with the index mare during the final stages of her fatal illness. The horse trainer, whose hands and arms had abrasions, attempted to force feed the mare by placing his bare hands with food into the sick mare's mouth. Both the stable hand and the horse trainer became ill 5–6 days after the death of the mare with fever, myalgia, headaches, lethargy, and vertigo. The stable hand remained lethargic for several weeks but eventually recovered. The horse trainer developed progressive respiratory failure and died. His autopsy findings were consistent with interstitial pneumonia, with focal alveolitis and syncytial formation [2]. An identical virus, which was ultimately named Hendra virus, was grown from samples from both the affected horses and the affected people [3].

Nipah virus was first recognized in a large human encephalitis outbreak in peninsular Malaysia [4–6]. The initial cases were identified among pig farmers who lived near the city of Ipoh within the state of Perak in late September 1998. Patients presented with fever and headache; many progressed to unconsciousness and death [7]. By December 1998, larger clusters of similar cases were reported within the Port Dickson District of Negeri Sembilan, 300 km south of Ipoh [8]. In March 1999, a novel paramyxovirus was isolated from the cerebrospinal fluid of an affected patient from Sungai Nipah village [9]. Immunostaining demonstrated the virus within the pathological lesions confirming it as the cause of the outbreak [4]. Ultimately, the Malaysian Ministry of Health reported 283 cases with 109 (39 %) fatalities [6]. Cedar virus, is a henipavirus isolated from urine of *Pteropus* bats in Australia and reported in 2012 [10]. Although Cedar virus readily infects guinea pigs and ferrets in the laboratory, unlike Hendra or Nipah virus it does not cause serious illness in these animals [10]. Human infection with Cedar virus has not been described.

RNA consistent with a novel henipavirus was identified from feces of *Eidolon helvum*, the African Straw-colored fruit bat in Ghana in 2008 [11]. Subsequent sampling of 6 bat species across 5 African countries have identified a diversity of henipavirus like RNA sequences, and the full genome of

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an apparently novel Henipavirus was characterized [12]. Human infection with African henipaviruses has not been described.

3 Methodology Used in Epidemiologic Analysis

3.1 Wild Animal Studies

The known henipaviruses are not part of the normal human microbiota. Their ecological niche is within old world fruit bats of the family *Pteropodidae* [13, 14]. Closely related henipaviruses have been identified among different *Pteropodidae*. Nipah virus infects *P. vampyrus, P. hypomelanus, P. lylei*, and *P. giganteus* [15–17]. Hendra virus infects all four *Pteropus* species in Australia [18]. RNA fragments suggestive of closely related henipaviruses were collected from feces of *Eidolon helvum*, a fruit bat from family *Pteropodidae* that lives in Africa [19]. These infections of genetically related *Pteropodidae* with genetically related henipaviruses suggests that these viruses coevolved with their *Pteropodidae* hosts [19, 20].

Although not part of the lifecycle of the henipaviruses, humans and many other mammals are susceptible to infection, infections that can have catastrophic consequences. When a human infection with henipavirus is recognized, an underlying question is how this bat virus got into a human.

3.2 Outbreak Investigations

Most of what is known of the epidemiology of human infection with henipaviruses comes from outbreak investigations. Investigators have conducted in-depth case studies to describe unique or illustrative situations. This has been particularly important for Hendra virus, because so few human infections have been recognized. Outbreak investigators have conducted case–control studies and evaluated cohorts of people who were potentially exposed to henipavirus to assess risk factors for transmission.

To complement outbreak investigations, scientists have conducted serological surveys to explore the frequency of unrecognized infection and the risk to groups who were not implicated in outbreak investigations.

3.3 Surveillance

Recurring Nipah virus outbreaks in Bangladesh have characterized the population at risk sufficiently to permit prospective surveillance for the virus in hospitals which have repeatedly identified cases [21]. Nipah virus represents less than 2 % of all cases of encephalitis presenting to these hospitals, so prospective individual level diagnostics are only conducted at the three facilities that have identified the most cases of Nipah virus during the 3 months, January through March, when the most cases have been identified. To efficiently identify outbreaks of Nipah virus in Bangladesh over a broader geographical area and wider seasonality, a complementary surveillance activity focuses on identifying clusters of patients with encephalitis, that is, at least two people who live within a 30-minute walk of each other and who within 2 weeks of each other developed symptoms of meningoencephalitis including fever, new seizures, or mental status changes [22].

3.4 Laboratory Diagnosis

3.4.1 Virus Detection

The most definitive confirmation of henipavirus infection is recovery of the virus from a patient sample. Both Hendra and Nipah virus grow readily on Vero cell culture [3, 4]. Henipavirus has been successfully cultured from human cerebrospinal fluid, respiratory secretions, and urine [9, 23, 24]. Both Nipah and Hendra virus can be identified through polymerase chain reaction (PCR) using either nested or real-time platforms. Different laboratories have used different primers. PCR has been used successfully on respiratory secretions, cerebrospinal fluid, blood, and urine [23, 24–28]. Two research groups have developed a recombinant vesicular stomatitis virus that expresses the F and G protein of Nipah virus (pseudotyped virus) [29–31]. This pseudovirus-based assay can be used safely in a biosafety level II laboratory, has high analytical sensitivity, and provides an alternative more rapid assessment of virus neutralization.

3.4.2 Measurement of Virus-Specific Antibodies

Although there are no commercially available tests for serological diagnosis of henipavirus in humans, several laboratories have developed enzyme-linked immunosorbent assays (ELISA) to detect antibody against Nipah and/or Hendra virus. To secure henipavirus antigen for the ELISA, some laboratories grow henipavirus-infected cells in tissue culture and then use various procedures to purify viral materials that are then used to detect antibody in samples [32–34]. Since most countries consider Nipah and Hendra virus as biosafety level IV pathogens, only laboratories with biosafety level IV facilities can produce reagents, and the disease occurs in places where there are few such laboratories. Alternatively, some laboratories have developed recombinant henipavirus antigens [35–38]. These recombinant-based ELISAs do not require a biosafety level IV laboratory to develop, but their test performance has not been evaluated against a wide diversity of samples. Using either viral culture-derived or recombinant henipavirus antigens, laboratories have developed indirect ELISA assays to detect Henipavirus-specific IgG antibodies and capture ELISA to detect Henipavirus-

specific IgM antibodies [32, 35]. These ELISA assays provide an efficient approach to detect antibody and to screen a high volume of samples, for example, in a surveillance system. However, since the system depends on antibody detection, its diagnostic sensitivity early in illness is suboptimal. Among patients tested within the first 4 days of illness, 30 % of those ultimately confirmed as infected with Nipah virus were anti-Nipah IgM antibody negative by ELISA [33].

3.4.3 Clinical Diagnosis of Henipavirus Infection

During outbreaks in Bangladesh, many people die from apparent Nipah virus infection before samples can be collected or before they develop IgM antibodies [39]. During such outbreaks, clinical assessment is sometimes sufficient for diagnosis. When previously healthy people develop characteristic symptoms of Nipah virus infection – fever with new onset seizures or mental status change – and when they are linked to a laboratory confirmed case, either because they shared a common exposure that was implicated in the transmission of Nipah or because of exposure to the respiratory secretions of a confirmed case, they are generally classified as probable cases [39]. The risk of misclassification is small, because the rapid onset of a fatal illness with symptoms of meningoencephalitis is uncommon. It is unlikely that such an uncommon event would occur from a different etiology during a Nipah outbreak.

4 Biological Characteristics

4.1 Morphology and Morphogenesis

Henipavirus particles are enveloped and pleomorphic, ranging in size from 40 to 1,900 nm and can vary from spherical to filamentous forms when imaged by electron microscopy [3, 40, 41] (Fig. 22.1).

The envelope carries surface projections composed of the viral transmembrane anchored fusion (F) and attachment (G) glycoproteins. Hendra virus possesses a double-fringed appearance of spikes, whereas Nipah virus appears to have a single fringe of projections [41]. Like other paramyxoviruses, the henipavirus genomes are an unsegmented, single-strand, negative-sense RNA [42, 43]. At the time of their discovery, the genomes of Hendra and Nipah virus were the largest among all members of the *Paramyxoviridae* family, one factor that justified their separation into their own genus, *Henipavirus* [44]. Henipavirus genomes conform to the "rule of six," i.e., the total number of nucleotides is evenly divisible by six [45], an important feature in the way the nucleocapsid (N) protein interacts with the viral genomic RNA [43]. The RNA genome in association with the N protein is referred to as the ribonucleoprotein core that has a characteristic herringbone appearance by electron microscopy. The ribonucleoprotein core is contained within a lipid bilayer (envelope) that is derived from the infected host cell during virus assembly and budding.

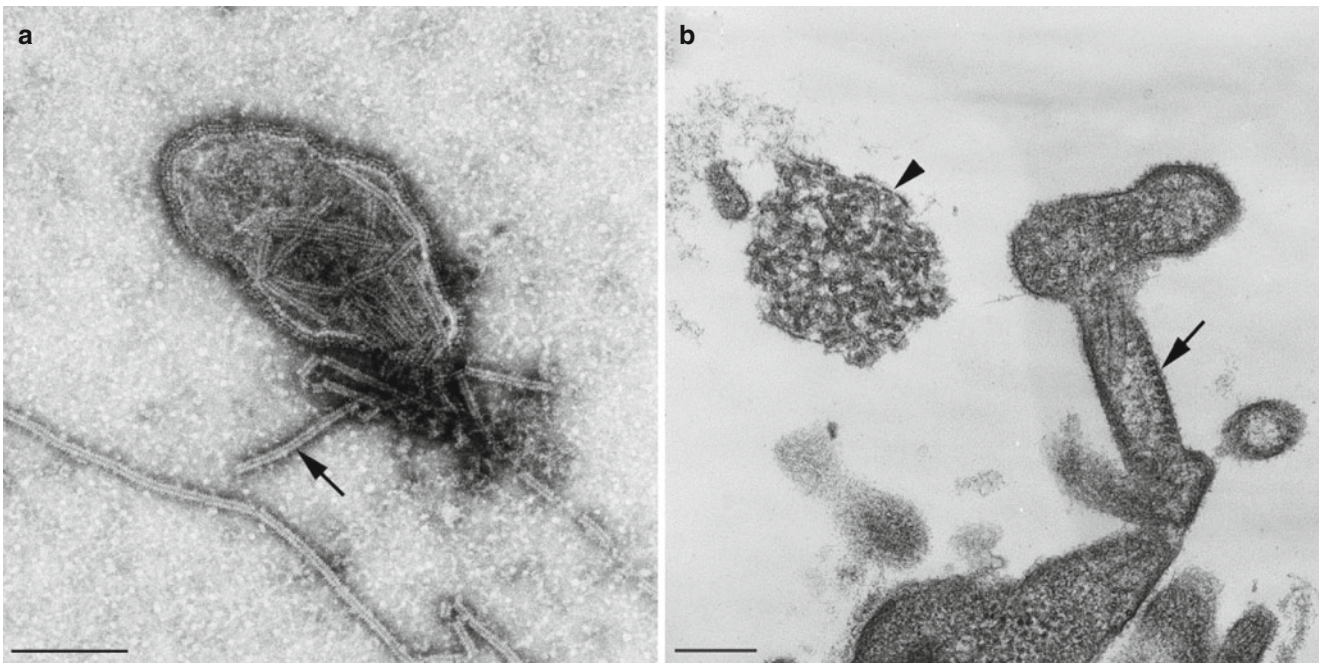

Fig. 22.1 Henipavirus particles visualized by electron microscopy. (**a**) Transmission electron micrograph of Hendra virus negative stained with 2 % phosphotungstic acid. *Arrow* indicates the nucleocapsids being released. Scale bar represents 200 nm. (**b**) Transmission electron micrograph of Nipah virus-infected Vero cells in thin section. *Arrow* indicates virus budding from the plasma membrane with underlying profiles of transversely sectioned nucleocapsids. *Arrowhead* indicates a released virus particle with internal nucleocapsids, the membrane is disrupted. Scale bar represents 200 nm (Images courtesy of the AAHL Biosecurity Microscopy Facility, Australian Animal Health Laboratory (AAHL) Livestock Industries CSIRO, Australia)

The relative gene order of the henipaviruses is conserved as compared to other paramyxoviruses with the N gene first, followed by P (phosphoprotein), M (matrix), F, G, and L (large/polymerase) genes (in a 3′–5′ order) [42]. The N, P, and L proteins form a complex that replicates the viral RNA; polymerase activity resides within L [43, 45]. The henipavirus P gene is also the largest among the paramyxoviruses and encodes the V and W proteins through a transcriptional editing mechanism that adds untemplated G nucleotides. An alternative start site within the P gene encodes the C protein [42]. The henipavirus M protein, which underlies the viral membrane, organizes viral proteins during virion budding from the host cell; the Nipah virus M protein can independently bud from expressing cells forming virus-like particles (VLPs) [46, 47]. Finally, the G and F envelope glycoproteins project from the surface of the virion as well as on infected cells and are essential for the binding and entry steps of virus infection (reviewed in [48]). The G glycoprotein attaches the virion to host cell via receptors and the F glycoprotein fuses the viral membrane with the host cell membrane (reviewed in [49]). The G and F glycoproteins are also the principal antigens to which virtually all henipavirus-neutralizing antibodies are directed and are the major components or targets of well-advanced vaccine and antiviral strategies (reviewed in [50]).

4.2 Physical Properties

As enveloped paramyxoviruses, Hendra and Nipah virus would be expected to be more labile to environmental factors as compared to non-enveloped virions; however, there is some evidence of persistence. A patient with confirmed Nipah virus infection in Malaysia who neither entered nor went near pig farms prior to his illness worked repairing pig cages [51]. His illness suggests that pig secretions or excretions remain infectious at least for hours and perhaps for days. Nipah virus spillover events in Bangladesh have been attributed to contaminated food [52, 53]. In the laboratory, henipavirus survived greater than 4 days at 22 °C in pH-neutral fruit bat urine and survived in various fruit juices from several hours to 4 days depending on temperature and pH [54].

5 Descriptive Epidemiology

5.1 Hendra

All four *Pteropus* species native to Australia commonly have serum antibody against Hendra virus [18] and three of the four have been confirmed to shed Hendra virus, at least occasionally, in their urine [55]. From 1994 to 2012, only seven human infections with Hendra virus have been recognized; 4 (57 %) died and 1 reportedly has serious, ongoing health problems (www.outbreak.gov.au). All confirmed human Hendra virus infections occurred among people who had close contact with a sick horse in Queensland, Australia. Serological studies among people who cared for ill horses, among healthcare providers who cared for Hendra virus-infected humans [23, 56], and among Australians with close contact with *Pteropus* bats have identified no evidence of asymptomatic infection [57]. Apparently, human infection with Hendra virus occurs rarely.

5.2 Nipah

5.2.1 Malaysia/Singapore

In the large Malaysian Nipah outbreak in 1998, cases occurred almost exclusively among people who worked on pig farms. Males and persons of Chinese ethnicity were more likely to be pig farmers in Malaysia and were more likely to be infected with Nipah virus [58]. Abattoir workers in Singapore who handled pigs imported from Malaysia during the Malaysian Nipah outbreak also became infected [59]. Among asymptomatic persons who lived or worked on pig farms where cases of human Nipah infection were identified, 11 % had antibody to Nipah virus [58]. The human outbreak of Nipah virus infection ceased after widespread deployment of personal protective equipment to people contacting sick pigs, restriction on livestock movements, and culling over 900,000 pigs [60]. Malaysian authorities confirmed 265 cases of human Nipah virus infection and 105 deaths during the outbreak [4]. Authorities in Singapore confirmed 11 cases and 1 death [5]. Since the outbreak ended in 2013, no human or porcine Nipah virus infections have been reported from Malaysia or Singapore.

5.2.2 Nipah Bangladesh/India

The epidemiology of Nipah virus in Bangladesh/India infection has been quite different from Malaysia. In 2001 in Siliguri, India, 47 patients with encephalitis were linked in a web of person-to-person transmission among patients and healthcare workers who had contact with human Nipah cases [26]. Over 70 % of people infected with Nipah virus in Bangladesh and India have died [26, 52]. In Bangladesh, all recognized Nipah outbreaks have occurred in rural communities. Both adults and children have been infected, including many children age 5–15 years [52]. Nipah cases have been remarkably clustered in both space and time [61]. All but one of the recognized human infections with Nipah virus in Bangladesh have occurred in the west, central, and northwest regions. Cases have occurred disproportionately in close proximity to major rivers. The two reported outbreaks from India have occurred within 50 km of the border with Bangladesh [26, 62], adjacent to areas where human cases have been recognized in Bangladesh (Fig. 22.2).

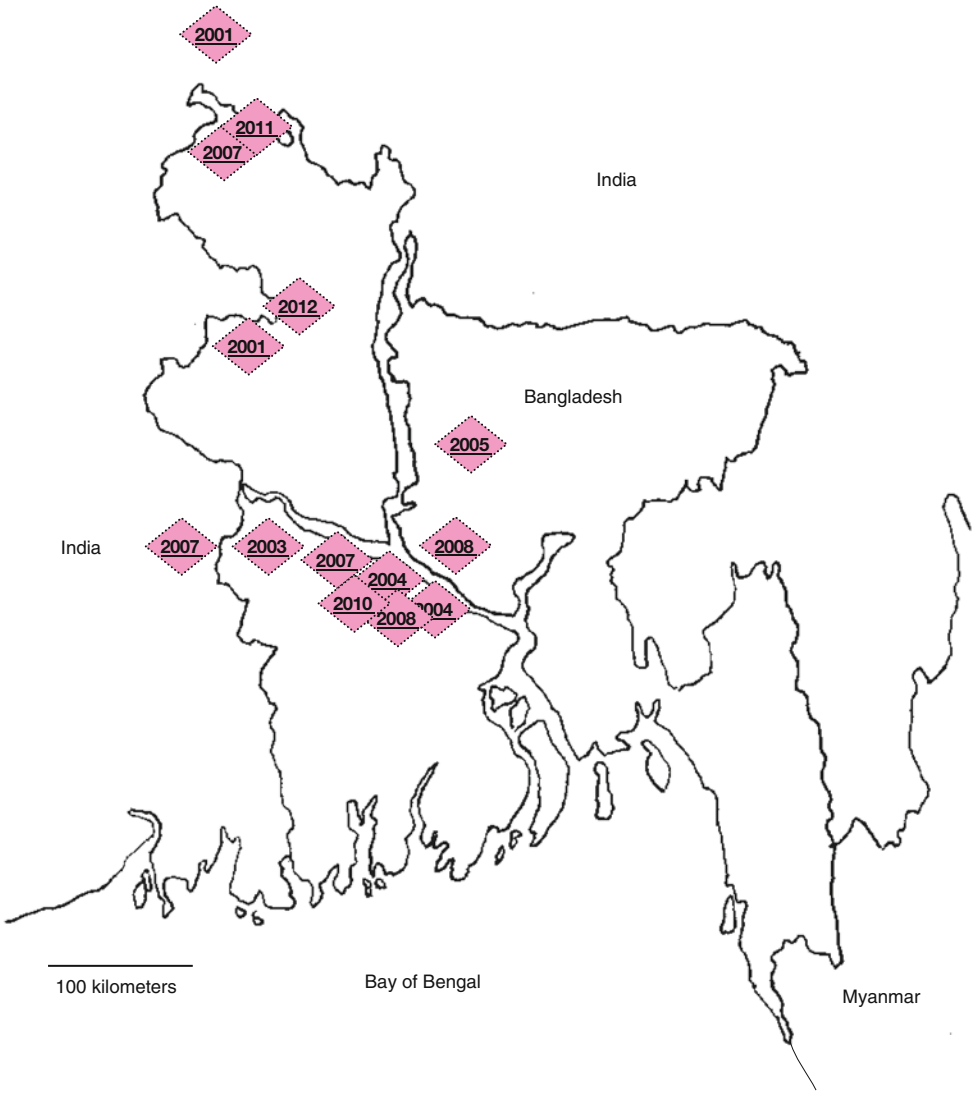

Fig. 22.2 Location of Nipah outbreaks in Bangladesh/India 2001–2012

Surveillance is particularly intense in these areas where outbreaks have repeatedly been identified, but the Government of Bangladesh maintains national surveillance for outbreaks and investigates clusters of various sorts of illness throughout the country. All of the outbreaks which have been confirmed to be Nipah through March 2012 have occurred in this more restricted region. From 2001 to 2014, human Nipah cases were identified every year except in 2002 and 2006. Primary cases of human Nipah infection are those apparently acquired through contact with contaminated non-human secretion/excretions. Among 121 primary cases in Bangladesh, all have been identified between December and April with 79 (65 %) identified in January.

Sero-surveys of communities affected by outbreaks in Bangladesh [63] have identified a much lower proportion of asymptomatic infections than sero-surveys in Malaysia during the outbreak. A study of 104 healthcare workers who cared for Nipah patients identified two nursing students who had IgG antibody against Nipah virus suggesting prior infection [64].

6 Mechanism and Route of Transmission

6.1 Hendra

Four species of *Pteropus* bats, *P. alecto, P. conspicillatus, P. poliocephalus,* and *P. scapulatus*, live in Queensland, Australia [55]. Because of changes in their native habitat, *Pteropus* bats in Queensland now frequently occupy trees in urban communities [65]. Hendra virus has been recovered from urine collected underneath bat roosts of each of these species [55]. Longitudinal collection of bat roost urine specimens suggests that bat colonies shed Hendra virus intermittently without a regular seasonal pattern [55]. Hendra virus

has also been recovered from uterine fluid [13]. Through January 2012, 67 equine infections with Hendra virus have been recognized [66], though the precise pathways that the virus moved from *Pteropus* bats to horses are unknown.

The first recognized Hendra virus infection occurred in a pregnant mare that became ill while staying in an open paddock and was moved to a stable in the Brisbane suburb of Hendra and died 2 days later [2]. Between 8 and 11 days after the mare's death, 18 other horses residing in or near the stable became ill. Among 18 horses with clinical illness, 14 died, 12 horses from the Hendra stable, one horse staying in the paddock adjoining the stable, and one horse living on a neighboring property that had very close contact with horses from the Hendra stable. The index mare was the apparent source of all the subsequently infected horses since they developed illness within one incubation period (8–11 days after the death of the index mare). Whether the virus was transmitted directly from horse to horse or whether transmission was facilitated by the activities of people caring for the horses is unknown. The absence of a successive wave of infection among horses and the rarity of horse-to-horse transmission in subsequent investigations suggest that Hendra virus super spreader horses are exceptional.

In addition to the two employees at the Hendra stable who had particularly close contact with the index mare during the final stages of her fatal illness, other exposures in Queensland that resulted in horse-to-human Hendra virus transmission included a farm worker who cared for two sick horses, one with acute respiratory distress, and the other with a rapid onset of neurological symptoms. After both horses died, the farm worker assisted a veterinary surgeon during the necropsy of the two horses [25]. Throughout caring for the horses and the necropsy, the farm worker never wore gloves, mask, or protective eyewear.

A veterinarian developed Hendra infection after conducting a limited autopsy on a 10-year-old horse that died of a rapidly progressive respiratory illness with large amounts of bloodstained frothy nasal secretions [67]. While not wearing gloves or other personal protective equipment, the veterinarian reached deep into the carcass to examine internal organs and became heavily contaminated with the horse's body fluids. The two autopsy assistants and an adult family member who held the dying animal's head and were exposed to frothy bloody nasal secretions did not become ill and were seronegative for Hendra virus infection [67].

A 33-year-old male veterinarian and a 21-year-old female veterinary nurse became infected with Hendra virus during an outbreak that affected five horses in a veterinary practice in Thornlands, Queensland [23]. Both the veterinarian and the nurse performed nasal cavity lavage on a horse during the 3 days before the horse developed symptoms of what was later confirmed to be Hendra virus infection.

Each of the recognized human cases of Hendra virus had intimate contact with a Hendra virus-infected horse, usually with heavy exposure to respiratory secretions and without wearing personal protective equipment. Other people with close contact with these same horses did not develop Hendra virus infection. These observations suggest that Hendra virus is not easily transmitted from horse to human. It apparently requires a horse that is an unusually efficient transmitter and a person with a high exposure to infectious secretions.

6.2 Nipah

6.2.1 Malaysia

There are two *Pteropus* species native to Malaysia, *Pteropus hypomelanus* which is smaller and prefers island habitats and *Pteropus vampyrus*. Both species commonly have antibodies against Nipah virus [15], and their colonies intermittently shed Nipah virus in their urine [68, 69].

The precise mechanism of transmission of Nipah virus from *Pteropus* bats to pigs in Malaysia is not confirmed, though the index farm was surrounded by native *P. vampyrus* habitat, and both the index farm and other pig producers commonly raised mangoes, a food attractive to *P. vampyrus*, on the same property where they were raising pigs. Mathematical modeling suggests that multiple spillovers from bats into the pig population would be necessary to create a dynamic population with sufficient susceptible pigs to sustain Nipah virus transmission within pigs for months [70].

Unlike the severe illness seen in Hendra virus-infected horses, most pigs infected with Nipah virus had mild illness. Among three pigs infected with Nipah virus through experimental oral inoculation or sharing a cage with an inoculated pig, all developed asymptomatic infections [71]. On farms the case fatality among adult-infected pigs ranged from <1 to 5 % [72]. A minority of Nipah virus-infected pigs developed more severe illness with fever, agitation, trembling, and twitching accompanied by rapid labored respirations, increased drooling, and a nonproductive loud barking cough [72]. Pathological examination of severely affected pigs showed extensive involvement of the lungs with a giant cell pneumonia with multinucleated syncytial cells containing Nipah virus antigen in the lungs and epithelial cells lining the upper airways [4]. Nipah virus was recovered from respiratory secretions of infected pigs, and Nipah virus antigen was detected in renal tubular epithelial cells [4, 71].

Humans infected with Nipah virus in Malaysia were more likely than controls to have direct contact with pigs that appeared sick and to have close contact with pigs through feeding pigs, processing baby pigs, handling dead pigs, and assisting in breeding, birthing, or medicating pigs [58]. Abattoir workers in Singapore who developed Nipah virus infection were more likely than controls to be exposed to pig urine or feces from pigs that had been imported from Malaysia during the Malaysian Nipah virus outbreak [5].

The isolation of Nipah virus from pigs' lungs and respiratory secretions and the observation that human cases of Nipah virus infection had more direct contact with pigs than controls suggests that Nipah virus was transmitted from infected pigs to humans through contaminated saliva and possibly urine.

All human Nipah virus infections in the outbreak in Malaysia/Singapore in 1998–1999 may have been linked to a single event of Nipah virus transmission from an infected bat to an immunologically primed pig population, leading to a sustained porcine epidemic which in turn led to a human epidemic. The genomic sequences from Malaysian Nipah virus isolates from pigs and people were nearly identical [4, 73].

There was little evidence of person-to-person transmission of Nipah virus in Malaysia. Although asymptomatic infections were identified among members of the households of cases [58], these infections may have resulted from shared exposures to infected pig secretions or excretions. A cohort study enrolled 363 healthcare workers from three hospitals that cared for Nipah patients [74]. Healthcare workers reported skin ($n=89$) or mucus membrane ($n=39$) exposure to body fluids of Nipah virus-infected patients and needlestick injuries ($n=12$). None reported any serious illness, encephalitis, or hospital admission. None of the initial 363 serum samples had detectable Nipah virus IgG or IgM antibody by ELISA. Among 293 serum samples collected 30 days later, three (1 %) were positive for Nipah virus IgG antibody, though none had detectable IgM and all three were negative for anti-Nipah virus-neutralizing antibodies.

6.2.2 Bangladesh/India

Pteropus giganteus is the single *Pteropus* species currently living in Bangladesh. These bats commonly have antibody to Nipah virus [17, 75] and urine collected from *P. giganteus* occasionally contains detectable Nipah virus RNA [76].

Drinking raw date palm sap is the most common pathway of Nipah virus transmission from *Pteropus* bats to people identified in outbreak investigations in Bangladesh [21, 53, 77, 78]. Human Nipah virus outbreaks in Bangladesh and India coincide with the cool dry date palm sap harvesting season [52]. At the beginning of the season, the bark is shaved off of one side of the tree (*Phoenix sylvestris*) and a small hollow bamboo tap is placed at the base [79]. In the late afternoon, the date palm sap harvester scrapes the area where the bark was removed so the sap can flow freely and ties a 2–4 l clay pot underneath the tap. During the night, the sap rises to the top of the tree, and some sap oozes out from where the bark is denuded, flows through the tap, and drips into the clay pot. At daybreak, sap collectors gather the clay pots and often sell some of their sap to village residents who drink it fresh in the morning.

Pteropus bats occasionally shed Nipah virus in their saliva [80–82]. Infrared photography confirms that *P. giganteus* bats commonly visit date palm trees during collection and lick the sap stream [83] (Fig. 22.3). Infrared cameras placed in the seven trees that were the source of fresh date palm sap drunk by the human Nipah virus cases in the Manikganj/Rajbari outbreak in 2008 identified an average of four *P. giganteus* bat visits where the bat licked the sap stream per tree per night [21].

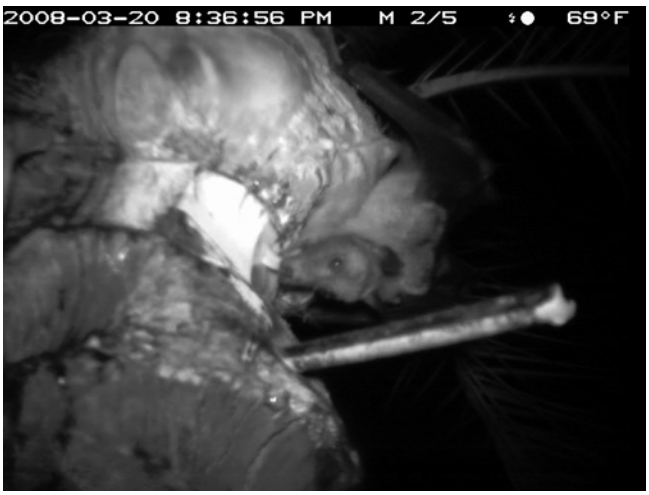

Fig. 22.3 Infrared photograph showing a *Pteropus* bat licking date palm sap from the shaved part of the tree

Nipah virus has not been isolated from a domestic animal in Bangladesh, but outbreak investigations have linked human Nipah virus cases to apparent domestic animal infections. The index case in the 2001 outbreak in Meherpur District developed illness on April 20, the latest post-winter onset of any confirmed Nipah virus outbreak in Bangladesh, past the end of the date palm sap season in most communities. Nipah virus cases in Meherpur were eight times more likely to report contact with a sick cow than controls [17]. In the Naogaon outbreak in 2003, Nipah virus cases were six times more likely than controls to report contact with a pig herd that visited the community 2 weeks before the human outbreak [84]. In 2004, a child developed Nipah virus infection 2 weeks after playing with two goats that developed an illness that began with fever and progressed to difficulty walking, frothing at the mouth, and death [61].

Person-to-person transmission of Nipah virus has been frequently recognized in Bangladesh/India. The two largest recognized outbreaks caused by person-to-person transmission include 47 linked cases in the Siliguri, India, 2001 outbreak mentioned above [26] and a person-to-person chain of transmission that infected 33 persons with Nipah virus in Faridpur, Bangladesh, in 2004 [27]. Nipah virus RNA has been frequently identified in the saliva of Nipah virus patients [24, 85]. In Faridpur, Nipah virus cases were more likely than controls to report touching a Nipah virus-infected patient who later died [27]. Similarly, Nipah virus-infected cases in Thakurgaon in 2007 were more likely than uninfected controls to have been in the same room when the index case was

coughing [75]. Across all recognized outbreaks in Bangladesh from 2001 to 2007, Nipah virus patients who had difficulty breathing during their illness were more likely to transmit Nipah virus than Nipah virus patients who did not have difficulty breathing (12 vs. 0 %, $p=0.03$) [52].

7 Pathogenesis and Immunity

7.1 Cellular Tropism and Host Range

A remarkable feature of the henipaviruses that separate them from all other paramyxoviruses is their exceptionally broad species tropism and ability to cause fatal disease in multiple vertebrate hosts including humans, monkeys, pigs, horses, cats, dogs, ferrets, hamsters, and guinea pigs, which, in addition to their bat reservoir hosts, spans 6 mammalian orders [81, 86–98]. The henipaviruses use host cell membrane proteins as entry receptors and bind to ephrin-B2 and ephrin-B3 using their G glycoproteins [99–102]. Human ephrin-B2 and ephrin-B3 are members of a large family of surface expressed glycoprotein ligands that bind to Eph receptor tyrosine kinases and both the ephrins and Eph receptors mediate bidirectional cell-cell signaling within the nervous, skeletal, and vascular systems [103, 104]. The ephrin-B2 and ephrin-B3 molecules are highly sequence-conserved proteins including among those hosts susceptible to henipavirus infection; the human, horse, pig, cat, dog, and flying fox have amino acid identities of 95–96 % for ephrin-B2 and 95–98 % for ephrin-B3 [105]. Ephrin-B2 expression is prominent in arteries, arterioles, and capillaries in multiple organs and tissues including arterial smooth muscle and human bronchiolar epithelial cells [106], while ephrin-B3 is found predominantly in the nervous system and the vasculature (reviewed in [107, 108]). The identification of ephrin-B2 and ephrin-B3 as entry receptors for Hendra virus and Nipah virus helps explain both the broad species tropism that the henipaviruses possess and the observed distribution of viral antigen within infected animals and people (reviewed in [89, 109]).

7.2 Immune Response

Henipavirus infection in people and other susceptible animals induces strong humoral responses. In humans with clinical Nipah virus encephalitis, anti-Nipah virus antibodies were detected in sera (71 %) and cerebral spinal fluid (CSF) (31 %) [7]; levels of virus-specific IgM antibodies were higher than IgG antibodies in CSF and serum [5, 9, 110]. Seroconversion of IgG against Hendra virus was observed in two human cases of Hendra virus infection in 2008 following an influenza-like illness that progressed to encephalitis [23].

Less is known about the memory responses or cell-mediated immune response to henipavirus infection though both are likely present. In Nipah virus challenge studies in African green monkeys when animals survive a severe infection and are rechallenged with very high doses of virus, they remain completely protected from subsequent infection and disease (TW Geisbert and CC Broder 2008, unpublished).

There are a few published reports on cytokine responses to henipavirus infection. In vitro infection of endothelial cells with Nipah virus induced increases in the secreted inflammatory chemokines, monocyte chemotactic protein-1 (MCP-1), interleukin-8 (IL-8), and interferon-inducible protein 10 (IP-10) or C-X-C motif chemokine 10 (CXCL10), and infected cell culture supernatants could induce monocyte and T-lymphocyte chemotaxis [111]. These pro-inflammatory chemokines produced by Nipah virus-infected endothelial cells in vitro are consistent with the prominent vasculitis observed in infections in vivo. In the hamster model of henipavirus infection, the development of neurological disease was correlated with disruption of the blood–brain barrier (BBB) and upregulation of tumor necrosis alpha (TNF-á) and interleukin 1 â (IL-1â) and IP-10 (CXCL10), and these inflammatory mediators are likely important in henipavirus pathogenesis [112]. Nipah virus-infected hamsters expressed IP-10 (CXCL10) mRNA in various organs with kinetics that followed the replication of virus. Elevated levels of CXCL10 were also identified in brain tissue of patients with fatal Nipah virus infection from the Malaysian outbreak [113].

7.3 Antagonism of the Host Interferon Response

Products of the P gene inhibit both double-stranded RNA signaling and interferon signaling and the P, V, W, and C proteins can all antagonize the host interferon response, features that are thought to contribute to viral pathogenicity. The details of these mechanisms have been reviewed elsewhere [114–116]. The W protein is the most potent interferon antagonist and P protein is the least [117]. However, a recent study conducted using live virus infection indicated that interferon signaling remains functional during henipavirus infection of human cell lines while interferon production was inhibited [118]. Further, henipavirus infection of bat cells does not induce interferon expression and interferon signaling is inhibited; these observations suggest that the control of virus infection and lack of disease in these natural hosts is mediated by mechanisms other than the interferon response [119]. In addition, recombinant Nipah virus individually lacking either the V, C, or W proteins antagonized the interferon response, and wild-type virus and in vivo Nipah virus lacking either the V or C protein were

significantly less pathogenic. The roles of these proteins in pathogenicity thus appear to be independent of their interferon-antagonist activity [120].

8 Patterns of Host Response

8.1 Henipavirus Infection in People

The initial site of henipavirus replication is ill-defined but is likely within the respiratory system followed by apparent hematogenous systemic spread [110]. Established infection is characterized by a widespread vasculitis and endothelial cell tropism resulting in multinucleated syncytial cells. There is prominent parenchymal cell infection and pathogenesis of many, if not most, major organs with the brain, lung, heart, kidney, and spleen significantly involved [9, 110, 121]. Clinically, severe infection in humans presents as a severe respiratory disease, encephalitis, or a combination of both [2, 7, 39]. The widespread endothelial cell infection and resulting vasculitis, thrombosis, and parenchymal cell infection in many organ systems, particularly in the CNS and respiratory system, contribute to fatal human henipavirus infection [42, 89, 110].

Human henipavirus infection can also result in a clinically quiescent period following a recovery from an acute infection, which can later recrudesce as encephalitis. For example, the second recognized fatal case of human Hendra virus infection occurred in an individual who experienced relapsed encephalitis 13 months after infection [25]. Similarly, among cases of human Nipah virus infection in the Malaysian outbreak, neurological disease frequently presented >10 weeks following recovery from acute encephalitis [122] and relapsed encephalitis has presented from several months to 11 years following infection [123–125]. An examination of the first two fatal human cases of Hendra virus infection, one from an acute respiratory disease and another from relapsed encephalitis, demonstrated that Hendra virus can cause both acute encephalitis without clinical signs or relapsed encephalitis similar to Nipah virus [121]. Relapsed encephalitis is presumably caused by the recrudescence of virus replication that is restricted to the central nervous system (CNS) [121, 123].

8.2 Natural and Experimental Henipavirus Infections of Animals

8.2.1 Infection in *Pteropus* Bats

No clinical disease caused by Hendra virus [13, 126] or Nipah virus [69, 127] infection has been reported in naturally infected fruit bats. Experimental infections with Hendra virus in *P. poliocephalus* and *P. alecto* [14, 128, 129] and with Nipah virus in *P. poliocephalus* and *P. vampyrus* [14, 81] also produced no clinical disease or gross pathological findings even with high doses (50,000 $TCID_{50}$) of virus inoculation; however, bats do seroconvert [81, 128, 129]. Transplacental transmission of Hendra virus in bats can also occur [129] but fetal tissues show no pathology. Virus shedding from experimentally infected bats also occurs but is rare and has only been observed from urine [14, 81].

8.2.2 Infection in Spillover Hosts

Evaluating henipavirus pathogenesis among various animal species has been critical for understanding their biology, for modeling human disease, and as platforms for testing vaccines and therapeutics. A major limitation to in vivo henipavirus infection studies is the requirement for BSL-4 containment which impacts the size and scope of animal challenge studies. These difficulties were compounded by the early observation that henipaviruses did not cause a pathogenic infection in mice, rats, and rabbits [95, 96]. Several animal modeling platforms of henipavirus infection accurately reflect the pathogenic processes seen in either naturally infected humans or in economically important livestock (horses and pigs).

All cases of natural Hendra virus spillover infections in Australia have been in horses, while Nipah virus infection had first occurred in pigs in Malaysia, although dogs, cats, and horses were also infected (reviewed in [130]). Hendra or Nipah virus infection in horses results in more severe disease than either virus causes in pigs (reviewed in [131]). Natural Hendra virus infection in horses is often severe and experimental infections are essentially uniformly fatal. The incubation period is 8–11 days and the animals initially become anorexic, depressed, with general uneasiness and ataxia, and become febrile with sweating. A severe and fatal respiratory disease will often rapidly progress. Neurological disease can also present but is less frequent [128, 132]. Infection is widespread with an endothelial cell tropism with syncytia [89], and virus can be recovered from a number of internal organs, including lung, and from saliva and urine [128, 133–135]. Experimental Nipah virus infection of horses has not been reported but a naturally infected horse had viral antigen and nonsuppurative meningitis [89].

In natural and experimental Nipah virus infection of pigs, the respiratory system is a primary target organ of virus replication and pathology, with viral antigen present in the respiratory epithelium and syncytia in small blood and lymphatic vessels [71, 89]. The involvement of the CNS appears less common, with meningitis or meningoencephalitis more common than encephalitis [71]. Nipah virus infection of Landrace piglets resulted in a mild clinical disease with fever and respiratory signs, but neurological disease could also appear [93]. Virus replication is seen in the respiratory system, the lymphoid tissues, and the CNS and was greatest in the respiratory system but syncytia were less frequent. Virus was recovered

from the respiratory, lymphatic, and nervous systems, and virus shedding was observed in nasal, pharyngeal, and ocular fluids. Experimental Hendra virus infection of pigs also resulted in a respiratory disease with possible CNS involvement. Hendra virus appeared to cause a more severe disease in pigs in comparison to Nipah virus, and Hendra virus shedding was noted in nasal, oral, rectal, and ocular fluids [97].

Cats were discovered to be susceptible to natural Nipah virus infection and disease in the Malaysian outbreak [89], and cats were later shown to be highly susceptible to infection and disease by both Hendra and Nipah virus. Hendra virus disease in cats is similar to that seen in horses, revealing widespread vasculitis and parenchymal lesions in the lungs and other tissues and organs [89, 133]. Experimental Nipah virus infection in the cat is similar to Hendra virus infection but with more extensive inflammation of the respiratory epithelium [71, 89, 91]. The systemic vasculitis seen in the cat model is consistent with the resulting pathology of Nipah virus infection in humans. In utero transmission of Nipah virus in cats has also been demonstrated with evidence of extensive viral replication in many tissues of a pregnant adult cat and in fetal tissues [136].

8.2.3 Hamsters and Ferrets

The golden hamster is the only small animal model of henipavirus infection that accurately reflects human pathology and was first described with Nipah virus challenge experiments [96] and later with Hendra virus [88]. Intranasally infected hamsters die 9–15 days later, displaying progressive signs of neurologic disease and breathing difficulties; viral genome is detectable in multiple organ systems and severe pathology is most evident in the brain [96]. Hendra virus infection of hamsters also yields pathology that resembles acute human Nipah virus cases [88], including widespread endothelial infection and vasculitis and parenchymal lesions, especially in the CNS. Henipavirus infection in hamsters with higher doses of either virus manifests greater respiratory disease, while lower doses yield greater neurological disease [112].

Ferrets are also an excellent model of henipavirus infection and disease [86, 137, 138]. Six to ten days after low dose oral-nasal challenge with Nipah virus, ferrets develop severe respiratory and neurological disease with widespread vasculitis and parenchymal lesions including the CNS neurons [86, 137]. Hendra virus-infected ferrets, with doses as low as 50 $TCID_{50}$, rapidly progressed with severe disease between 6 and 9 days following infection [138]. Clinical signs and Hendra virus-mediated disease were essentially identical to those observed with Nipah virus infection. Overall, the ferret model reproduces all the hallmarks seen in henipavirus-infected people.

8.2.4 Nonhuman Primates

The African green monkey accurately reproduces human Hendra virus and Nipah virus-mediated disease [87, 92]. Henipavirus infection of African green monkeys results in a uniformly lethal disease with low dose challenge by intratracheal inoculation within 7–10 days. Virus infection yields widespread vasculitis and endothelial and arterial smooth muscle cell syncytia formation in virtually all organs and tissues [87, 92]. Subjects develop severe respiratory disease; lungs show significant congestion, hemorrhage, polymerized fibrin, and viral antigen [87]. Monkeys infected with either Nipah or Hendra virus also exhibit neurological disease, along with vascular and parenchymal lesions in the brain including infection of neurons and significant involvement of the brainstem.

9 Control and Prevention

9.1 Preventing Henipavirus Outbreaks

Preventing henipavirus transmission from bats to domestic animals or humans depends on adopting regular practices that reduce transmission risk. A high index of suspicion for infection in intermediate hosts including horses in Queensland and pigs in Southeast Asia can identify potentially high-risk situations when increased attention to infection control can be lifesaving. Regular surveillance for henipavirus infection among domestic animals can better characterize the magnitude of risk and help to focus preventive efforts.

Healthcare providers should maintain a high index of suspicion for human infection with henipavirus among people who are at high risk for infection including veterinary healthcare providers in Queensland, people who raise pigs in Southeast Asia, and people in South Asia who either drink raw date palm sap or who recently provided care for persons with fever and mental status changes. Early recognition of human cases can identify high-risk situations and help to prevent additional infections.

9.1.1 Preventing Hendra Virus Spillover

The first step to prevent human infection with Hendra virus is to prevent Hendra virus infection in horses. Although the precise pathway the virus moves from *Pteropus* bats to horses is not defined, through 2009 all infected horses were stabled in open paddocks [139]. Prudent steps to minimize horse exposure to *Pteropus* saliva, urine, and birth products include placing horse feed and water containers under shelter and removing horses from open paddocks when nearby flowering and fruiting trees are attracting flying foxes [140]. Sick horses should be isolated from other horses [140].

Next, all persons who work with sick horses in Queensland and the surrounding area should recognize that a sick horse may be shedding Hendra virus. The very high levels of exposure to respiratory secretions and other body fluids among

human cases of Hendra virus infection, while most attendants were uninfected, suggest that modest steps to improve infection control, if consistently applied, could prevent transmission. People who have contact with horses in areas where Hendra virus infections have been recognized should minimize their contact with equine blood and body fluids especially when a horse develops a rapidly progressive febrile illness characterized by respiratory distress and frothy nasal discharge. Horse handlers should regularly wash their hands with soap and isolate sick horses from other horses and people [141]. If Hendra virus infection is suspected, horse handlers and veterinary personnel should wear personal protective equipment to protect their clothing, skin, and face from contact with horse blood or body fluids.

9.1.2 Preventing Nipah Virus Transmission

People who raise pigs in areas where *Pteropus* bats live should remove trees that are attractive to *Pteropus* bats from the immediate area where pigs are being raised.

In Bangladesh, the government is encouraging people to avoid drink fresh date palm sap, because of the risk of Nipah virus. Researchers have been exploring deploying barrier skirts around date palm sap trees during collection to prevent physical access of bats to the sap [79, 83]. These have been quite effective in excluding the bats, but only a small minority of date palm sap collectors are using barrier skirts. Combined efforts to discourage fresh sap consumption, or, if people insist on drinking fresh sap, to ensure that such sap was collected from a tree protected by a date palm skirt, are currently being evaluated.

Preventing person-to-person henipavirus transmission is part of a broader effort to reduce transmission of dangerous saliva-transmitted pathogens in low-income countries. In high-income countries, healthcare providers are professionals, trained on procedures to reduce risk of infection, and provided with disposable personal protective equipment to reduce their risk. In low-income countries, where families are the primary care providers [142, 143], they have no training in infection control, and they neither have the access to nor can afford infection control supplies. An active area of research is to identify practical strategies for low-resource settings that can reduce the risk of saliva-transmitted infections.

9.2 Limiting Human Disease

9.2.1 Antiviral Strategies

Ribavirin, a ribonucleoside analog with broad antiviral activity, was used in the Nipah virus outbreak in Malaysia. The morbidity and mortality among treated patients were significantly lower than was observed among untreated patients earlier in the outbreak [144], though it is unclear how much of this improved survival can be attributed to ribavirin. Chloroquine, an antimalarial drug first demonstrated to block the proteolytic processing of Hendra virus-F [145], was later shown to inhibit henipavirus infection in vitro [146]. Both ribavirin and chloroquine have been evaluated in vivo in animal challenge models. Ribavirin only delayed Nipah virus disease and death and had no benefit against Hendra virus infection in hamsters [147, 148]. Ribavirin therapy also only delayed Hendra virus disease and pathogenesis by 1 or 2 days in African green monkeys [92]. Chloroquine treatment, alone or in combination with ribavirin, had no therapeutic benefit in ferrets challenged with Nipah virus or in hamsters challenged with either Nipah virus or Hendra virus [137, 147, 148]. Ribavirin is still considered a possible treatment for henipavirus infection, though there is little additional evidence of benefit. In 2008, two Hendra virus-infected patients showed no discernible benefit after treatment with ribavirin [23], and combined treatment with chloroquine and ribavirin was unsuccessful in one Hendra virus-infected person in 2009 [149].

PolyIC$_{12}$U, a strong inducer of interferon-α and interferon-β production and a specific activator of the toll-like receptor 3 pathway (reviewed in [150]), blocked Nipah virus replication in cell culture and was tested in vivo against Nipah virus infection in the hamster model [148]. Continuous administration of polyIC$_{12}$U for 10 days beginning at the time of Nipah virus challenge prevented lethal disease in five of six animals. Further studies using TLR3 agonists such as PolyIC$_{12}$U as immunotherapeutic agents appear warranted.

Heptad peptide-based membrane fusion inhibitors corresponding to either of the heptad repeat (HR) domains of a paramyxovirus F glycoprotein can potently block virus infection in vitro (reviewed in [151]). HR-2 peptides have been used more often and act by blocking the formation of the 6-helix bundle structure in F which is required for virus and host cell membrane merger. A 36 amino acid HR-2-based F glycoprotein sequence (Nipah virus-FC2) [152] was the first one tested and it is analogous to the approved HIV-1-specific drug enfuvirtide (Fuzeon™). An HR-2 peptide derived from the human parainfluenza virus type-3 (hPIV3) F glycoprotein has also proved to be a potent Hendra virus fusion inhibitor [153]. A sequence-optimized and cholesterol-tagged hPIV3-based HR-2-derived peptide was tested in the Nipah virus hamster model [154] and showed it could penetrate the CNS and exhibit some effective therapeutic activity against Nipah virus. Peptide fusion inhibitors as henipavirus therapeutics are actively being investigated.

9.2.2 Passive Immunization Strategies

The henipavirus envelope glycoproteins are the major targets of virus-neutralizing antibodies (reviewed in [155]). Challenge studies carried out in hamsters demonstrated that protective passive immunotherapy with either Nipah virus G- or F-specific polyclonal antiserums or mouse monoclonal anti-

bodies (mAbs) was possible [88, 156, 157]. Using recombinant antibody techniques, henipavirus-neutralizing human mAbs specific for the G glycoprotein were isolated from a naïve human phage-displayed antibody library [158]. One mAb, m102, exhibited strong cross-reactive neutralization activity against both Hendra virus and Nipah virus and was affinity maturated yielding mAb m102.4 and converted to IgG1 format for production in a CHO-K1 cell line. The m102.4 mAb has exceptionally potent neutralizing activity and with 50 % inhibitory concentrations below 0.04 against Nipah virus and 0.6 μg/ml against Hendra virus [159]. The epitope recognized by m102.4 maps to the ephrin receptor binding site [160], and it neutralizes all known isolates of Hendra virus and Nipah virus [86].

The efficacy of m102.4 treatment in vivo has been examined in both the ferret and African green monkey models of Hendra virus and Nipah virus infection. In a pre- and postexposure Nipah virus challenge study in the ferret, animals were given a single 50 mg dose (~25 mg/kg) at 24 h before (pre-) or 10 h following (post-) challenge by intravenous infusion. Virus was administered oronasally as a 5,000 $TCID_{50}$ dose of Nipah virus (tenfold the minimal infectious dose – 50 %). Control ferrets became febrile with severe clinical illness and were euthanized by day 8, while one subject in the pre-group and all in the post-group were febrile with depression, but by day 10 fevers began to abate. At 13 days postinfection, 2/3 animals in the pre-group progressed with disease and were euthanized, whereas all other ferrets (3/3 in the post-group and 1/3 pre-group) were free of any disease signs and remained well until study end point. Control subjects revealed severe systemic pathology, and the two pre-group-treated animals which experienced a delayed disease course had markedly reduced pathology, but there was no pathology seen in any of the surviving ferrets [86]. Similar findings have been obtained in a Hendra virus challenge study in ferrets followed by m102.4 postexposure therapy (J Pallister and CC Broder 2012, unpublished).

The m102.4 human mAb has also recently been examined in the African green monkey henipavirus challenge model [161] as postexposure passive immunotherapy. Fourteen monkeys were challenged intratracheally with Hendra virus, and 12 animals were infused twice with a 100 mg dose (~20 mg/kg) of m102.4 beginning at 10, 24, or 72 h postexposure with the second infusion ~48 h later. All 12 animals that received m102.4 survived infection, whereas the untreated control subjects succumbed to severe systemic disease by day 8. Animals in a 72 h treatment group, where mAb therapy began 3 days after lethal challenge, exhibited neurological signs but recovered by day 16. At study end, there was no evidence of Hendra virus-specific pathology in any of the m102.4-treated animals. Further, no infectious Hendra virus could be recovered from any of the m102.4-treated animals. Essentially identical findings have been noted in a Nipah virus challenge and m102.4 postexposure therapy study conducted in African green monkeys (TW Geisbert and CC Broder 2014, submitted).

During the 2010 Hendra virus spillover occurrence in Queensland, Australia, two individuals considered to be at high risk of Hendra virus infection were offered m102.4 therapy on a compassionate use basis even though no human safety testing had been carried out and it was not recommended for use in humans. There were no adverse reactions resulting from infusion of the mAb and both individuals remain well. In July 2012, a third asymptomatic individual in Australia also received a 20 mg/kg dose of m102.4 following high-risk Hendra virus exposure, also with no adverse reaction [162].

9.2.3 Active Immunization Strategies

Active immunization strategies against Nipah virus infection was first examined in hamsters with an attenuated vaccinia virus strain (NYVAC) using recombinants prepared with either the Nipah virus F and G glycoprotein genes [156]. The vaccine demonstrated complete protection from Nipah virus-mediated disease but with evidence of Nipah virus replication and an anamnestic antibody response in challenged animals. Recombinant canarypox-based vaccine (ALVAC) candidates, encoding Nipah virus F and G glycoprotein genes, for use in pigs were tested by immunization of 4-week-old pigs twice within 2 weeks [163]. Each ALVAC vector was tested alone and in combination, and piglets were challenged intranasally with Nipah virus. All pigs were protected from Nipah virus-mediated disease by either vector alone or in combination. The individual ALVAC-F- and ALVAC-G-vaccinated animals had only low levels of detectable virus shedding by nucleic acid analysis but no infectious virus could be recovered, while the combined vaccination with ALVAC-F/G appeared superior with neither infectious virus nor Nipah virus RNA being detected. These data suggest that an ALVAC-G or combination vector formulation could serve as a protective vaccine against Nipah virus disease in pigs but the combination of vectors would be needed to assure the prevention of virus shedding [163].

A subunit vaccine strategy for henipaviruses has been extensively explored using a soluble oligomeric form of the G glycoprotein (sG) [164] and Hendra virus-sG is now commercially deployed as a livestock vaccine [165]. Hendra virus-sG, produced by recombinant expression in mammalian cell culture and properly N-linked glycosylated [166], retains its oligomeric form and ability to bind ephrin receptors [99]. Hendra virus-sG elicits potent cross-reactive neutralizing antibody responses in a variety of animals and presents more cross-reactive epitopes (anti-Nipah virus G antibodies) as compared to sG from Nipah virus [167]. Henipavirus sG vaccination was first tested in the Nipah virus cat model with both Hendra virus-sG and Nipah virus-

sG administered as three 100 μg doses 3 weeks apart. Both immunogens induced a completely protective humoral immune response against lethal subcutaneous Nipah virus challenge [91]. A follow-up study examined lower levels of pre-challenge neutralizing antibody titers together with a high-dose oronasal challenge of Nipah virus [168]. Two doses of either 50, 25, or 5 μg of Hendra virus-sG formulated spaced by 3 weeks were tested. All vaccinated animals were completely protected from a high-dose oronasal challenge of Nipah virus (50,000 $TCID_{50}$). Hendra virus-sG immunization has also been tested in the ferret model [138]. Ferrets were immunized twice with a 20-day interval with either a 100, 20, or 4 μg dose of Hendra virus-sG. Animals were challenged oronasally with a 5,000 $TCID_{50}$ dose (tenfold the minimal infectious dose 50 %), and all vaccinated ferrets remained free of any signs of fever or clinical disease. Postmortem examination of vaccinated animals showed all subjects completely normal except one of four animals in the lowest dose group. There was no evidence of virus or viral genome in any tissues or samples from animals in the 100 and 20 μg vaccine groups. Only a very low level of genome was detected in the nasal washes in one animal in the 4 μg group. No virus was recovered from any vaccinated animal.

Recently, Hendra virus-sG vaccination of African green monkeys followed by intratracheal Nipah virus challenge [169] or Hendra virus challenge (Geisbert and Broder, unpublished) has demonstrated complete protection from henipavirus infection and disease with no recoverable virus or evidence of virus shedding. The application of Hendra virus-sG as an equine vaccine against Hendra virus infection has been tested as a strategy to prevent both infection and virus shedding [170]. Several vaccination and Hendra virus challenge studies have been carried out in Australia; at the high-containment biological safety level 4 (BSL-4) facilities of the Animal Health Laboratories (AAHL), Commonwealth Scientific and Industrial Research Organisation (CSIRO), in Geelong. The Hendra virus-sG was used to immunize horses (2 doses) and both a high and a low dose of Hendra virus-sG antigen were examined. Horses were challenged with a high oronasal dose of Hendra virus (2×10^6 $TCID_{50}$), and to date, all vaccinated horses have remained clinically disease-free with no evidence of virus replication or virus shedding following virus challenge [165]. Development of the equine vaccine against Hendra virus was a collaborative effort among investigators of the US and Australian Government laboratories and private industry. On November 1, 2012, the Hendra virus horse vaccine called Equivac HeV® was released for use in Australia, and it is the first vaccine licensed and commercially deployed for public use against a BSL-4 agent. Preclinical development of Hendra virus-sG in vaccine formulations that could potentially be suitable for use in humans is in progress.

10 Unresolved Problems

The contribution of strain differences within henipaviruses to observed epidemiological differences is an open question. Are some strains of henipavirus more likely to infect humans, more likely to cause a primary respiratory disease and more likely to cause person-to-person transmission than other strains? If strain differences confer different infectious competencies, what are the genetic characteristics that underlie these competencies? We do not yet have enough strains of henipavirus, paired with detailed epidemiological information to resolve these questions, but continued careful outbreak investigation and collection of additional isolates may provide additional insight. Collecting additional strains may also clarify how much genetic variation occurs within the henipavirus genome. Ultimately, a better understanding of genetic diversity and the relationship between genomic differences and epidemiology would permit a sound assessment of the risk of spillover of a henipavirus strain capable of sustained person-to-person transmission.

Studies in non-*Pteropus* bats from China and Ghana [171, 172], pigs in Ghana [173], and cattle and goats in Bangladesh [174] have identified antibodies against henipavirus, but the serum neutralizes neither Nipah nor Hendra virus-infected cells. These serological results as well as the PCR fragments of henipavirus identified in feces of *E. helvum* suggest the existence of other henipaviruses. Isolating the specific viruses and exploring their ecology and the impact of such infections on animals including humans would broaden our understanding of the diversity and risk of henipaviruses.

While human infections with Hendra and Nipah virus have a remarkably high case fatality rate, human infection is rare. Australian animal handlers regularly care for sick horses without complications. Bangladeshi villagers have been enjoying raw date palm sap for generations with only a very small proportion of people enjoying this local delicacy suffering any adverse effect. This rarity of adverse outcomes means that most persons at risk for henipavirus infections never observe a connection between their own behavior and a health outcome. This represents a substantial barrier to adopting and maintaining behaviors to reduce their risk.

This small number of human infections also means that with limited budgets and competing priorities, prevention efforts have to be extremely low cost in order to be cost-effective. Although the m102.4 mAb has been remarkably effective in animal studies and is presently in preclinical development stages in both the United States and Australia, there have been insufficient financial commitments from either government or private industry to support further developmental costs including human safety testing trials.

Because of the high economic value of competition horses, the high mortality associated with Hendra virus infection, and the subsequent risk to people living in a high-income country,

there is a potential private market among horse owners for an effective livestock vaccine against Hendra virus. Indeed, Hendra virus-sG subunit vaccine is now sold as an equine vaccine. If henipavirus infections are recurrently recognized to infect domestic pigs, it's possible that pig producers will adopt vaccination, but the lower economic value of pigs and the lower severity of henipavirus infection in pigs means that many pig owners will be reluctant to invest in vaccine to protect their pigs. The rapid reproductive rate of pigs also makes routine immunization logistically demanding and so more expensive.

The Hendra virus-sG subunit immunogen has shown remarkable cross-protective efficacy in four mammalian species, and recent preliminary data suggests that the Hendra virus-sG subunit vaccine could be developed for human use. Because of concerns that henipaviruses may be adapted as a bioterror agent [175], there is some potential market for such a vaccine. A human vaccine would also be useful to protect the small number of laboratory workers engaged in studies with henipaviruses, biologists working with bats, and veterinarians and staff working with sick horses in Australia, but developing such a vaccine would require substantial additional investment. A human henipavirus virus vaccine is unlikely to be developed by private industry unless a clear and substantial demand from governments concerned with the threat of bioterrorism or deploying a vaccine strategy to reduce pandmic risk ensures a market. The incidence of Nipah virus in Bangladesh is too low for a vaccination program to be cost-effective in the foreseeable future if the sole objective is preventing an average of 20 human cases per year. However, if the risk of emergence of a strain of Nipah virus capable of efficient person to person transmission is high enough, then vaccination may represent a cost effective investment in pandemic prevention.

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Competing Interests C.C.B is a US federal employee and is coinventor on patents relating to human monoclonal antibodies against Hendra and Nipah viruses and soluble forms of Hendra and Nipah envelope glycoproteins and vaccines; assignees are the United States of America as represented by the Department of Health and Human Services (Washington, DC) and the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. (Bethesda, MD).

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Paramyxoviruses: Measles

23

William J. Moss and Diane E. Griffin

1 Introduction

Measles is one of the most important infectious diseases of humans and has caused millions of deaths since its emergence as a zoonotic disease thousands of years ago. For infectious disease epidemiologists, measles has served as an exemplary model of an acute infectious disease conferring lifelong immunity to reinfection. Kenneth Maxcy, the second chair of the Department of Epidemiology at the Johns Hopkins University School of Public Health, wrote in 1948, "The simplest of all infectious diseases is measles" [1]. Despite the apparent simplicity, much has been learned about measles in the 60 years since Maxcy's chapter on the epidemiology of infectious diseases. Detailed investigations of the virology, immunology, epidemiology, and transmission dynamics have shown measles to be a much more complex disease than Maxcy's statement suggests. This ignorance, however, has not impeded global measles control. Remarkable progress has been made in reducing measles morbidity and mortality through measles vaccination programs. This achievement attests to the enormous public health significance of measles vaccines. However, this progress is threatened by failures to maintain high levels of measles vaccine coverage and population immunity. Strategies for the global eradication of measles are currently being considered.

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2 Historical Background

Measles virus is closely related to rinderpest virus, a recently eradicated Morbillivirus pathogen of cattle, and measles likely evolved as a zoonotic infection in communities where cattle and humans lived in close proximity. Measles virus is believed to have become established as a human pathogen approximately 5,000–10,000 years ago when communities achieved sufficient population size in Middle Eastern river valley civilizations to maintain virus transmission [2]. The earliest extant descriptions of measles are those of Abu Becr, also known as Rhazes, who wrote a treatise on measles and smallpox in the tenth century. Interestingly, he considered measles to be the more severe of the two diseases. Linguistic evidence suggests that measles was recognized as a distinct disease between 450 and 650 AD. In the mid-eighteenth century, Francis Home attempted to prevent measles through inoculation of blood obtained from the skin of an afflicted individual, analogous to Jenner's approach to preventing smallpox.

The first to explicitly document the contagious nature of measles was the Danish physician Peter Panum during an outbreak of measles on the sparsely populated Faroe Islands in 1846. Through careful documentation of clinical cases, Panum provided accurate measurement of the incubation period for measles and evidence of the long-term protective immunity conferred by measles [3]. Specifically, Panum observed that adults who acquired measles during the prior outbreak six decades earlier were protected from disease, despite absence of exposure to measles between epidemics.

Attenuated and killed measles vaccines were introduced in the 1960s after successful isolation and growth of measles virus in tissue culture by John Enders [4] and further attenuation of measles vaccine virus by Maurice Hilleman. As a consequence of high levels of measles vaccine coverage, the WHO Region of the Americas interrupted indigenous transmission of measles virus in 2002. The Measles Initiative (now the Measles and Rubella Initiative) was founded in

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2001 as a partnership committed to reducing global measles mortality and has been instrumental in providing technical and financial support for measles control activities.

3 Methods for Epidemiologic Analysis

3.1 Clinical and Laboratory Diagnosis of Measles

Epidemiologic studies of measles require accurate diagnosis and valid case series. The characteristic clinical features of measles are of sufficient sensitivity and specificity to have high predictive value in regions where measles is endemic and allow for epidemiologic analyses of measles case series over many decades. However, laboratory confirmation is necessary, particularly where measles virus transmission rates are low or in immunocompromised persons who may not develop the characteristic clinical manifestations. Infection with rubella virus, parvovirus B19, human herpes virus 6, and dengue viruses may mimic measles. Detection of IgM antibodies to measles virus by enzyme immunoassay (EIA) is the most commonly used method to diagnose measles [5]. Alternatively, seroconversion using IgG-specific EIA or virus neutralization assays can be used to diagnose measles based on testing serum or plasma obtained during acute and convalescent phases. Serologic surveys to detect measles virus-specific IgG antibodies using blood or oral fluid samples provide age-specific data on population immunity and the average age of infection, from which the force of infection can be derived [6].

Measles virus can be isolated in tissue culture from white blood cells, respiratory tract secretions, and urine, although the ability to isolate measles virus diminishes quickly after rash onset. Amplification and detection of measles virus RNA by reverse transcription polymerase chain reaction (RT-PCR) from blood, urine, and nasal discharge is highly sensitive in detecting measles virus RNA and allows sequencing of the measles virus genome for molecular epidemiologic studies.

3.2 Molecular Epidemiology

Although measles virus is considered to be monotypic, variability within the genome is sufficient to allow for molecular epidemiologic investigations. Genetic characterization of wild-type measles viruses is typically based on sequence analysis of the genes coding for the nucleocapsid (N), hemagglutinin (H), and phospho (P) proteins [7, 8]. One of the most variable regions of the measles virus genome is the 450-nucleotide sequence at the carboxy-terminal of the N protein, with up to 12 % variability between wild-type viruses. The World Health Organization recognizes eight clades of measles virus (designated A through H) and 24 subclades or genotypes (Fig. 23.1) [9]. New genotypes may be identified with enhanced surveillance and molecular characterization. As measles control efforts intensify, molecular surveillance of circulating measles virus strains can be used to document interruption of measles virus transmission and identify the source and transmission pathways of measles virus outbreaks [10, 11]. Six genotypes have not been reported since at least 2000, and an additional five genotypes have not been reported since 2006, despite enhanced surveillance efforts [9], and may be inactive. A global database for measles virus genotypes was developed by the World Health Organization LabNet to collate information generated from enhanced measles virus surveillance activities (http://www.who-measles.org/Public/Web_Front/main.php).

3.3 Modeling Measles Virus Transmission Dynamics

Because of characteristic epidemic cycles and historical case series, measles has served as a model for understanding the transmission dynamics of an acute infectious disease and the impact of vaccination strategies. Mathematic modeling of measles dynamics was first attempted 100 years ago by Sir William Hamer [12]. The basic principle underlying early models was the law of mass action, in which the periodicity of measles incidence was described as a function of the changing number of infectious and susceptible persons as they contact one another. The number of susceptible persons, in turn, was determined by the introduction of new susceptibles through birth and the removal of susceptibles through the acquisition of protective immunity following infection. However, the epidemic cycles generated by these simple deterministic models approached a steady state, losing their periodicity. The introduction of stochastic (chance) and seasonal processes resulted in models of measles epidemics that closely resembled observed data. Further development of these models allowed for predictions of the impact of vaccination strategies on various epidemiologic characteristics of measles virus transmission, including the increase in the average age of infection and the interepidemic period [13]. More sophisticated models of measles virus transmission dynamics have investigated the role of nonlinear dynamics in temporal and spatial patterns of measles incidence [14], traveling waves of infection originating in large cities and spreading to small towns [15], seasonality [16], and the loss of spatial synchrony in measles epidemics following the introduction of measles vaccination [17].

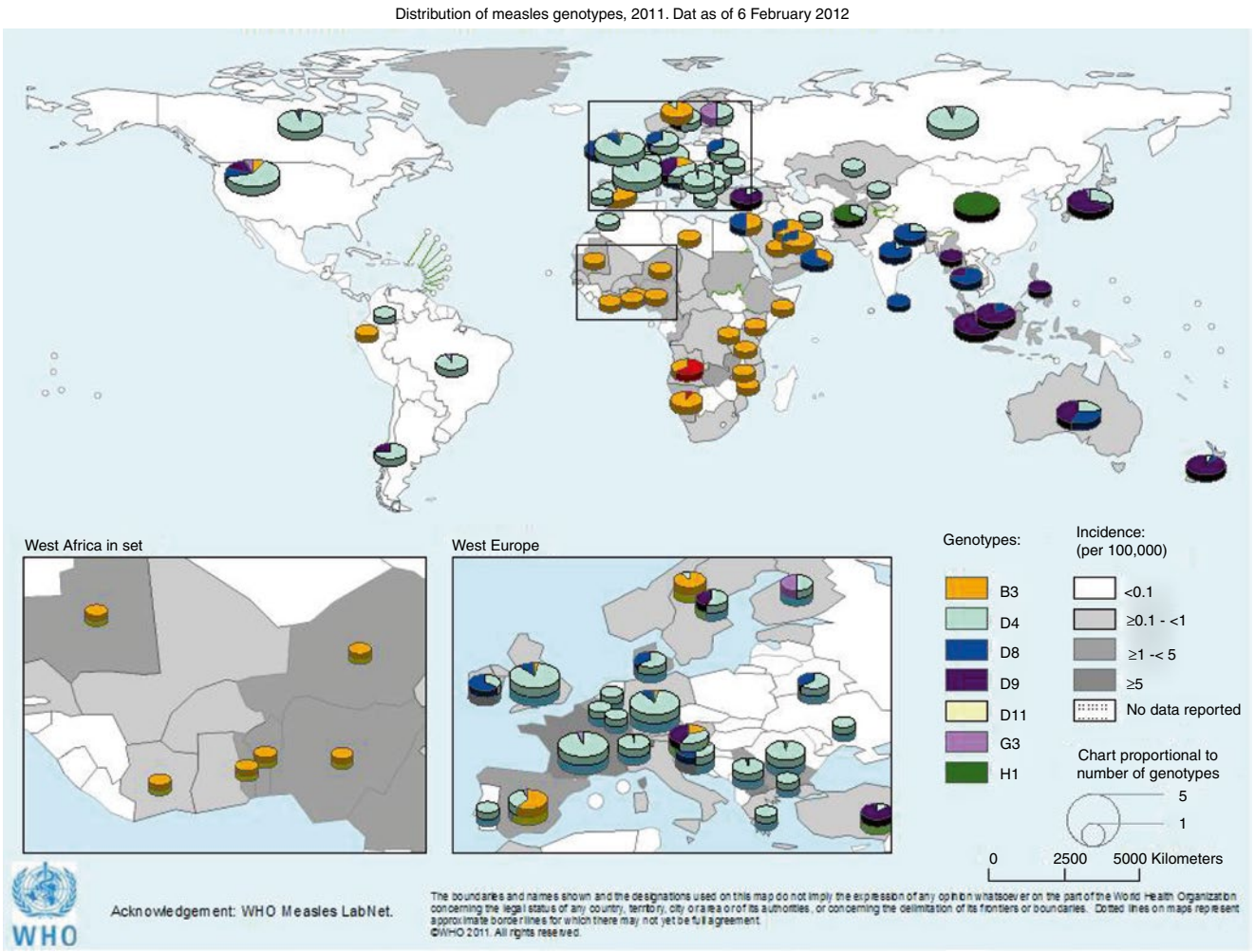

Fig. 23.1 Distribution of measles virus genotypes, 2011 (Source: World Health Organization http://www.who.int/immunization_monitoring/diseases/measles_monthlydata/en/index1.html)

4 Biologic Characteristics

Measles virus is a spherical, nonsegmented, single-stranded, negative-sense RNA virus and a member of the *Morbillivirus* genus in the family of Paramyxoviridae. Although RNA viruses have high mutation rates, measles virus is considered to be an antigenically monotypic virus, meaning that the surface proteins responsible for inducing protective immunity have retained their antigenic structure across time and space. The public health significance is that measles vaccines developed decades ago from a single measles virus strain remain protective worldwide. Measles virus is killed by ultraviolet light and heat, and attenuated measles vaccine viruses retain these characteristics, necessitating a cold chain for transporting and storing measles vaccines.

The measles virus RNA genome consists of approximately 16,000 nucleotides and is encapsalated and enclosed in a lipid-containing envelope derived from the host cell. The genome encodes eight proteins, two of which (V and C) are nonstructural proteins expressed from the P gene. Of the six structural proteins, P, large protein (L), and N form the nucleocapsid housing the viral RNA. The H, F, and matrix (M) proteins, together with lipids from the host cell membrane, form the viral envelope. This relatively simple combination of proteins and ribonucleic acid has evolved to be one of the most highly infectious, directly transmitted agents known and the cause of millions of human deaths.

In terms of understanding the epidemiology and control of measles, the two surface proteins F and H are most important. The H protein interacts with F to mediate fusion of the viral envelope with the host cell membrane [18]. The primary function of the H protein is to bind to the host cellular receptors for measles virus. The H protein elicits strong immune responses, and the lifelong immunity that follows infection is due primarily to neutralizing antibodies against H.

Three cellular receptors have been identified: CD46, CD150 (SLAMF1), and nectin-4. CD46 is a complement regulatory molecule expressed on all nucleated cells in humans. SLAMF1, an acronym for signaling lymphocyte activation molecule family member 1, is expressed on activated T and B lymphocytes and antigen-presenting cells. Nectin-4, the most recently identified receptor, is an adherens junction protein found on epithelial cells [19, 20]. The distribution of these host proteins determines the tissue tropism and cell types infected by measles virus. Wild-type measles virus enters cells primarily through the cellular receptor SLAMF1, and most vaccine strains can use CD46 as a receptor, although wild-type measles virus may use both CD46 and SLAMF1 as receptors during acute infection [21]. Nectin-4 permits measles virus to enter the basolateral surface of respiratory epithelial cells.

5 Descriptive Epidemiology

5.1 Disease Burden

The disease burden due to measles has decreased as a result of several factors. Measles mortality declined in developed countries in association with economic development, improved nutritional status, and supportive care, particularly antibiotic therapy for secondary bacterial pneumonia. Remarkable progress in reducing measles incidence and mortality has been, and continues to be, made in resource-poor countries as a consequence of increasing measles vaccine coverage, provision of a second dose of measles vaccine through supplementary immunization activities (SIA), and efforts by the World Health Organization, the United Nations Children's Fund (UNICEF), and their partners to target endemic countries for accelerated and sustained measles mortality reduction. Specifically, this targeted strategy aimed to achieve greater than 90 % measles vaccination coverage in every district and ensure that all children received a second dose of measles vaccine. Provision of vitamin A as a part of polio and measles SIAs has probably contributed further to the reduction in measles mortality.

In 2003, the World Health Assembly endorsed a resolution that urged member countries to reduce the number of deaths attributed to measles by 50 % compared with 1999 estimates by the end of 2005. This global public health target was met, with estimated measles mortality reduced by 60 % from an estimated 873,000 deaths in 1999 (uncertainty bounds 634,000–1,140,000) to 345,000 deaths in 2005 (uncertainty bounds 247,000–458,000) [22]. Further reductions in global measles mortality were achieved by 2008, during which there were an estimated 164,000 deaths due to measles (uncertainty bounds 115,000 and 222,000 deaths) (Fig. 23.2) [23]. Using a refined model to estimate measles mortality, the number of deaths attributed to measles declined 74 % from an estimated

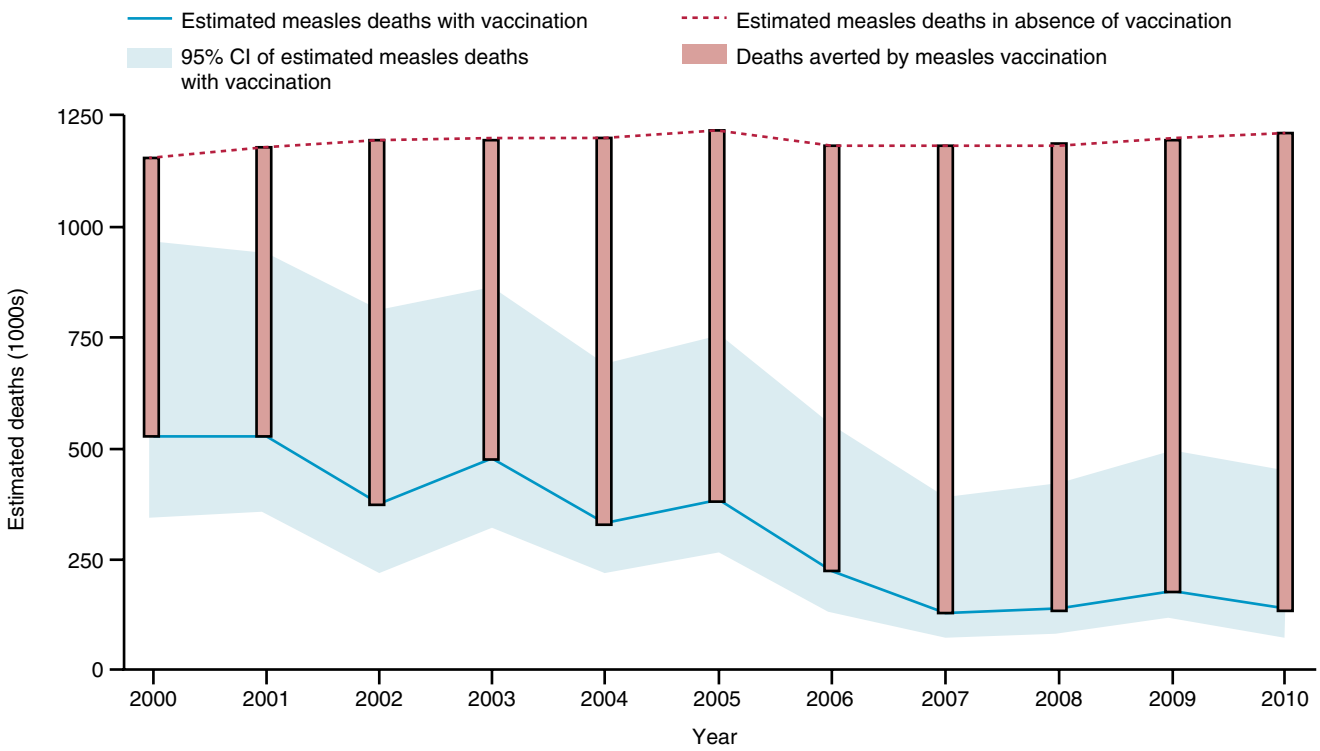

Fig. 23.2 Global estimated measles nortality and measles deaths averted (Source: World Health Organization [23])

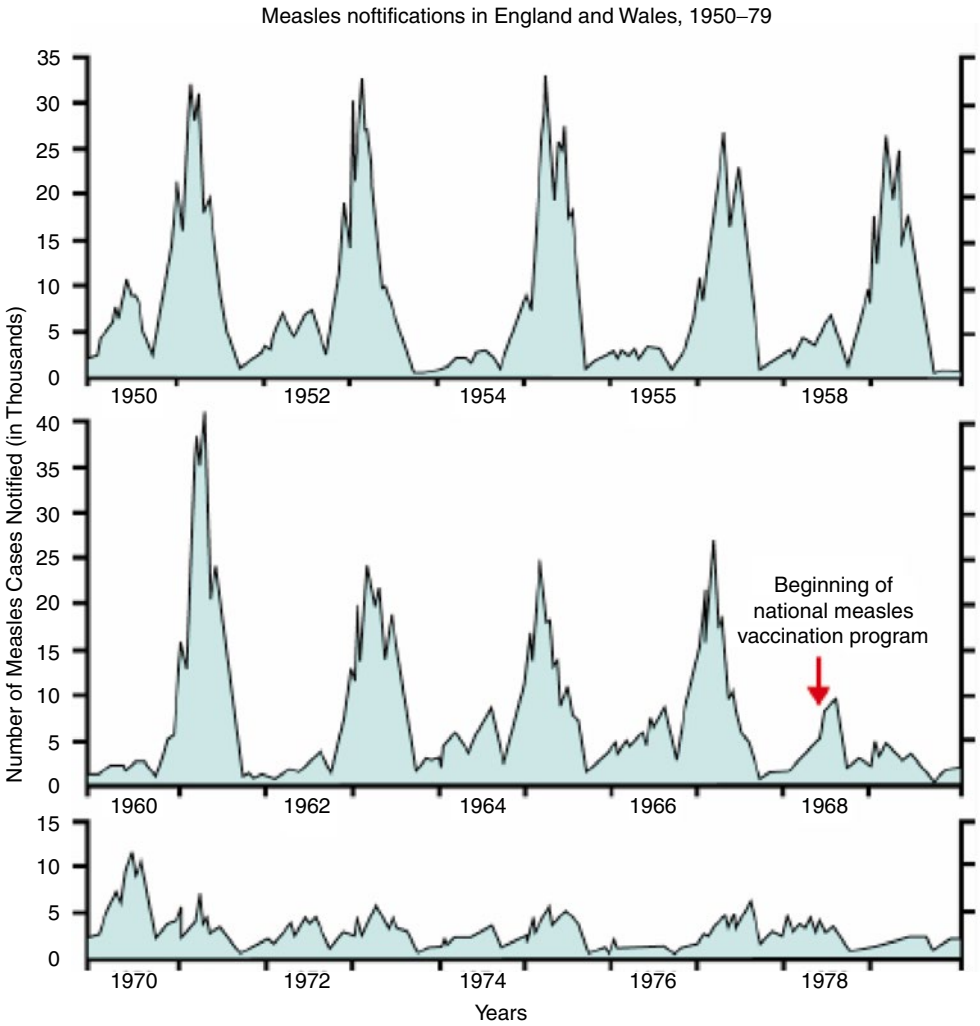

Fig. 23.3 Epidemic cycles of measles cases in England and Wales by week, 1950–1979. The *arrow* indicates the beginning of the national measles vaccination program in 1968 (Source: Fine and Clarkson [26])

535,000 in 2000 (95 % confidence intervals 347,200–976,400) to an estimated 139,000 deaths in 2010 (71,200–447,800) [24]. Prior to this effort, an estimated 30 million cases of measles occurred each year, with more than one million deaths.

5.2 Geographic Distribution

Measles is a global disease but was absent from the Americas prior to contact with Europeans. Measles, in combination with smallpox, likely was responsible for large numbers of deaths of Native Americans, facilitating European conquest [25]. Progress in measles elimination efforts has resulted in the interruption of measles virus transmission in large geographic regions, including the Americas. Because of its mode of transmission and high infectivity, measles virus is most readily maintained in densely populated urban settings. Migration of infected persons to rural areas results in outbreaks in susceptible rural populations too small to sustain measles virus transmission. An extreme example occurs in isolated island populations where periodic introduction of measles virus results in widespread but self-limited outbreaks.

5.3 Temporal Patterns and Seasonality

Measles incidence has a typical temporal pattern characterized by yearly seasonal epidemics superimposed upon longer epidemic cycles of 2–5 years or more (Fig. 23.3). These epidemic cycles have been well documented over many decades in different geographic locations and have been characterized analytically in both simple and sophisticated mathematic models. In temperate climates, annual measles outbreaks typically occur in the late winter and early spring. These annual outbreaks are likely the result of social networks facilitating transmission (e.g., congregation of children at school) and environmental factors favoring the viability and transmission of measles virus [26]. In the tropics, measles outbreaks have variable relationships with rainy seasons, which combined with high birth rates result in highly irregular, large measles outbreaks [16].

Measles cases continue to occur during the interepidemic period in large populations but at low incidence. The longer cycles occurring every several years result from the accumulation of susceptible persons over successive birth cohorts and the subsequent decline in the number of susceptibles following an outbreak. In the absence of a vaccination program, these longer epidemic cycles tend to occur every 2–4 years. Measles vaccination programs that achieve coverage rates in excess of 80 % extend the interepidemic period to 4–8 years by reducing the number of susceptibles.

5.4 Reservoir

Humans are the only reservoir for measles virus, a fact important for the potential eradication of measles. Nonhuman primates may be infected with measles virus and develop an illness similar to measles in humans, with rash, coryza, and conjunctivitis. However, populations of wild monkeys are not of sufficient size to maintain measles virus transmission [2, 27].

5.5 Incubation Period

The incubation period for measles, the time from infection to clinical disease, is approximately 10 days to the onset of fever and 14 days to the onset of rash, with a median of 12.5 days (95 % confidence intervals 11.8–13.3 days) [28]. The incubation period may be shorter in infants or following exposure to a large inoculum of virus, and it may be longer in adults. During this seemingly quiescent period, the virus is replicating and infecting target tissues. The incubation period is best measured during outbreaks in which the time of exposure to the index case can be precisely determined.

5.6 Infectious Period

The infectious period is more difficult to measure than the incubation period as it requires careful observation of the contacts of exposed persons prior to the onset of rash. Generally, persons with measles are infectious for several days before and after the onset of rash, when titers of measles virus in the blood and body fluids are highest. The fact that measles virus is contagious prior to the onset of recognizable disease hinders the effectiveness of quarantine measures. Detection of measles virus in body fluids by a variety of means, including identification of multinucleated giant cells in nasal secretions or the use of RT-PCR, suggests the potential for prolonged infectious periods in persons immunocompromised by severe malnutrition or human immunodeficiency virus (HIV) infection [29, 30]. However, whether detection of measles virus by these methods indicates prolonged contagiousness is unclear.

5.7 Average Age of Infection

The average age of measles virus infection depends upon the rate of contact with infected persons, the rate of decline of protective maternal antibodies, and the vaccine coverage rate. Young infants in the first months of life are protected against measles by maternally acquired IgG antibodies. An active transport mechanism in the placenta is responsible for the transfer of IgG antibodies from the maternal circulation to the fetus starting at about 28 weeks' gestation and continuing until birth [31]. Three factors determine the degree and duration of protection in the newborn: (1) the level of maternal anti-measles virus antibodies, (2) the efficiency of placental transfer, and (3) the rate of catabolism in the child. Although providing passive immunity to young infants, maternally acquired antibodies can interfere with the immune responses to the attenuated measles vaccine by inhibiting replication of vaccine virus. In general, maternally acquired antibodies are no longer present in the majority of children by 9 months of age, the time of routine measles vaccination in many countries [32]. The half-life of anti-measles antibodies was estimated to be 48 days in the United States and Finland, but it is shorter in developing countries. Women with vaccine-induced immunity tend to have lower anti-measles virus antibody concentrations than women with naturally acquired immunity, and their children may be susceptible to measles at an earlier age [33]. Infants born to HIV-infected women may have lower levels of protective maternal antibodies independent of their HIV infection status and also may be susceptible to measles at a younger age [34].

In densely populated urban settings with low vaccination coverage rates, measles is a disease of young children. The cumulative distribution can reach 50 % by 1 year of age, with a significant proportion of children acquiring measles virus infection before 9 months, the age of routine vaccination. As measles vaccine coverage increases, or population density decreases, the age distribution shifts toward older children. In such situations, measles cases predominate in school-age children. Infants and younger children, although susceptible if not protected by immunization, are not exposed to measles virus at a rate sufficient to cause a large disease burden in this age group. As vaccination coverage increases further, the age distribution of cases may be shifted into adolescence and young adults, necessitating targeted measles vaccination programs for these older age groups.

5.8 Infectivity

Measles virus is one of the most highly contagious, directly transmitted infectious agents, and outbreaks can occur in populations in which fewer than 10 % of persons are susceptible. Chains of transmission commonly occur among household contacts, school-age children, and healthcare workers.

The contagiousness of measles virus is best expressed by the basic reproductive number R_o, which represents the mean number of secondary cases that arise if an infectious case is introduced into a completely susceptible population [35]. R_o can be empirically measured, although the introduction of measles virus into a completely susceptible population is rare. In the 1951 measles epidemic in Greenland, the index case attended a community dance during the infectious period resulting in a R_o of 2000 [36]. R_o also may be estimated from the average age of infection (A), the life expectancy (L), and the duration of protection from maternally acquired antibodies (M) using the following equation:

$$R_o = \frac{L\text{-}M}{A\text{-}M}$$

The average age of infection can be inferred from age-specific seroprevalence data in the absence of vaccination. The estimated R_o for measles virus is 12–18 [13], in contrast to only 5–7 for smallpox virus and polioviruses. The high infectivity of measles virus implies that a high level of population immunity is required to interrupt measles virus transmission.

5.9 Herd Immunity Threshold

Interruption of measles virus transmission does not require that all persons be immunized and protected. If a sufficient proportion of the population is immune, the probability that an unprotected person will encounter an infectious individual is reduced to almost zero. Protection of unvaccinated persons by a reduction in the risk of exposure is referred to as herd immunity [37, 38], and the level of population immunity necessary to interrupt transmission is known as the herd immunity threshold (H). The herd immunity threshold can be derived using analytical models of infectious disease dynamics from the equation

$$H = 1\text{-}1/R_o$$

where R_o is the basic reproductive number [13]. A number of assumptions are made in deriving this formula, including the unrealistic assumption of homogenous mixing of the population (i.e., an individual has an equal chance of coming into contact with any other individual). Nevertheless, this simple equation provides a means of assessing the level of population immunity required to interrupt transmission based upon a measure of infectivity (R_o). For measles, with a R_o of 12–18, the herd immunity threshold is 93–95 %. This does not represent the level of vaccine coverage but the proportion of the population protected against measles. This level of population immunity cannot be achieved with a single dose of measles vaccine, for which the primary vaccine failure rate is about 15 % when administered at 9 months of age.

5.10 Critical Community Size

To provide a sufficient number of new susceptibles through births to maintain measles virus transmission, a population size of several hundred thousand persons with 5,000–10,000 births per year is required [2]. Measles virus is believed to have become established in human populations about 5,000–10,000 years ago when human populations achieved sufficient size in the Middle Eastern river valley civilizations to maintain virus transmission. The critical community size is a key parameter for the persistence of measles virus transmission in insular populations [2, 39].

6 Mechanisms and Routes of Transmission

Measles virus is transmitted primarily by respiratory droplets small enough to traverse several feet but too large to remain suspended in the air for long periods of time. The symptoms induced during the prodrome, particularly sneezing and coughing, enhance transmission. Measles virus also may be transmitted by the airborne route, suspended on small particles for a prolonged time [40]. Direct contact with infected secretions can transmit measles virus, but the virus does not survive long on fomites as it is quickly killed by heat and ultraviolet radiation.

7 Pathogenesis and Immunity

7.1 Pathogenesis of Measles Virus Infection

Respiratory droplets from infected persons serve as vehicles of transmission by delivering infectious virus to respiratory tract mucosa of susceptible hosts. During the incubation period, measles virus replicates and spreads within the infected host. In the standard model of measles virus pathogenesis, viral replication occurs initially in epithelial cells in the upper respiratory tract and the virus spreads to local lymphatic tissue (Fig. 23.4). Replication in local lymph nodes is followed by viremia and the dissemination of measles virus to many organs, including lymph nodes, skin, kidney, gastrointestinal tract, and liver, where the virus replicates in epithelial and endothelial cells as well as in lymphocytes, monocytes, and macrophages. In a rhesus macaque model, the predominant cell types infected by measles virus were $CD150^+$ cells and dendritic cells [41]. Recently, an additional model of measles virus pathogenesis was proposed in which measles virus enters respiratory epithelial cells from infected lymphocytes and monocytes through the basolateral surface [42, 43]. Virus then buds from the apical surface, allowing for respiratory transmission.

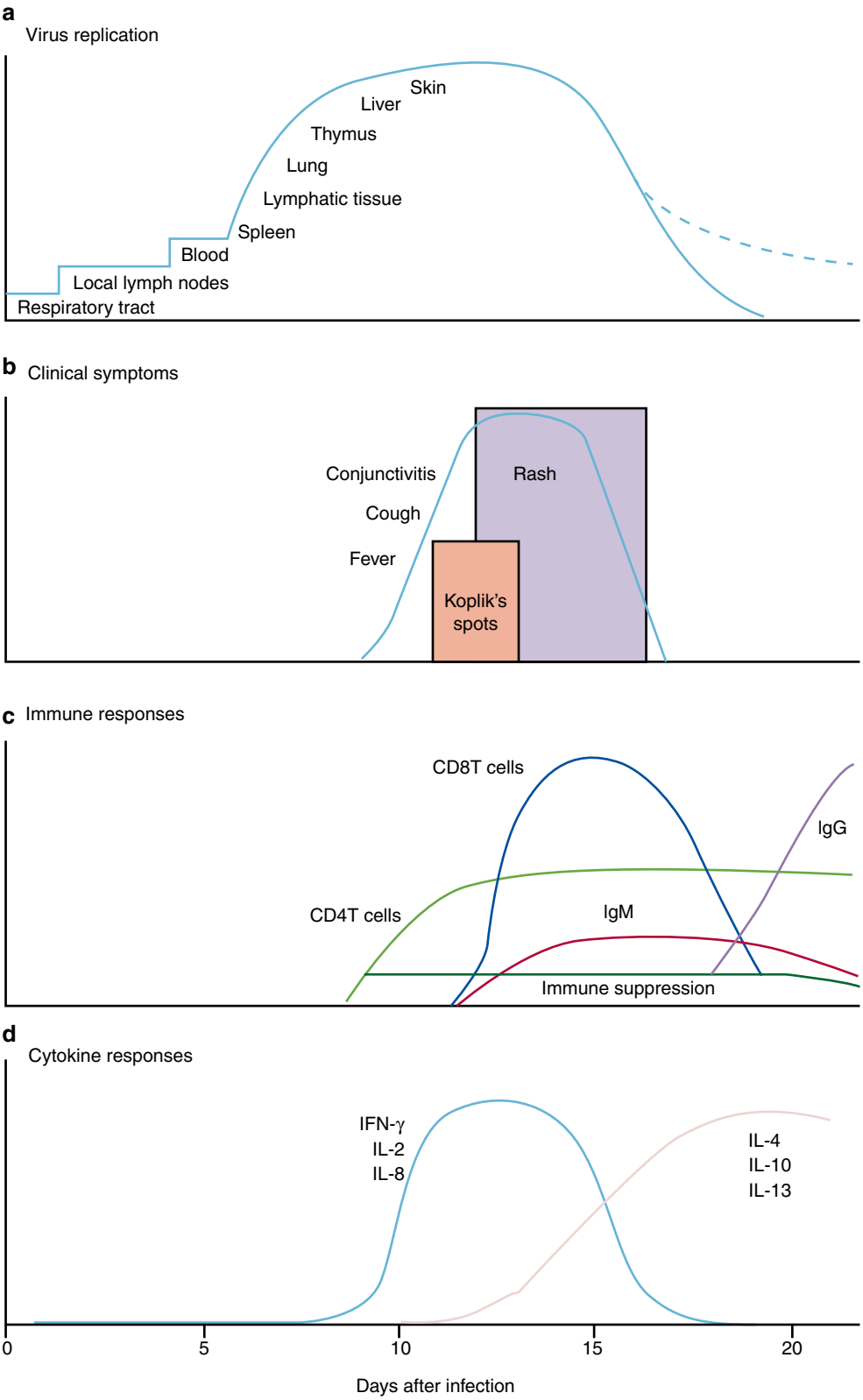

Fig. 23.4 Basic pathogenesis of measles virus infection. (**a**) Virus infection starts in the respiratory tract and then spreads to infect multiple organs including lymphoid tissue, liver, lungs, and skin. Virus clearance begins with the onset of rash. Clearance of infectious virus is complete 20 days after infection but viral RNA persists at multiple sites. (**b**) Clinical signs and symptoms begin about 10 days after infection with prodromal symptoms of fever, conjunctivitis and appearance of Koplik's spots followed by the maculopapular rash that lasts 3–5 days. (**c**) The rash is a manifestation of the adaptive immune response with infiltration of CD4+ and CD8+ T cells into sites of virus replication and initiation of virus clearance. There is a rapid activation, expansion, and then contraction of virus-specific CD8+ T cells. The CD4+ T cell response appears at the same time, but activation is prolonged. Measles-virus specific IgM appears with the rash and is commonly used to confirm the diagnosis of measles. This is followed by the sustained synthesis of measles-virus-specific IgG. Immune suppression is evident during acute disease and for many weeks after recovery. (d) Cytokines and chemokines that are produced during infection in sufficient quantities to be found in increased concentrations in plasma are of several distinct types. Shortly after infection, the chemokine IL-8 is increased. During rash, IFN-γ and IL-2 are produced by activated type 1 CD4+ T cells and by CD8+ T cells. After resolution of the rash, type 2 and regulatory CD4+ T cells produce IL-4, IL-10, and IL-13. *Dashed line* viral RNA, *IFN* interferon, *IL* interleukin.

Although measles virus infection is clinically inapparent during the incubation period, the virus is actively replicating and the host immune responses are developing. Evidence of these processes can be detected. During the incubation period, the number of circulating lymphocytes is reduced (lymphopenia). Measles virus can be isolated from the nasopharynx and

blood during the later part of the incubation period and in the several-day prodromal period prior to the onset of rash when levels of viremia are highest. The prodrome ends with the appearance of the measles rash. The rash results from measles virus-specific cellular immune responses and marks the beginning of viral clearance from blood and tissue. Clearance of infectious virus from the blood and other tissues occurs within the first week after the appearance of the rash, although measles virus RNA can be detected in body fluids of some children for at least 3 months using a PCR-based assay [29, 44].

7.2 Immune Responses to Measles Virus

Host immune responses to measles virus are essential for viral clearance, clinical recovery, and the establishment of long-term immunity. Early nonspecific (innate) immune responses occur during the prodromal phase of the illness. These innate immune responses contribute to the control of measles virus replication before the onset of more specific (adaptive) immune responses [45]. The adaptive immune responses consist of measles virus-specific humoral (antibody) and cellular responses. The protective efficacy of antibodies to measles virus is illustrated by the immunity conferred to infants from passively acquired maternal antibodies and the protection of exposed, susceptible individuals following administration of anti-measles virus immune globulin [46]. The first measles virus-specific antibodies produced after infection are of the IgM subtype (Fig. 23.4). The IgM antibody response is typically absent following re-exposure or revaccination and serves as a marker of primary infection. IgA antibodies to measles virus are found in mucosal secretions. The most abundant and most rapidly produced antibodies are against the N protein, and the absence of antibodies to N protein is the most accurate indicator of seronegativity to measles virus. Although not as abundant, antibodies to H and F proteins are responsible for virus neutralization and are sufficient to provide protection against measles.

Evidence for the importance of cellular immunity to measles virus clearance is demonstrated by the ability of children with agammaglobulinemia (congenital inability to produce antibodies) to fully recover from measles, whereas children with severe defects in T-lymphocyte function often develop severe or fatal progressive disease [47]. $CD4^+$ and $CD8^+$ T lymphocytes are activated in response to measles virus infection (Fig. 23.4) [48]. $CD4^+$ T cells secrete cytokines capable of modulating the humoral and cellular immune responses (Fig. 23.4) [49]. The initial predominant Th1 response (characterized by IFN-γ) is essential for viral clearance, and the later Th2 response (characterized by IL-4) promotes the development of measles virus-specific antibodies. $CD8^+$ T cells are cytotoxic and important for control and clearance of infectious virus [50, 51].

The duration of protective immunity following wild-type measles virus infection is generally thought to be life long [52]. The immunologic mechanisms involved in sustaining high levels of neutralizing antibody to measles virus are not completely understood, although general principles of immunologic memory probably govern this process. Immunologic memory to measles virus includes both continued production of measles virus-specific antibodies by long-lived plasma cells in the bone marrow and the circulation of measles virus-specific $CD4^+$ and $CD8^+$ T lymphocytes [53]. Although immune protection is best correlated with the levels of anti-measles virus antibodies, long-lasting cellular immunity almost certainly plays an important role in protection from infection and disease.

Measles vaccines also induce both humoral and cellular immune responses. Antibodies first appear between 12 and 15 days after vaccination and peak at 21–28 days. IgM antibodies appear transiently in blood, IgA antibodies are predominant in mucosal secretions, and IgG antibodies persist in blood for years. Vaccination also induces measles virus-specific T lymphocytes. Although both humoral and cellular responses can be induced by measles vaccine, they are of lower magnitude and shorter duration compared to those following wild-type measles virus infection.

7.3 Immune Suppression

The intense immune responses induced by measles virus infection are paradoxically associated with depressed responses to unrelated (non-measles virus) antigens, lasting for several weeks to months beyond resolution of the acute illness [54]. This state of immune suppression enhances susceptibility to secondary bacterial and viral infections causing pneumonia and diarrhea and is responsible for much of the morbidity and mortality attributed to measles. Delayed-type hypersensitivity (DTH) responses to recall antigens, such as tuberculin, are suppressed [55], and cellular and humoral responses to new antigens are impaired following measles virus infection [56]. Reactivation of tuberculosis and remission of autoimmune diseases have been described after measles and are attributed to this state of immune suppression.

Abnormalities of both the innate and adaptive immune responses have been described following measles virus infection. Transient lymphopenia with a reduction in $CD4^+$ and $CD8^+$ T lymphocytes occurs in children following measles virus infection. Functional abnormalities of immune cells have also been detected, including decreased lymphocyte proliferative responses [57]. Dendritic cells, major antigen-presenting cells, mature poorly, lose the ability to stimulate proliferative responses in lymphocytes, and undergo cell death when infected with measles virus in vitro [58]. The dominant Th2 response in children recover-

ing from measles can inhibit Th1 responses and increase susceptibility to intracellular pathogens [54]. Engagement of CD46 on monocytes suppresses IL-12 production, a cytokine important for Th1 responses [59]. Engagement of CD46 and CD3 on $CD4^+$ T cells induces production of high levels of IL-10 and transforming growth factor (TGF)-β, an immunomodulatory and immunosuppressive cytokine profile characteristic of regulatory T cells [60]. The role of these cytokines in the immune suppression following measles is supported by in vivo evidence of elevated levels of IL-10 in the plasma of children after measles virus infection [61].

8 Patterns of Host Response

8.1 Clinical Disease and Complications

Clinically apparent measles begins with a prodrome characterized by fever, cough, coryza (runny nose), and conjunctivitis (Fig. 23.4). Koplik's spots, small white lesions on the buccal mucosa inside the mouth, may be visible during the prodrome and allow the astute clinician to diagnose measles prior to the onset of rash. The prodromal symptoms intensify several days before the onset of rash. The characteristic erythematous and maculopapular rash appears first on the face and behind the ears and then spreads in a centrifugal fashion to the trunk and extremities (Fig. 23.5). The rash lasts for 3–4 days and fades in the same manner as it appeared. Malnourished children may develop a deeply pigmented rash that desquamates or peels during recovery [62].

In uncomplicated measles, clinical recovery begins soon after appearance of the rash. Unfortunately, complications occur in up to 40 % of measles cases, and the risk of complication is increased by extremes of age and malnutrition [62]. Complications of measles have been described in almost every organ system. The respiratory tract is a frequent site of complication, with pneumonia accounting for most measles-associated deaths [63]. Pneumonia is caused by secondary viral or bacterial infections or by measles virus itself. Pathologically, measles virus infection of the lung is characterized by multinucleated giant cells that form when measles virus proteins on the surface of infected cells facilitate cell fusion. Other respiratory complications include laryngotracheobronchitis (croup) and otitis media (ear infection). Mouth ulcers, or stomatitis, may hinder children from eating or drinking. Many children with measles develop diarrhea, which further contributes to malnutrition. Eye disease (keratoconjunctivitis) is common after measles, particularly in children with vitamin A deficiency, and was a frequent cause of blindness.

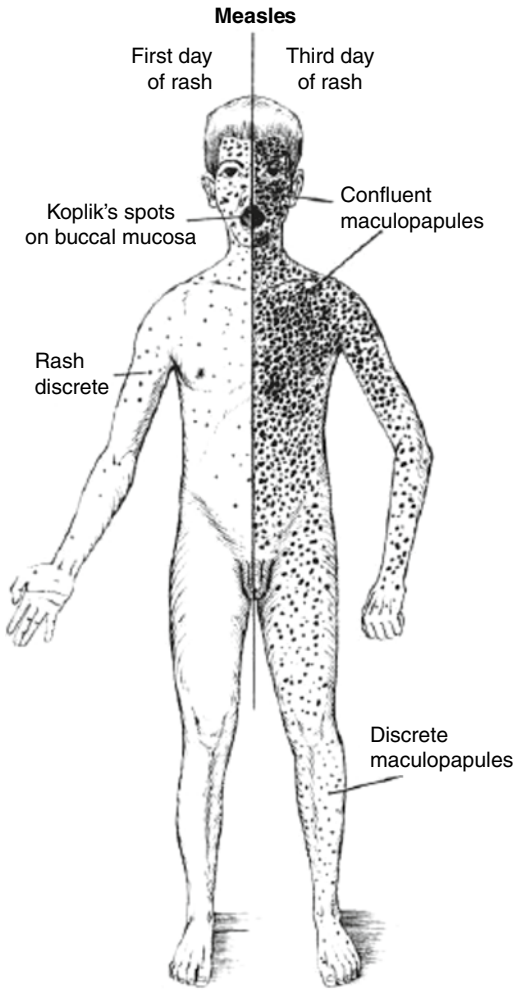

Fig. 23.5 Development and distribution of measles rash (Source: Perry and Halsey [69]. Krugman S. St. Louis: Mosby, 1958; Infectious Diseases of Children)

Because the rash of measles is a consequence of the cellular immune response, persons with impaired cellular immunity, such as those with the acquired immunodeficiency syndrome (AIDS), may not develop the characteristic measles rash. These persons have a high case fatality and may develop a giant cell pneumonitis caused by measles virus. T-lymphocyte defects due to causes other than HIV infection, such as cancer chemotherapy, also are associated with increased severity of measles.

Rare but serious complications of measles involve the central nervous system. Post-measles encephalomyelitis complicates approximately 1 in 1,000 cases, mainly older children and adults. Encephalomyelitis occurs within 2 weeks of the onset of rash and is characterized by fever, seizures, and a variety of neurologic abnormalities. The finding of perivenular

demyelination, the induction of immune responses to myelin basic protein, and the absence of measles virus in the brain suggest that post-measles encephalomyelitis is an autoimmune disorder triggered by measles virus infection. Other central nervous system complications that occur months to years after acute infection are measles inclusion body encephalitis (MIBE) and subacute sclerosing panencephalitis (SSPE). In contrast to post-measles encephalomyelitis, MIBE and SSPE are caused by persistent measles virus infection. MIBE is a rare but fatal complication that affects individuals with defective cellular immunity and typically occurs months after infection. SSPE is a slowly progressive disease characterized by seizures, progressive deterioration of cognitive and motor functions, followed by death that occurs 5–15 years after measles virus infection. It most often occurs in persons infected with measles virus before 2 years of age.

8.2 Measles Mortality and Case Fatality

The measles case fatality proportion is highest at extremes of age. Vaccinated children, should they develop disease after exposure, have less severe disease and significantly lower mortality rates. Vaccination programs, by increasing the average age of infection, shift the burden of disease out of the age group with the highest case fatality (infancy), further reducing measles mortality.

Measles case fatality proportions vary widely, depending upon the average age of infection, nutritional status of the population, measles vaccine coverage, and access to health care [64]. In developed countries, such as the United States, fewer than 1 in 1,000 children with measles die. In endemic areas in sub-Saharan Africa, the measles case fatality proportion may be 5 %. Measles is a major cause of child deaths in refugee camps and in internally displaced populations. Measles case fatality proportions in children in complex emergencies have been as high as 20–30 %. During a famine in Ethiopia, measles alone or in combination with wasting accounted for 22 % of 159 deaths among children younger than 5 years of age and 17 % of 72 deaths among children aged 5–14 years [65].

8.3 Nutritional Status

Measles and malnutrition have important bidirectional interactions. Measles is more severe in malnourished children, although separating the independent effects of nutritional and socioeconomic factors is often difficult. Children with severe malnutrition, such as those with marasmus or kwashiorkor, are at particular risk of death following measles. Measles, in turn, can exacerbate malnutrition by decreasing intake (particularly in children with mouth ulcers), increasing metabolic demands, and enhancing gastrointestinal loss of nutrients as a consequence of a protein-losing enteropathy [66]. Measles in persons with vitamin A deficiency leads to severe keratitis, corneal scarring, and blindness [67].

8.4 Sex Differences

Interestingly, some data suggest that measles mortality may be higher in girls. Among persons of different ages and across different regions, measles mortality in girls was estimated to be 5 % higher than in boys [68]. Although older historical data and recent surveillance data from the United States do not support this conclusion [69], if true, the higher mortality in girls is in contrast to most infectious diseases in which disease severity and mortality is highest in males. Supporting the hypothesis of biologic differences in the response to measles virus was the observation that girls were more likely than boys to have delayed mortality following receipt of high-titer measles vaccine [70]. The underlying mechanisms are likely differences in immune responses to measles virus between girls and boys, although no cogent biological theory has been developed [71].

8.5 Host Genetics

All persons without preexisting protective immunity are believed to be susceptible to infection with measles virus, and geographic differences in disease severity and mortality are almost certainly due to environmental and nutritional factors rather than genetic differences. Nevertheless, genetic factors (e.g., genes regulating cytokine production) may explain some of the differences in response to measles virus between individuals. The host genetics underlying immune responses to measles vaccine have been more extensively studied and suggest that polymorphisms in human leukocyte antigen (HLA) genes are associated with differences in antibody responses [72].

9 Control and Prevention

9.1 Measles Vaccines

The best means of preventing measles is active immunization with measles vaccine [73]. The first attenuated measles

vaccine was developed by passage of the Edmonston strain of measles virus, isolated by John Enders, in chick embryo fibroblasts to produce the Edmonston B virus [74]. Licensed in 1963 in the United States, this vaccine was protective but also induced fever and rash in many vaccinated children. Further passage of the Edmonston B virus produced the more attenuated Schwarz vaccine that was licensed in 1965 and currently serves as the standard measles vaccine in much of the world. The Moraten strain (meaning "more attenuated Enders" and licensed in 1968) was developed by Maurice Hilleman and is used in the United States. Attenuated measles vaccine strains have mutations that distinguish them from wild-type viruses [75] and exhibit decreased tropism for lymphocytes [76].

The recommended age of first vaccination varies from 6 to 15 months and is a balance between the optimum age for seroconversion and the probability of acquiring measles before that age [77]. The proportions of children who develop protective levels of antibody after measles vaccination are approximately 85 % at 9 months of age and 95 % at 12 months of age [78]. Two doses of measles vaccine are necessary to achieve sufficiently high levels of population immunity to interrupt transmission [35]. The first dose is typically administered through the primary healthcare system. The World Health Organization recommends that the first dose of measles vaccine be administered at 9 months of age [73], although countries in which the risk of measles is low frequently provide the first dose at 12–15 months of age. Two strategies to administer the second dose of measles vaccine are through the primary healthcare system or mass immunization campaigns called supplementary immunization activities, an approach first developed by the Pan American Health Organization (PAHO) for South and Central America and modeled after polio eradication strategies [79]. These campaigns are used to deliver other health interventions, including insecticide-treated bed nets for prevention of malaria, vitamin A, antihelminthic drugs, and other vaccines, such as rubella vaccine.

The duration of vaccine-induced immunity is at least several decades if not longer [52]. Secondary vaccine failure rates have been estimated to be approximately 5 % at 10–15 years after immunization, but are probably lower when vaccination is given after 12 months of age [80]. Decreasing antibody concentrations do not necessarily imply a complete loss of protective immunity, as a secondary immune response usually develops after re-exposure to measles virus, with a rapid rise in antibody titers without overt clinical disease.

9.2 Measures of Protection

Measles vaccine efficacy under study conditions, or effectiveness under field conditions, is measured as 1 minus a measure of the relative risk in the vaccinated group compared to the unvaccinated group (VE = 1 − RR). A number of field methods and study designs can be used to measure measles vaccine efficacy [81, 82]. For example, measles vaccine efficacy can be estimated by measuring the proportion of measles cases occurring in vaccinated persons and the proportion of the population that is vaccinated. Measles vaccine efficacy (VE) then can be calculated using the following equation:

$$\mathrm{VE} = \frac{\mathrm{PPV} - \mathrm{PCV}}{\mathrm{PPV} - (\mathrm{PCV} \times \mathrm{PPV})}$$

where PPV is the proportion of the population that is vaccinated against measles and PCV is the proportion of measles cases that are vaccinated.

Immunologic markers of protective immunity are commonly used to assess measles vaccines. Measurement of antibodies to measles virus by the plaque reduction neutralization assay is best correlated with protection from infection and remains the gold standard for determination of protective antibody titers. Neutralizing antibody levels of 120 mIU/mL (or 200 mIU/mL depending upon the WHO reference serum used) are considered protective following vaccination [83].

9.3 Optimal Age of Vaccination

The optimal age of measles vaccination is determined by consideration of the age-dependent increase in seroconversion following measles vaccination and the average age of infection. In regions of intense measles virus transmission, the average age of infection is low and the optimal strategy is to vaccinate against measles as young as possible. However, both maternally acquired antibodies and immunologic immaturity reduce the protective efficacy of measles vaccination in early infancy [84]. In many parts of the world, 9 months is considered the optimal age of measles vaccination and is the age recommended by the Expanded Programme on Immunization (EPI). Most countries following the EPI schedule administer measles vaccine alone, although more countries are introducing combined measles and rubella vaccines as rubella control programs expand. In communities with intense measles virus transmission, a significant proportion of children may acquire measles before 9 months of age. Under some circumstances, provision of an early dose of measles vaccine at 6 months of age (e.g., in outbreaks or to HIV-infected children) is appropriate. In contrast, in regions that have achieved measles control or elimination, and where the risk of measles in infants is low, the age of measles vaccination is increased to ensure that a higher proportion of children develop protective immunity. For example, in the United States, the first dose of measles vaccine is administered at 12–15 months of age, as a combined measles, mumps, and rubella (MMR) vaccine.

9.4 Surveillance and Outbreak Investigation

Disease surveillance is an important component of measles control programs, providing estimates of measles incidence and mortality, the effectiveness of the control program, and information to support targeted interventions [85]. Case-based surveillance with laboratory confirmation of suspected cases should be the goal. Not all suspected cases in an outbreak need to be laboratory confirmed if they can be epidemiologically linked to a confirmed case. As the incidence of measles declines, other viral causes of fever and rash may be mistaken for measles. Many surveillance programs test specimens for rubella virus-specific IgM antibodies. Although confirmation typically requires detection of anti-measles virus IgM antibodies in plasma or serum, less invasive specimen collection methods, including oral fluid swabs and dried blood spots collected on filter paper, can also be used [5].

10 Unresolved Problems

10.1 Measles Virus Persistence

Recent observations of measles pathogenesis in a rhesus macaque model, in conjunction with observations of prolonged detection of measles virus RNA in children [29, 44], suggest that measles virus RNA persists in peripheral blood mononuclear cells for up to 4 months after infection and is associated with a biphasic T-cell response with peaks at 7–25 days and 90–110 days [86]. These observations suggest measles may not be as acute a viral infection as once understood. Persistent measles virus replication could explain the prolonged state of immune suppression and the live-long immunity following infection [87].

10.2 Ideal Measles Vaccine

The ideal measles vaccine would be inexpensive, safe, heat-stable, and immunogenic in neonates or very young infants and administered as a single dose without needle or syringe [88]. The age at vaccination would ideally coincide with other vaccines in the EPI schedule to maximize compliance and share resources. Finally, a new vaccine should not prime individuals for atypical measles upon exposure of immunized individuals to wild-type measles virus (a complication of formalin-inactivated measles vaccines) [89] and should not be associated with prolonged immunosuppression, adversely affecting immune responses to subsequent infections (a complication of high-titer measles vaccines) [90].

Several candidate vaccines with some of these characteristics are undergoing development and testing. Naked cDNA vaccines are thermostable and inexpensive and could theoretically elicit antibody responses in the presence of passively acquired maternal antibody. DNA vaccines encoding either or both the measles H and F proteins are safe, immunogenic, and protective against measles challenge in naive, juvenile rhesus macaques [91]. A different construct, containing H, F, and N genes and an IL-2 molecular adjuvant, provided protection to infant macaques in the presence of neutralizing antibody [92, 93]. Alternative techniques for administering measles virus genes, such as alphavirus [86], parainfluenza virus [94], or enteric bacterial [95] vectors, are also under investigation. Oral immunization strategies have been developed using plant-based expression of the measles virus H protein in tobacco [96], and dry powder attenuated measles vaccine delivered by inhalation was shown to induce protective immunity in rhesus macaques [97].

10.3 Measles Eradication

In the WHO Region of the Americas, intensive vaccination and surveillance efforts interrupted endemic measles virus transmission [98], and all five remaining WHO regions set measles elimination targets of or before 2020. Progress in reducing measles incidence and mortality in sub-Saharan Africa [99] led to the proposal to eliminate measles in the WHO African region by 2020 [100].

The elimination of measles in large geographic areas, such as the Americas, suggests that global eradication is feasible with current vaccination strategies. Potential barriers to eradication include the following: (1) lack of political will, (2) difficulties of measles control in densely populated urban environments, (3) the HIV epidemic, (4) waning immunity and the potential transmission from subclinical cases, (5) transmission among susceptible adults, (6) the risk of unsafe injections, and (7) unfounded fears of disease caused by measles vaccine [101, 102]. Whether the threat from bioterrorism precludes stopping measles vaccination after eradication is a topic of debate, but, at the least, a single-dose rather than a two-dose measles vaccination strategy could be adopted.

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Paramyxoviruses: Mumps

24

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1 Introduction

Mumps is an acute contagious disease caused by a nonsegmented negative strain RNA virus in the paramyxovirus family. Infection with the mumps virus typically results in unilateral or bilateral parotitis, frequently accompanied by more significant complications, such as meningitis, pancreatitis, or orchitis. Symptoms usually resolve within 2 weeks of onset; serious complications are rare. Approximately one-third of infections are unrecognized or present with nonspecific or primarily respiratory symptoms. Prior to implementation of national mumps immunization programs, more than 90 % of most populations had serologic evidence of exposure to the virus by adolescence. Following widespread use of mumps-containing vaccine, disease incidence declined sharply, particularly in countries with a two-dose vaccine program. However, over the past few years there has been resurgence in mumps cases globally, even in highly vaccinated populations.

2 Historical Background

Mumps was first described by Hippocrates in the fifth century as an illness accompanied by swelling around one or both ears and, in some cases, painful swelling of one or both testes. Central nervous system (CNS) involvement was first described in the literature by Hamilton in 1790 [1]. The etymological origin of the term mumps is unclear but may stem from the English noun, *mump*, meaning lump, in reference to the distinctive facial swelling that accompanies the disease, or, alternatively, the name may derive from the mumbling speech of patients with parotitis.

Although in modern times mumps is generally thought of as a disease of childhood, historically it was considered to be an illness that affected armies during times of mobilization. Mumps was a major cause of morbidity among Confederate soldiers during the American Civil War [2] and was the leading cause of days lost from active duty by US troops in France during World War I [3, 4]. The annual hospital admission rate for mumps among US soldiers at that time was 55.8 per 1,000, exceeded only by influenza and gonorrhea [5]. The highest reported rate of mumps in the US Army (75.5 in 1,000) occurred in 1918 and declined markedly to 6.9 in 1000 in 1943. Although mumps incidence was greatly reduced during World War II relative to preceding wars, it remained a major problem, having a frequency three times higher than that of the next most common condition, measles [4]. Mumps outbreaks continued to occur in military settings until the implementation of routine MMR immunization of military recruits [6].

A viral etiology of the disease was suggested in several experiments conducted in animals in the early part of the 1900s [7–9], but it was not until 1934 that mumps was determined to be caused by a virus present in the saliva of infected patients [10, 11]. In these studies, Johnson and Goodpasture injected filtered, bacteria-free material from the saliva of patients with epidemic parotitis into the Stenson's ducts of rhesus monkeys and produced nonsuppurative parotitis. A diluted emulsion from the parotids of these monkeys caused parotitis in susceptible children but not in immune controls. Ten years following this landmark study, Habel [12] and Enders [13, 14] cultivated the virus in developing chick embryos, leading to the development of an inactivated vaccine that was tested in humans a few years later [15]. The first live attenuated mumps virus vaccines were used in the late 1950s in the Soviet Union [16] and in the early 1960s in the United States (US) [17].

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3 The Agent

Mumps virus is a member of the genus *Paramyxovirus* in the family Paramyxoviridae, genus *Rubulavirus*, which includes human parainfluenza viruses 2, 4a, and 4b; parainfluenza virus 5 (formerly simian virus 5); Mapuera virus; porcine rubulavirus (La Piedad Michoacan Mexico virus); simian virus 41; and tentative genus members Menangle virus and Tioman virus. The virus is readily inactivated by heat, formalin, ultraviolet light, and other agents.

The enveloped mumps virion is polymorphic and contains a negative-sense RNA genome of 15,384 nucleotides encoding seven genes that generally parallel those of the other paramyxoviruses. These include the nucleocapsid (N) gene, V gene, matrix (M) gene, fusion (F) gene, small hydrophobic (SH) gene, hemagglutinin-neuraminidase (HN) gene, and the large (L) gene [18]. Each gene encodes a single protein, with the exception of the V gene, a faithful transcript of which produces the nonstructural V protein, whereas cotranscriptional addition of non-templated guanine residues produces the phosphoprotein (P) and I protein [19, 20]. The N protein coats the viral RNA to form the ribonucleocapsid, which complexes with the P and L proteins, which together provide the polymerase activity responsible for transcription, replication, methylation, capping, and polyadenylation [21–24]. The M, F, SH, and HN proteins are all associated with the viral envelope. The M protein is involved in viral assembly, presumably by linking the N proteins of the ribonucleocapsid with the cytoplasmic tails of the HN and the F proteins that span the host cell-derived viral envelope [25]. The HN protein is responsible for cellular attachment during the initial infection process as well as release of nascent virions from the cell surface postinfection [26, 27]. The HN and F proteins work cooperatively to facilitate fusion of the virion membrane with the host cell membrane and introduce the viral nucleocapsid into the host cell. These two viral proteins also induce cell-to-cell fusion following infection [21, 28]. The V and SH proteins are accessory proteins, acting as antagonists of the host antiviral response, with the former interfering with the interferon response [29–31] and the latter interfering with the TNF-α-mediated apoptotic signaling pathway [32]. Studies of the role of the I protein in virus replication and infection are lacking.

The HN and F glycoproteins, being exposed on the virion surface, are the main targets of the humoral immune response. Only antibodies directed to these two proteins have been shown to neutralize virus infectivity and to confer protection [33–36]. Although mumps virus is serologically monotypic [37], antigenic differences among the various strains of mumps virus have been demonstrated in laboratory studies [38–42], and there is some evidence that such differences may allow for breakthrough infections in persons previously immunized, particularly upon waning of antibody levels [40, 41, 43, 44].

4 Methodology Involved in Epidemiologic Analysis

4.1 Sources of Data

The epidemiologic study of mumps is limited by the frequency of asymptomatic infection, the lack of serological testing in cases of parotitis with other etiologies misdiagnosed as mumps, and the failure to report cases. Large outbreaks, as well as specific studies, have provided information on incidence and complications. The experiences during World War I and World War II have contributed greatly to our understanding of the epidemiology of the disease [3, 4, 45, 46]. Mumps was first made a nationally reportable disease in 1922 but was removed from the list of reportable diseases in 1950. Mumps was reinstated as a notifiable disease in 1968 following the licensure of a live attenuated mumps virus vaccine. The number of cases of mumps reported each week is published in the Morbidity and Mortality Weekly Reports by the Centers for Disease Control and Prevention (CDC), and incidence data (derived from the National Notifiable Diseases Surveillance System) are summarized annually [47].

4.2 Serological Surveys

Over the years, the seroprevalence of mumps immunity has been assessed in a number of serological surveys using various methods available at the times the studies were conducted [6, 48–61].

4.3 Laboratory Methods

The diagnosis of mumps can be confirmed by isolation of the virus, by detection of viral RNA, or, with some limitations, by serological testing for virus-specific antibodies. The latter also forms the basis for assessing susceptibility to mumps infection and responses to vaccination.

4.3.1 Virus Isolation and RNA Detection

The virus can be recovered from most body fluids and secretions with variable degrees of success. The highest success rates have been achieved with buccal swabs obtained from the area proximal to the Stensen's duct. The virus can also be readily detected in saliva and, in cases of mumps meningitis, from the cerebrospinal fluid (CSF). Rates of success with other clinical specimens, such as urine and semen, are lower. Regardless of the source material, virus detection rates dramatically decline after the first week of symptoms. Despite the apparent high frequency of viremia during mumps (based on the evidence of wide dissemination of the virus), mumps virus or viral RNA has rarely been identified in blood or serum [62, 63], possibly related to the presence

of anti-mumps virus antibodies, which begin to appear at the time of symptoms [64].

For virus isolation, a number of cell lines can be used, the most permissive of which are Vero and CaCo-2 [65]. Cultures typically display syncytia formation within 5 days of incubation with mumps virus, followed by lysis; however, reliance on cytopathic effects alone is not a reliable means of confirming the presence of mumps virus since some mumps strains are non-cytopathic, and many of the viruses in the differential diagnosis of mumps cause cellular pathology indistinguishable from that induced by mumps virus. Instead, confirmation by PCR amplification of viral RNA is often required. Because of this need, and the ability to directly assay clinical material by PCR without an intervening in vitro culture step, virus isolation by culture is often unnecessary [44, 66–68]. The success of virus isolation or PCR-based detection may be influenced by vaccination status. While mumps virus RNA has been detected in >80 % of clinically diagnosed cases in children with a history of 0 or 1 dose of mumps-containing vaccine [44, 69, 70], only about 30 % of mumps cases involving children with a history of two doses of vaccine may test positive [66, 71]. While the reason for this phenomenon has not been established, it is likely due to an attenuated course of virus growth and spread in fully vaccinated individuals.

Data from PCR-based detection of the viral genome is also used for molecular epidemiological purposes. Such analyses are usually based on the sequence of the 316-nucleotide-long SH gene, the most variable in the genome and therefore the genomic region used for genotype classification. Presently, 12 different mumps virus genotypes have been assigned based on the SH gene sequence, designated A–N (excluding E and M, which have been merged with genotypes C and K, respectively) [72–74].

4.3.2 Serological Tests

The humoral response to mumps infection follows a course typical of that to respiratory viruses. Salivary IgA develops 5–7 days after infection and is followed by virus-specific serum IgA and IgM, detectable within a few days of symptom appearance. Levels of IgA and IgM peak 1–2 weeks later and decline over the next several weeks [43, 64, 75–78]. During this time, low-avidity serum IgG is produced [64, 77, 79]. Upon a second exposure to mumps virus, a high-avidity, virus-neutralizing IgG is produced, whereas IgM and IgA antibodies are often Undetectable [43]. Although virus-specific IgG can persist for many years to a lifetime, it appears that in the absence of periodic natural boosting, antibody levels and antibody effectiveness decline over time [37, 80–85], possible resulting in the susceptibility of persons previously immune.

Historically, serological testing has been the gold standard for a laboratory confirmation of mumps; however, in the present era of high vaccine coverage, unless a significant rise in virus-specific IgG can be demonstrated using paired acute and convalescent serum samples, results of serological testing must be carefully considered. Before the widespread use of vaccine, enzyme immunoassay (EIA) detection of virus-specific antibody was reliable for confirming mumps, but in the present era where nearly everyone has a history of mumps vaccination, both cases and non-cases would be expected to be IgM negative and IgG positive. Thus, a negative IgM result no longer rules out mumps, and a positive IgG result no longer confirms mumps. It should be noted that some EIAs are highly sensitive, and although IgM is not produced in high amounts in the anamnestic immune response, it is nonetheless present. Thus, the detection of IgM in a person with mumps with a history of vaccination is not an evidence of primary vaccine failure [86].

While the EIA remains the most commonly used serology test, it is worth noting that detection of IgG by EIA does not imply the presence of protective antibody in that both virus-neutralizing (protective) and non-neutralizing antibodies are detected by this method. Seroconversion can be demonstrated by EIA even in the absence of demonstrable neutralizing antibody [87]. For the purpose of assessing protective immunity, only assays that measure levels of virus-neutralizing antibody, such as the plaque reduction neutralization assay or the microneutralization assay, are relevant. Other once prevalent techniques such as complement fixation (CF), radioimmunoassay (RIA), hemagglutination inhibition (HI), immunofluorescence assay (IFA), and hemolysis-in-gel (HIG) are no longer used due to their time-intensive nature, relative insensitivity, and/or problems with cross-reactions with other paramyxoviruses [77, 87–92].

Mumps antibody can also be detected in the CSF of patients with mumps meningitis. Antibody in CSF is detectable within a few days of onset of illness and peaks within 2 weeks. Elevated CSF–serum IgG antibody ratios from samples collected on the same day indicate mumps infection because mumps antibodies are uncommon in the CSF in the presence of other infections [93–96].

Skin tests are unreliable and should not be used to assess immunity to mumps [97, 98].

5 Descriptive Epidemiology

5.1 Incidence and Prevalence

Within a decade after the December 1967 licensure of mumps vaccine in the United States, the number of reported cases declined by nearly 90 % from 152,209 in 1968 when mumps was reinstituted as a reportable disease to 16,817 cases in 1978 (Fig. 24.1). Mumps previously had been a reportable disease between 1922 and 1950. Following a period of a record low incidence in the early 1980s, a resurgence

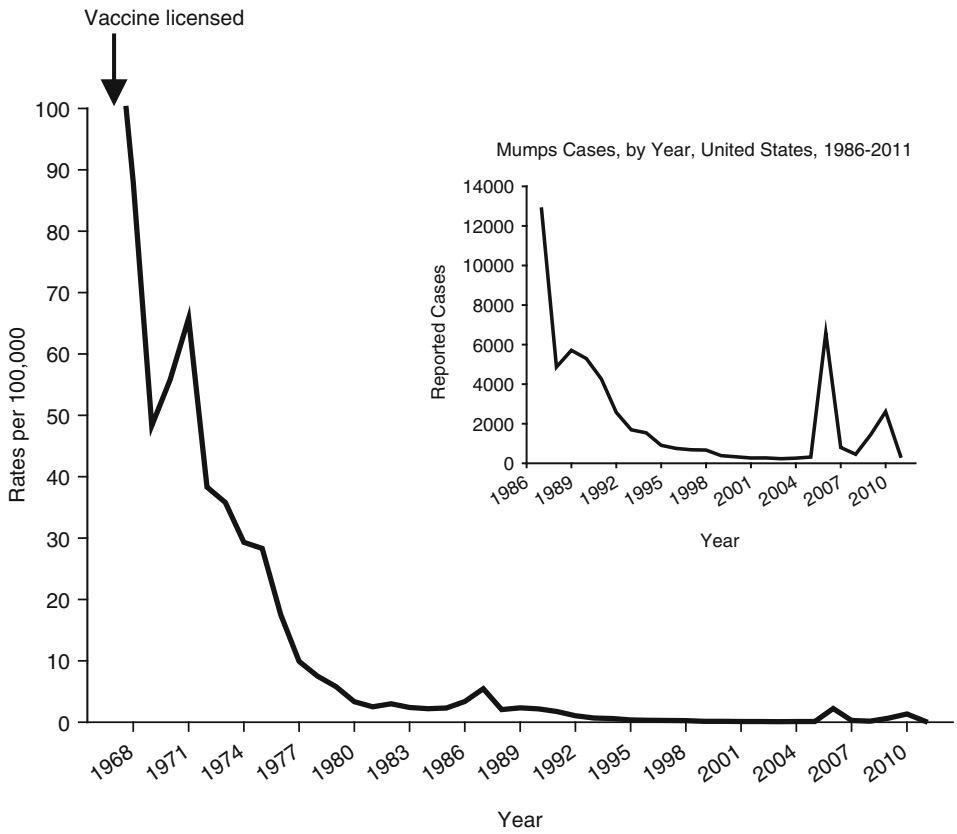

Fig. 24.1 Reported cases per 100,000 population per year. United States, 1968–2011. (Data from Centers for Disease Control and Prevention)

of mumps occurred in the United States between 1986 and 1987, which resulted in an almost fivefold increase in the annual incidence rate per 100,000 population (1985, 1.1; 1987, 5.2) [99]. This resurgence was attributed primarily to the low vaccination coverage of adolescents and young adults who were born between 1967 and 1977 [100, 101]. Although the vaccine was licensed in 1967, its use was limited until 1977 when it was recommended for routine use in young children. By 2003, the number of cases of mumps reported to the CDC reached an all-time low of 231 cases, representing a 99.9 % decrease in the number of cases from the prevaccine era. Mumps incidence remained at historic lows until 2006 when the United States experienced its largest mumps epidemic in 19 years with 6,584 cases reported, mostly in the Midwest (Fig. 24.1, inset) [102–104]. Unlike the 1986–1987 mumps resurgence, the mumps outbreaks in 2006 were not due to unvaccinated cohorts, as nearly all cases occurred in persons with a history of vaccination. The number of reported cases precipitously declined in the 2 years that followed but then spiked again in 2009–2010 with focal outbreaks in New York and New Jersey [105]. Provisional data for 2011 indicates a return to pre-outbreak levels.

The number of cases of mumps reported each year is generally considered to be far less than what actually occurs. Underreporting results in part from the mild nature of the disease and the associated lack of need to seek medical care in many instances. A serological survey conducted in Florida [55] highlighted an infrequent number of physician contacts and the failure of physicians to report cases; only 27 % of the 126 cases identified were seen by a physician and only 6 % were reported. The estimated annual incidence rate based on this study was ten times the rate based on national surveillance figures. Low reporting efficiency was identified in several other studies [3, 106, 107]. In addition, the high rate of subclinical mumps infections, estimated to approach 50 % in several studies [108, 109], contributes to a gross underestimation of the true incidence of infections. On the other hand, cases reported as mumps based on the presence of parotitis without laboratory confirmation may not be mumps, since there are other causes of parotitis. However, this is not a concern for cases of parotitis reported during an outbreak or for cases epidemiologically linked, since only mumps virus causes outbreaks of parotitis.

5.2 Survey Data

Numerous population surveys have been carried out using questionnaires and interviews, skin tests, and serological tests to assess patterns of susceptibility and immunity in communities.

In the 1960s, Harris and coworkers [110, 111] used questionnaires to study the incidence of mumps among health professionals, university faculty members, and households. During this time, which was before the wide use of mumps vaccine, cases were most likely among pediatricians and least likely among the general university faculty. Among households, the peak incidence of mumps was found to occur at 7 years of age, and 74 % of the study population reported mumps before 10 years of age.

An early study by Henle et al. [52] in 1951 showed that 70–80 % of persons with a history of mumps had positive findings on the CF test and skin tests. Although these tests are now known to be insensitive or unreliable, fewer than 2 % of those who tested positive subsequently acquired mumps.

Early serological surveys were conducted in military populations. Serosurveys in the prevaccine era from 1951 to 1962 using the CF or HI tests showed that 46–76 % of army recruits in the United States were immune to mumps [48, 56, 57]. Later serosurveys [49, 58, 59] conducted more than two decades after the licensure of mumps vaccine and using the more sensitive EIA found that 84–88 % of military recruits were immune. In contrast to World War I data that showed increased susceptibility among men from rural areas [112], no significant urban–rural differences were found in the later serosurveys conducted in 1962 and in 1989 [48, 49]. Kelley and coworkers [49] found that black, non-Hispanic recruits were more likely to be seropositive than those from other racial or ethnic groups.

Serosurveys also have been conducted in civilian populations of all ages. Retrospective tests on sera in the World Health Organization (WHO) Serum Bank at Yale University showed a wide variation in the proportion of seropositive findings according to geographic area in the prevaccine era [50]. Among children 5–9 years of age, mumps antibody was not found in any of the children from Alaska and in only 15 % of those from Tahiti. In contrast, antibody was detected in more than half of 5–9-year-olds from New Haven, Connecticut (52 %), the Cape Verde Islands (55 %), the Bahamas (74 %), and Iceland (79 %). Among persons of all ages residing in remote islands off Alaska, only 7 % of residents of St. Paul Island [113] and 12 % of those from St. George Island [114] were seropositive when assessed in the late 1960s. Similarly, mumps antibody was found in an average of only 33 % of members of isolated Indian tribes in South America [115]. In comparison, mumps antibody was found in 90 % of the residents of New Haven and 88 % of medical students tested in another seroprevalence study using the neutralization test [97].

Kenny et al. [116] measured mumps-neutralizing antibody in sera collected between 1970 and 1973 in children 1–15 years old in the Dominican Republic, Honduras, the Republic of Panama, and the United States. The proportion of seropositive 1–3-year-old children in the Middle American countries and in the United States was similar (20–25 %). In the older age groups, significantly higher rates of seropositivity were found among US children compared to children in the Middle American countries. Among children 4–6 years old, 70 % of children in the United States had mumps antibody compared to approximately 55 % of children in the Middle American countries ($p < 0.05$).

Variation in the average age of infection has been found in other serosurveys from other parts of the world. In St. Lucia, 70 % of unvaccinated children were found to be seropositive by 4 years of age [117]. In contrast, a majority of children from the Netherlands [118] and from Scotland [119] remained susceptible at this age. In the Netherlands, most children develop mumps between 4 and 6 years of age, and by 14 years of age, 90 % are seropositive. Before vaccination campaigns were initiated in Spain, 53 % of 3–5-year-old and 61 % of 6–7-year-old children were immune [120]. The lack of immunity was associated with rural residency, low socioeconomic status, and lack of school attendance or siblings. In the United Kingdom, before routine mumps vaccination was initiated in 1988, most cases were in children 6–7 years of age [121]. Subsequent to the routine vaccination program, the average age of infection has decreased, perhaps because of increased contacts among preschool age children [122]. Serological data from England and Wales from 1990 showed that 70 % of 1–2-year-olds and 73 % of 3–4-year-olds were immune [123].

Data from the 1999–2004 US National Health and Nutrition Examination Survey (NHANES) involving participants aged 6–49 years [60] revealed an overall age-adjusted mumps seroprevalence of 90.0 %, with a lower confidence limit of 88.8 %, well below the 92 % level estimated to be required for herd immunity [124, 125]. Seropositivity was higher (93.4 %) among participants in the earliest birth cohort (1949–1956) and lowest (85.7 %) among those born from 1967 through 1976. Seroprevalence among persons born from 1977 to 1986 and from 1987 to 1998 (the youngest cohort in the study) was 90.1 and 90.3 %, respectively. Seroprevalence was higher among US-born non-Hispanic blacks (96.4 %) and non-US-born Mexican Americans (93.7 %). Sex, education, and birthplace were also found to be independent predictors in some cohorts. As discussed in Sect. 9, seroprevalence in young children is likely higher now than indicated by the 1999–2004 NHANES, given that the median coverage with two doses of MMR vaccine among kindergartners during the 2011–2012 school year in the United States was estimated to be approximately 95 % [126].

5.3 Epidemic Behavior and Contagiousness

Mumps outbreaks typically occur wherever children and young adults aggregate, as in schools, military barracks, and other institutions. Mumps is less contagious than measles or

chickenpox. Hope-Simpson [127] demonstrated the infectiousness of mumps by assessing the incidence of cases resulting from every exposure of a susceptible person in the home and concluded that the susceptible–exposure attack rate for mumps was 31.1 % compared to 75.6 % for measles and 61.0 % for chickenpox. The higher rate of subclinical infection with mumps may account for an underestimation of the true infectiousness of the virus; however, the higher average age at infection observed for mumps [128] supports the less efficient transmission of the virus.

Outbreaks in virgin populations have been used to estimate the contagiousness of mumps. In three isolated island groups off Alaska, where no outbreak of mumps had occurred for more than 50 years and approximately 90 % of persons tested were seronegative [113, 114, 129], clinical mumps occurred in 35–65 % of the population and an additional 20–24 % had subclinical infections following the introduction of mumps virus.

5.4 Geographic Distribution

Mumps occurs worldwide with the exception of isolated island groups and remote, sparsely populated areas. In the United States, mumps outbreaks typically occur when a sufficient number of susceptible persons accumulate to support an outbreak. It follows that the incidence is higher in more densely populated areas. Recent data has shown that mumps outbreaks can even occur within highly vaccinated populations, particularly among groups of individuals in close contact, such as university campus environments. Investigations of three different mumps outbreaks in the United States occurring in 2006, all on college/university campuses, revealed that 96–100 % of cases occurred in persons with a history of vaccination during childhood, most with two doses [66, 130, 131]. Mumps outbreaks among high two-dose vaccinated populations were also identified in close-contact environments in other countries [132–134]. There is ample evidence of waning of mumps immunity postvaccination [81, 83–85, 135–140], and this is likely a significant contributory factor in these outbreaks.

Nucleotide sequencing of clinical isolates (see Sect. 4.3.1) indicates that virus genotypes D and G predominately circulate in the Western hemisphere, whereas genotypes F, C, and I predominately circulate in the Asia-Pacific region and genotypes B, H, J, and K in the Southern hemisphere (Fig. 24.2). Co-circulation of multiple genotypes within a region, or even within an outbreak, does occur. Some have suggested that

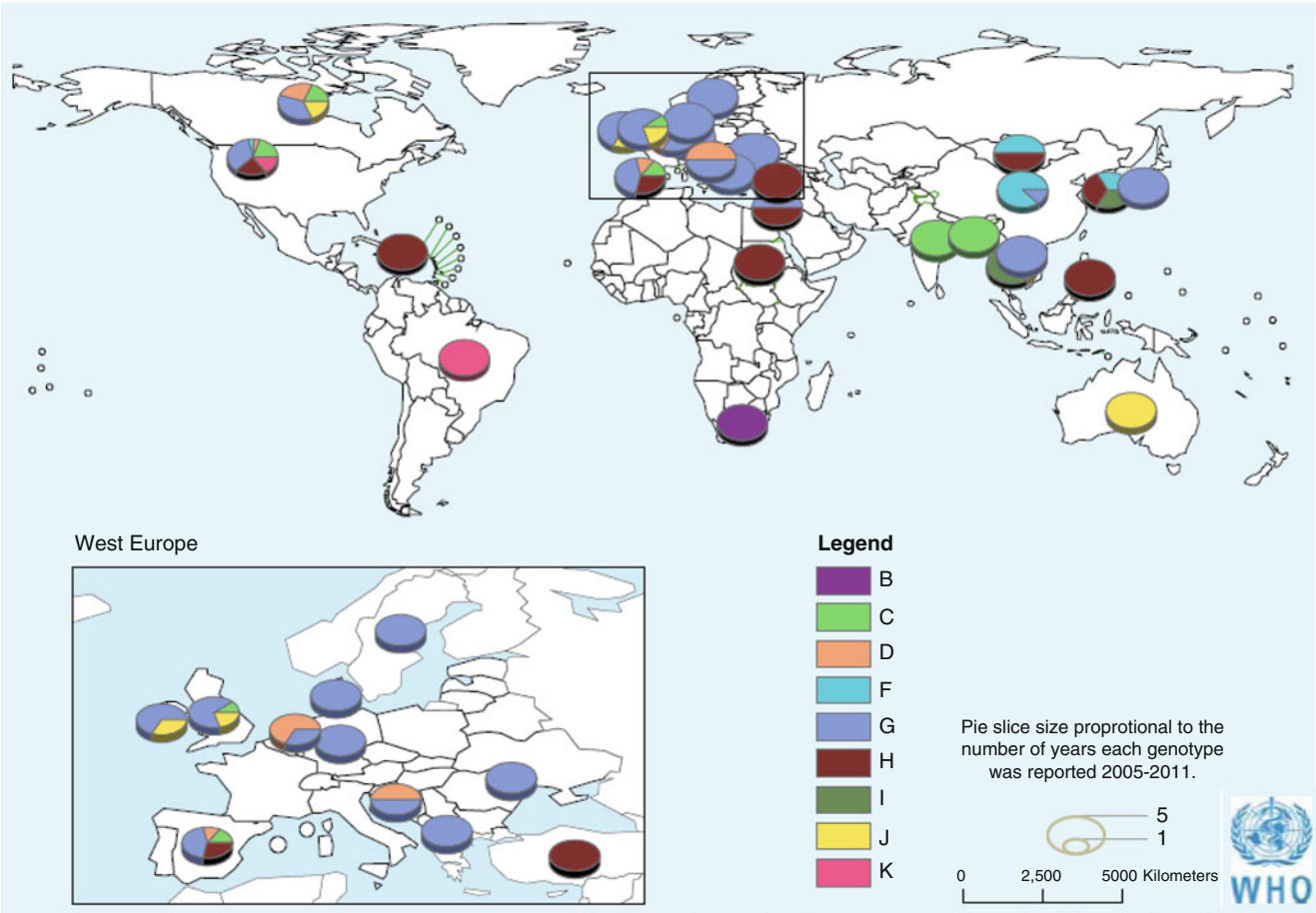

Fig. 24.2 Distribution of reported mumps genotypes, 2005–2011. (Source: courtesy of WHO, with permission http://www.who.int/immunization_monitoring/diseases/mumps/en/index.html, accessed September 11, 2012)

certain virus genotypes are more virulent than others [141–143]; however, this has not been adequately examined and remains to be demonstrated.

5.5 Temporal Distribution

In the North Temperate Zone, mumps occurs more frequently in winter and spring than at other times of the year. This pattern has not changed since the wide use of vaccine and the associated dramatic reduction in incidence. Seasonal differences are not apparent in tropical areas. Epidemiologic reports suggest an interepidemic period for mumps of approximately 3 years [121, 122, 144].

5.6 Age Distribution

In the prevaccine era, the largest proportion of cases occurred in children 5–9 years of age. Although mumps vaccine was licensed in the United States in 1967, it was not until 1977 that the Advisory Committee on Immunization Practices (ACIP) recommended routine vaccination of all children 1 year of age or older [145]. In the years following, the average age of cases began to increase, reflecting protection in younger populations. This shift to older age groups was first observed in 1982 [146]. A more marked shift occurred during a resurgence of mumps in 1986–1987 when a disproportionately high number of cases were reported among older children and adolescents aged 10–19 years and among young adults [147–149]. Between 1988 and 1993, persons 15 years of age and older have accounted for more than one-third of reported cases with known age compared to an estimated 8 % in this age group during the 5 years following licensure [150, 151]. By 1992, the highest age-specific incidence shifted back to the 5–9-years-old age group, remaining in this age group until the multistate mumps outbreak in 2006 when the age-specific incidence shifted again to older age groups [103, 152] before returning to the younger age group after the outbreak [153, 154]. This pattern again repeated itself during the recent mumps outbreaks in New York and New Jersey in 2009–2010 [105]. Mumps incidence by age group since 1990 is presented in Fig. 24.3.

The 1986–1987 resurgence reflected underimmunization of the cohort born between 1967 and 1977, a period of time when mumps vaccine was not administered routinely to children and the risk for exposure to mumps was decreasing [146]. More recent outbreaks, which have occurred in highly vaccinated populations, are thought to be due to waning immunity (see Sect. 5.4).

5.7 Gender Distribution

During non-outbreak years when only sporadic cases are reported, a trend toward higher attack rates is apparent among males than females (Fig. 24.4), likely a consequence

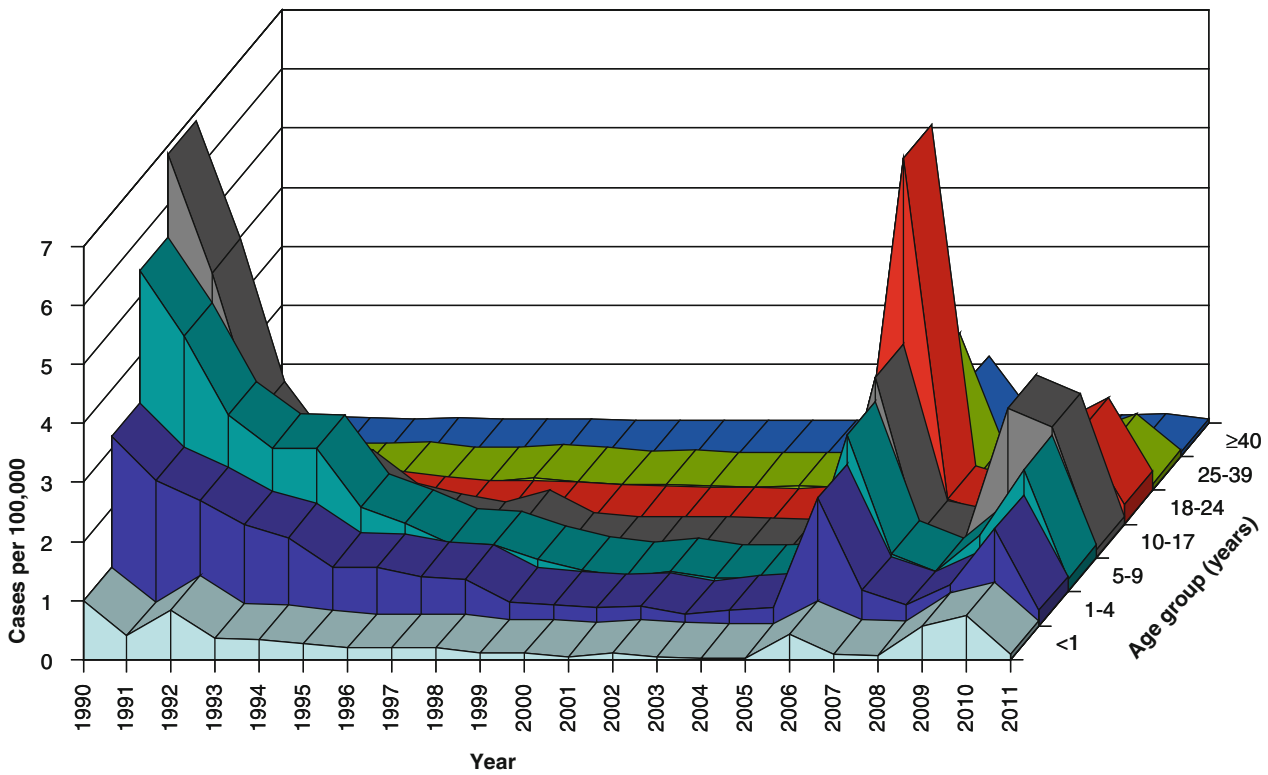

Fig. 24.3 Annual mumps incidence by age group, 1990–2011, as reported to the CDC. Figure courtesy of Albert Barskey, National Center for Immunization and Respiratory Diseases, CDC

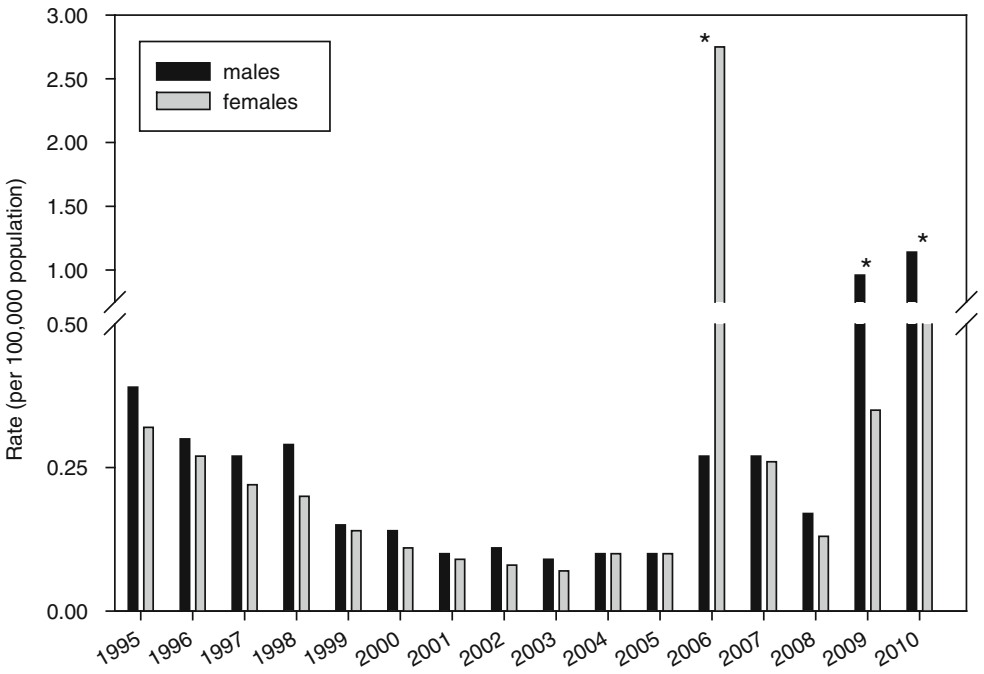

Fig. 24.4 Mumps incidence by gender, as reported to the CDC, 1995–2010, from Summary of Notifiable Diseases (http://www.cdc.gov/mmwr/mmwr_nd/index.html). * Denotes years during which large focal outbreaks significantly contributed to the total number of reported cases

of mumps in postpubertal males being frequently complicated by orchitis, and, thus, cases among males are more likely to be seen by physicians and to be reported. However, during mumps outbreaks, several investigations found higher attack rates in females [131, 139, 155–157]. This was particularly apparent during the multistate outbreak of mumps in the United States in 2006. It has been postulated that higher attack rates among females may be a reflection of gender differences in social behaviors facilitating increased transmission or exposure, but this is not clear [131, 155]. In contrast, during the 2009–2010 mumps outbreaks in the United States, attack rates were much higher among males, but this was a consequence of the outbreak setting, all male schools within orthodox Jewish communities in New York and New Jersey [105]. Fewer than 3 % of cases associated with the 2009–2010 mumps outbreaks occurred among persons outside this community.

Factoring out gender-specific manifestations (e.g., orchitis, oophoritis, mastitis), rates of complications are similar in males and females, with the exception of neurological manifestations, which appear with a 3:1 or greater male–female ratio [117, 158–160].

5.8 Race and Occupation

Historically, incidence rates among the black population tended to be higher than among the white population. This was first shown in the military and has been demonstrated subsequently in studies of civilian outbreaks [161, 162]. Based on data from 28 states in which race and ethnicity were reported for at least one-half of the cases, incidence rates among black persons ranged from 1.2 to 8.2 times those of other racial groups during the 4-year period of 1990–1993 [151]. Since that time, the trend has changed with somewhat higher rates of infection among whites (Fig. 24.5). In non-outbreak years, incidence rates among other racial/ethnic populations have been significantly higher than among blacks and whites. However, caution must be used when drawing conclusions from these data as different racial/ethnic populations might have different patterns of access to health care, regional differences in reporting compliance may exist, and many outbreaks may target racially/ethnically concentrated populations due to circumstances unrelated to race/ethnicity.

Persons engaged in occupations that involve contact with persons in age groups or settings associated with the highest incidence of mumps are likely to have frequent exposures to the mumps virus. A large mail survey conducted in the late 1960s when mumps was primarily a disease of young children showed that the risk for acquiring mumps from an occupational exposure was highest among practicing pediatricians and lowest among university staff [110]. In this study, the risk of mumps among teachers was related inversely to the age of their students. With recent outbreaks mostly occurring on university campuses, it would stand to reason that occupational exposure is now higher among university staff than among practicing pediatricians; however, this has not been examined.

5.9 Occurrence in Different Settings

Any setting in which a pool of susceptible persons are in close contact facilitates the transmission of the mumps virus.

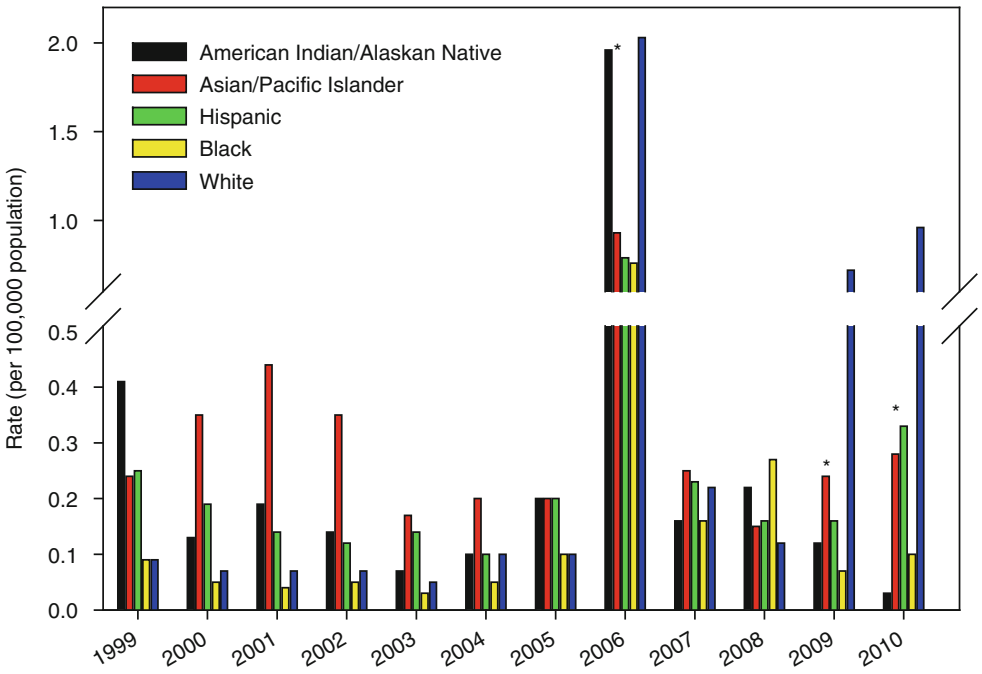

Fig. 24.5 Mumps incidence by race/ethnicity, as reported to the CDC, 1999–2010

5.9.1 Families and Schools

Families and schools have had an important role in the transmission of mumps [106]. Children exposed to mumps at school introduce the virus into the household where it may spread to susceptible family members. Prior to routine vaccination of young children, the younger children in a family usually acquired mumps at an earlier age than their oldest sibling. During that time period, most outbreaks occurred in school settings, with peak incidence in children during the first 5 years of school attendance. In the present era of decades of high vaccine coverage among young children, peak incidence has shifted to older age groups, presumably due to waning immunity in those individuals. Indeed, the time since the last vaccination has been linked to declining levels of mumps virus-specific antibodies [81, 83, 135, 136], decreased vaccine effectiveness [85, 137, 138], and an increased odds of being a case [84, 139, 140].

5.9.2 Military

Historically, epidemiologic data were derived largely from studies of military populations (see Sect. 2). The seasonality of mumps was recognized by the high proportion (70 %) of cases occurring during winter and spring. The incidence was higher among blacks than among whites. Mumps was associated with high rates of hospitalization and days lost from duty. Mortality was low, although deaths resulting from secondary infections such as pneumonia were more common during World War I than in later times when effective treatments for these complications became available. Seroprevalence studies carried out among military recruits in the prevaccine era found larger proportions of susceptible individuals [48, 56, 57] than were found in later serosurveys [49, 58, 59] (see Sect. 5.2). Mumps outbreaks in military settings continued into the postvaccine era [163, 164] but appeared to have tapered off by the mid-1990s, coinciding with the US military's practice of routinely administering MMR vaccine to all recruits without regard to prior vaccination status [6, 165]. The exception to this was the Air Force, which targeted MMR vaccination to recruits lacking serologic evidence of immunity to measles or rubella [49, 166].

5.9.3 Hospitals

Mumps is a relatively uncommon cause of nosocomial disease. Sporadic cases resulting from transmission in hospitals have been documented, including transmission from asymptomatic staff [98, 167–169]. Data from a study in an area with widespread outbreaks of mumps suggested that the introduction of mumps into hospitals by employees or by patients is likely when local incidence is high [170]. In a retrospective cohort study conducted at a hospital in Chicago, in a state reporting 795 cases of mumps during the 2006 multistate mumps outbreak, nine mumps cases were found to have resulted in 339 exposures, 98 % of whom were hospital staff [171]. The cost to the institution, which experienced ongoing transmission for 4 weeks, was calculated at $29,199 per mumps case.

5.10 Other Factors

Socioeconomic status has not been an important risk factor for mumps, except among impoverished populations where overcrowded living conditions facilitate the transmission of mumps virus as well as other infective agents.

6 Mechanisms and Routes of Transmission

Humans remain the only known reservoir of mumps virus. Although a virus with over 90 % similarity to mumps virus at the amino acid level was recently identified in bats [172], the only established route of transmission of mumps is person-to-person. In 1948, Henle and colleagues transmitted mumps to mumps-naïve children by following both oral and nasal routes of inoculation, suggesting that infection is spread through droplets containing mumps virus infecting the upper respiratory tract [109]. The spread of mumps among persons in close contact also suggests this mode of transmission. Mumps virus may be present in the saliva of infected individuals for up to 7 days before onset of clinical illness [173] and 8–9 days following onset; however, it is usually present from 2 to 3 days before and 4–5 days after onset of symptoms [174]. The virus also is present in the saliva of persons with inapparent infections [109]. Outbreak investigations performed during the prevaccine era demonstrate that most transmissions occur before symptom appearance or during the first few days of symptom onset [106]. Transmission of certain vaccine strains of the virus has also been demonstrated, but is an uncommon event [175–181]. In addition to horizontal transmission, data from animal studies and from case reports of humans provide evidence that mumps virus can also be transmitted transplacentally [182–186].

7 Pathogenesis and Immunity

During the incubation period of 16–18 days (range, 12–25 days) following exposure, it is believed by inference from related viruses that the mumps virus multiplies in the upper respiratory mucosa and spreads to the regional lymph nodes, resulting in transient viremia. Despite presumed viremia, the virus has only been infrequently isolated from the blood [63], possibly due to the coincident development of humoral antibody. Both through experimental infection of animals, laboratory studies on human samples, and in vitro studies, viremia appears to be predominately cell associated, with activated T lymphocytes being the most likely infected cell type [63, 187–189]. Cell-free viremia has been suggested by the isolation of virus from clarified human serum on one occasion [63], but this has not been subsequently reported.

The occurrence of viremia during mumps explains the wide dissemination of the virus, as indicated by multiple organ involvement. Parotitis or other salivary gland involvement is evident in nearly all clinical cases, a function of the clinical case definition being based on salivary gland swelling. Viral replication in the parotid gland produces periductal interstitial edema and local inflammation, primarily involving lymphocytes and macrophages [190]. Serum and urine amylase levels may be elevated as a result of inflammation and tissue damage in the parotid gland [191]. Viral excretion in saliva clears with the appearance of mumps-specific secretory IgA antibody about 5 days after onset [109, 173, 174].

Involvement of the CNS is common and may occur whether or not parotitis is present [192]. More than one-half of clinical mumps infections involve the CNS as measured by pleocytosis of the CSF [193, 194], although only a fraction of these cases present with overt CNS symptoms. The virus can be recovered from the CSF early in the course of meningitis [192, 195]. Experimental infection in hamsters suggests that mumps virus enters the CSF through the choroid plexus, permitting distribution of the virus through the ventricular pathways and the subarachnoid space [189]. Based on experimental infection studies, virus-infected choroidal and ependymal epithelia become inflamed and slough off into the CSF, a postulated mechanism for obstructive hydrocephalus and aqueductal stenosis commonly observed in laboratory animals infected intracerebrally [196–198]. Notably, hydrocephalus is a complication of mumps in humans, although relatively uncommon [199, 200]. While encephalitis can also develop, it too is rare, occurring in less than 0.5 % of clinical cases [158, 159, 201]. Encephalitis is probably a result of viral penetration of the brain parenchyma by spread from contiguous ependymal cells that line the ventricular cavities of the brain [18]. Viral entry into neurons allows the virus to spread widely along neuronal pathways. In addition to the evidence for viral persistence from laboratory studies of cell cultures [202–205] and from animal models [189, 206], cellular responses and oligoclonal humoral responses have been shown to persist within the CNS of patients, implying continued antigenic stimulation and suggesting the persistence of the virus [18, 207–209]. The association of such persistence with late-occurring progressive CNS disease has been suggested [210].

Mumps epididymo-orchitis is also common, occurring in up to 30 % of cases in postpubescent males. Epididymo-orchitis is believed to occur by direct invasion of testicular cells by the virus, as evidenced by the recovery of the virus from the semen and testis during orchitis [211, 212], although an indirect immune-mediated mechanism also has been postulated [213]. The pathology in cases of epididymo-orchitis involves both Leydig and germ cells and can lead to altered levels of hormones, including decreased testosterone production [214–216]. Hypofertility can result but is uncommon. Sterility is exceptionally rare.

Renal involvement occurs frequently and is mild. One group of investigators detected viruria in 80 % of specimens collected within the first 5 days [217], and in another study of young servicemen, viruria persisted in some patients for as long as 25 days following onset [218]. All of these men had some abnormal renal function tests; however, none had generalized edema or hypertension, and all had negative cultures and normal renal function at the end of the study.

Involvement of the pancreas is characteristic of experimental mumps, and human pancreatic beta cell cultures are permissive to the virus [219]. Pancreatic involvement in patients with mumps is generally limited to epigastric pain; however, extensive damage can occur, resulting in hemorrhagic pancreatitis, but this has rarely been reported [220].

Although uncommon, transient or permanent deafness is one of the distinctive features in mumps, and mumps virus is the most frequent cause of acquired unilateral sensorineural hearing loss in children. The pathogenesis of deafness in mumps is unclear but likely involves retrograde penetration of the virus from cervical lymph nodes into the perilymphatic fluid, resulting in infection of the cochlea and causing damage to the organ of Corti and the tectorial membrane [221–225]. No pathogenic link has been demonstrated between deafness in mumps and other complications.

Humoral immunity is most likely the primary mode of protection. Studies of infections by related viruses demonstrate cellular responses to also be important, particularly in the recovery and long-term protection from disease; however, the significance of cell-mediated immune responses in mumps infections is unclear. In persons with severely compromised T cell responses, the course of mumps was found to not differ from that seen in healthy individuals [226], suggesting a limited, if any, role of cellular immune responses in mumps symptoms and duration. Similarly, neither severe symptoms of mumps nor a protracted course of illness has been reported in persons with AIDS. Despite this, in vitro lymphocyte proliferative responses to mumps antigen occur in seropositive individuals [227] and are largely dependent on T lymphocytes with IgG receptors [228].

8 Patterns of Host Response

8.1 Common Clinical Features

Mumps virus infections may be asymptomatic or associated only with nonspecific or respiratory symptoms in more than one-half of persons infected [108, 229]. Inapparent infection may be more common in adults than in children, and parotitis may be more common in children. Among persons with clinically apparent disease, host responses vary, depending on which organs may be affected. The major clinical manifestations of mumps and their frequency are presented in Table 24.1, gleaned from a review of data from 47 mumps outbreaks involving at least 20 cases.

Swelling and tenderness of the salivary glands are common, primarily the parotid glands; however, sublingual and submandibular glands also may be involved. Parotitis may be unilateral or, more commonly, bilateral, peaking on about the third day of illness and resolving within 10 days. A prodromal period may precede the parotitis by several days with nonspecific symptoms, including fever, malaise, myalgia, headache, and anorexia. In some patients other glandular tissues may be infected, causing epididymo-orchitis [230], oophoritis, mastitis [231], pancreatitis [220], or thyroiditis [232]. Pleocytosis of the CSF is common [193, 194], but only in a fraction of such cases is aseptic meningitis

Table 24.1 Major clinical manifestations of mumps

	Frequency (%)			
Manifestation	Mean	Range	90 % confidence interval	Number of studies
Glandular				
Parotitis or other salivary gland swelling	98	83–100	96,99	49
Epididymo-orchitis [1]	13	1–39	11,16	46
Oophoritis	4	<1–17	1,7	9
Mastitis [2]	10	<1–31	2,18	6
Pancreatitis	4	<1–27	1,6	22
Neurologic				
Meningitis [3]	5	<1–33	3,7	35
Encephalitis	0.7	<1–2	0.1,1	8
Other				
Myocarditis [4]	6	1–15	2,10	5
Nephritis	0.4	<1–1	0,1	2
Deafness (transient or permanent)	2	<1–7	<1,4	9

Data sourced from Vaccine, 6th ed., Rubin and Plotkin, Mumps Vaccine, p.420, Copyright Elsevier 2013, with permission

[1] Male patients 12 years of age or older

[2] Female patients 12 years of age or older

[3] Includes symptoms described as severe headache and nuchal rigidity

[4] Based on electrocardiogram abnormalities

Table 24.2 Incidence of infection and clinical disease in a virgin soil outbreak of mumps in 561 residents of St. Lawrence Island, Bering Sea

Feature		Number	Percent
Mumps infections[a]		460[b]	82
Males	All infections	300[b]	53
	Clinical infection	205	68
	Asymptomatic infection	95	32
Females	All infections	261[b]	47
	Clinical infection	158	61
	Asymptomatic infection	103	40
Both sexes	All infections	561	100
	Clinical infection	363[b]	65
	Asymptomatic infection	198[b]	35
Total infection rate[c]			85
Clinical mumps	Any manifestation	363[b]	65
	Salivary gland swelling	344	95
	Stiffness or neck	40	11
	Scrotal swelling[d]	52[d]	25
	Swelling of breasts[e]	24	15

Data derived from Philip et al. [129]
[a]Based on serological data
[b]Of total population of 561
[c]An additional 3 % had disease without antibodies
[d]Of cases in males
[e]Of cases in females

clinically apparent, much less so for encephalitis. Transient renal abnormalities also are common, but they are seldom accompanied by clinical manifestations and, thus, are rarely reported [218]. Mumps virus can cross the placenta and infect the fetus [185], and mumps has been associated with spontaneous abortions and intrauterine fetal death [233] but not congenital malformations [234].

An investigation of mumps in a seronegative population of Eskimos living on St. Lawrence Island in the Bering Sea in the 1950s provided rates of clinical findings in serologically confirmed cases (Table 24.2) [129]. Mumps infection was confirmed in 82 % of the 561 residents. Orchitis and mastitis were age related with sharp increases in frequency at puberty. Orchitis was bilateral in 37 % of cases. There were a few women who had lower abdominal pain suggesting oophoritis. In a few cases there were symptoms of thyroiditis. Delirium, vomiting, and high fever were associated with stiff neck in some of the patients, but all recovered. One of four deaths reported during the outbreak occurred 2 days following the onset of parotitis in an infant; however, the cause of death was not determined. There were three spontaneous abortions among eight women with clinical mumps in the first trimester of pregnancy and one abortion among 12 women with inapparent infections. No stillbirths or miscarriages occurred among women who acquired infection after the first trimester, and there were no congenital malformations in infants born to mothers with mumps during pregnancy.

8.2 Involvement of the Central Nervous System

Mumps virus is as an important cause of aseptic meningitis and encephalitis [235–241]. The high frequency of involvement of the CNS in mumps infection demonstrated by numerous studies conducted over the past 50 years has led to CNS involvement frequently being considered part of the natural history of the disease.

Many studies of CNS involvement have categorized aseptic meningitis and encephalitis together under the term "meningoencephalitis" rendering it difficult to differentiate between these two CNS complications that have markedly different clinical courses and prognoses. Variability in incidence rates also reflects the case definition used, the skill of the observers, the age distribution of the population, whether cases are hospitalized or are diagnosed as outpatients, and the frequency of the use of lumbar puncture.

CSF pleocytosis most likely occurs in 40–65 % of patients with mumps, but symptoms of meningeal irritation are evident in fewer than 30 % of closely followed cases [193, 242]. Reported rates of meningitis diagnosed during outbreaks range widely from less than 1 % to approximately 20 % (Table 24.1).

The CSF profile consists of normal opening pressure, apredominance of lymphocytes with cell counts commonly between 200 and 600/mm^3, elevated protein in up to 70 % of patients, and moderately low glucose concentrations in up to 30 % of patients [159, 195, 243–247]. Aseptic meningitis associated with mumps is a benign disease that resolves spontaneously and is not associated with long-term morbidity. There is no correlation between the level of CSF pleocytosis and the severity of the illness or outcome [159, 246, 248].

Encephalitis complicating mumps is much less frequent than aseptic meningitis, probably occurring in 0.1 % of cases [159, 201]. Mumps had been considered the most common cause of viral encephalitis until the early 1980s when the incidence of mumps was greatly reduced and other viruses such as herpes simplex, enteroviruses, arboviruses, and varicella-zoster virus became the leading cause of encephalitis. Although mumps encephalitis may be a severe disease, most patients completely recover [195, 243, 249, 250]. Postencephalitis ataxia, behavioral changes, and electroencephalographic abnormalities, should they occur, typically resolve within a few weeks [195]. Rarely, permanent neurological sequelae can result from mumps encephalitis, including behavioral disorders, seizure disorders, cranial nerve palsies, muscle weakness, ataxia, chronic headaches, aqueductal stenosis, and hydrocephalus [159, 192, 196, 200, 244, 245, 248, 251, 252]. Myelitis or polyneuritis also may follow mumps encephalitis [253–256]. Overall, the mortality rate associated with mumps encephalitis is less than 2 % and is higher in adults than in

children. Neuropathologic findings at autopsy are variable, and many reports come from unconfirmed cases. In most carefully examined cases, there is evidence of both cellular destruction, suggesting a direct effect of the virus, and demyelinization, suggesting an autoimmune process [257].

Among children with mumps, CNS disease is three- to fourfold more common in males than in females [159, 195, 242–247].

Sensorineural deafness is an important sequelae of mumps and may occur in the absence of meningitis or encephalitis [258, 259]. Deafness usually has a sudden onset, is unilateral in about 80 % of cases, and is usually thought to be permanent [260–263], although one prospective study of 398 servicemen being treated for mumps at a military hospital found hearing loss due to mumps to be transient in most cases [259]. The incidence of hearing loss associated with mumps has been estimated to range from 0.05 per 1,000 cases [260] to over 40 per 1,000 cases [259], which may be an underestimate since inapparent mumps can cause deafness [264]. The virus may cause direct damage to the cochlea and to cochlear neurons [223, 265, 266]. Mumps virus has been isolated from the perilymph of a patient with mumps and sudden unilateral deafness [221].

8.3 Involvement of the Heart

Acute myocarditis may result from various viral infections, including mumps [267–274]. Among persons hospitalized for mumps, 3 % of children and 7 % of adults show transient electrocardiographic abnormalities involving the ST segment or atrioventricular conduction defects [267, 268]. Electrocardiographic abnormalities suggestive of myocarditis have been detected in 1–15 % of clinical cases, typically between days 5 and 10 of the illness [275], but clinically apparent cardiac complications are rare. When it does occur, myocarditis is usually self-limited; however, it may be catastrophic [267, 268, 271, 276]. Mumps has also been implicated in cases of endocardial fibroelastosis. Some investigators have suggested that maternal intrauterine infection may result in endocardial fibroelastosis based on positive mumps skin test results; however, the data is inconclusive [273, 277–280]. Nonetheless, mumps virus RNA has been detected in endomyocardial biopsies or autopsy samples from children with endocardial fibroelastosis [274, 281].

8.4 Orchitis and Sterility

Mumps may be complicated by orchitis in over one-third of postpubertal males (Table 24.1). In most cases, testicular swelling and pain is unilateral, but bilateral orchitis is common. Follow-up of men who had orchitis during the large outbreaks of mumps that occurred during World War I and World War II did not show an association with impotence or sterility. Although testicular atrophy occurs in approximately one-third of the cases of mumps orchitis [282], an effect on quality or quantity of sperm is infrequent [282, 283], and sterility rarely occurs [211, 215]. A small increased risk of testicular cancer following mumps orchitis has been reported [230, 284, 285], but the significance of this observation is unknown.

8.5 Mumps and Diabetes

Pancreatic involvement in mumps has been linked to diabetes mellitus, but a causal relationship remains to be proved [227, 286, 287]. Notably, pancreatic damage has not been documented in case reports of diabetes in children following mumps infection [288–295]. It has been speculated that mumps and diabetes have a related periodicity based on the occurrence of outbreaks of diabetes months or years following outbreaks of mumps [286]. These studies were uncontrolled and relied on the clinical diagnosis of mumps without supporting laboratory tests. In one study, mumps accounted for 8–22 % of the 30 % of cases of insulin-dependent diabetes mellitus in which a viral infection may have been the precipitating event [296]. Another study found a seasonal relationship of diabetes with coxsackievirus but not with other viruses [297]. In a subsequent study, the association of mumps and coxsackievirus with diabetes could not be confirmed [298]. Less than 1 % of cases of juvenile-onset diabetes followed recent infections with mumps virus in a sample of 1,663 diabetic patients in England [299]. A study in pregnant women found no association between the presence of mumps, coxsackie B or respiratory syncytial virus antibodies, and diabetes [300]. Other investigators also have failed to find a relationship between antibodies to mumps or other viruses and diabetes mellitus [301–303].

8.6 Other Complications

A variety of other acute and delayed complications have been reported following mumps infection. Arthropathy is a rare complication that occurs predominantly in young men. Gordon and Lauter [304] reviewed the literature on mumps arthritis from the first description in 1850 through the early 1980s. They noted that a total of 32 well-documented cases of mumps arthritis had been reported in the literature since 1924 when 6 cases (0.44 %) were reported during an outbreak of 1,334 cases of mumps in Paris. A retrospective survey of 2,482 patients with mumps in England and Wales

failed to identify any cases with arthritis. Arthropathy following mumps may be manifest as arthralgia without signs of inflammation; as polyarticular arthritis, which is often migratory; or as monoarticular arthritis of the knee, hip, or ankle. The arthropathy may last from 2 days to 6 months and is self-limited without sequelae. Mumps virus can replicate in human joint tissue in vitro [305, 306], and one study reported detection of mumps virus RNA in the synovial fluid in 3 of 42 patients with early rheumatoid arthritis [307]; however, there are no reports of isolation of the virus from affected joints of patients.

Viruria is a common finding in patients with mumps [308]. In one study all 20 patients with laboratory-confirmed mumps had abnormal renal function at some time during the acute infection, but all had normal renal function and negative urine cultures within 24 days [218]. Clinically apparent nephritis is uncommon and generally benign [309–312]; however, fatal cases have occurred [309, 313]. Nephritis may result from direct infection of the kidney or immune complex glomerulonephritis [313].

Ocular manifestations of mumps also have been reported, including involvement of the lacrimal gland, which affected 20 % of soldiers in a 1903 epidemic, and optic neuritis, which may be associated with involvement of other sites in the CNS [314]. For unknown reasons, such complications of mumps are now rarely reported. Keratitis, iritis, conjunctivitis, scleritis, endonitis, corneal endotheliitis, and central retinal vein occlusion have been reported rarely.

Although the persistence of mumps virus has been suggested as a factor in neurological and muscular disorders of unknown cause, there is no compelling evidence for a causal association [315–317].

9 Control and Prevention

Given the high rate of asymptomatic infections and that transmissions often occur before symptom appearance or during the first few days of symptom onset [106], case isolation is of limited value in preventing the spread of disease. Where indicated, the recommended period of patient isolation is 5 days following parotitis onset [318], a guideline based on recent data showing that fewer than 15 % of patients continue to shed virus beyond day 4 of symptom onset [319].

Standard immune globulin or gamma globulin preparations have been found to be of little value for postexposure prophylaxis [114, 320], although some success has been achieved with mumps virus-specific immune globulin preparations, but only when administered early after the outbreak [320, 321]. Nonetheless, the value of mumps-specific immune globulin to public health was considered to be not high enough to warrant routine use and the product is no longer available in the United States or most other countries [322, 323]. Thus, vaccination remains the only practical control measure.

The first mumps vaccines were formalin-inactivated virus preparations, produced within a year of the 1945 publications of Habel [12] and Levens and Enders [324] reporting the propagation of mumps virus in embryonated eggs. These products were found to be safe and effective and were widely used [15, 325–329], although the duration of protection was relatively short-lived. In the United States, inactivated mumps vaccine was used from 1950 to 1978.

The observation that vaccines using killed antigens did not produce long-lasting protection led to the development of live attenuated mumps virus vaccines in the 1950s in the Soviet Union [16] and in the 1960s in the United States [17]. This effort was stimulated by the earlier work of Habel [12, 330] and Enders and Levens [14] demonstrating that live virus that had undergone continuous passage in chick embryo produced inapparent infections, but good immune responses in monkeys. At least ten different live attenuated mumps vaccine strains have been used in various countries around the world [331].

A live attenuated mumps virus vaccine using the Jeryl Lynn strain of mumps virus (named after the child from whom the strain was first isolated) was developed in 1965 and licensed in the United States in 1967 [17, 332–334]. This remains the only live mumps virus strain used in the United States and is the most widely distributed vaccine globally. Extensive prelicensure field trials were carried out by Stokes et al. [332] in Philadelphia and by others in several field studies [333–337]. Overall seroconversion rate was 97 % among children and 93 % among adults. Efficacy was approximately 95 % based on a follow-up period of 20 months. Vaccine-induced antibody persists for decades [338–341], although there is ample evidence of declining antibody titers with time postvaccination [81, 82, 135]. Whether such declines in virus-specific antibody levels can lead to breakthrough infections is a matter of controversy [80, 84, 85, 130, 140].

Mumps vaccine administered after exposure does not appear to provide clinical protection or alter the severity of the disease [336]. However, evidence from observational studies suggests that mass vaccination or "ring vaccination" during a mumps outbreak may help contain the disease and terminate the outbreak [161, 342]. This approach, although unlikely to prevent infection in those already exposed, will act to prevent infection in susceptible contacts.

Mumps vaccine is often administered in combination with measles, mumps, and rubella (MMR) vaccine or in combination with measles, mumps, rubella, and varicella (MMRV) vaccine. In the United States, mumps vaccine is no longer available in monovalent formulation. Incorporation of mumps vaccine into multivalent formulations with other viruses was found not to affect mumps vaccine safety or immunogenicity [331, 343–348].

In 1967, when the live attenuated mumps virus vaccine was licensed in the United States, mumps control was not a high priority in the public health community, as the disease was considered primarily a mild inconsequential disease of childhood. At that time, the Advisory Committee on Immunization Practices (ACIP) recommended that the newly licensed mumps vaccine be considered for use in children approaching puberty, for adolescents, and for adults who have not had mumps, but was not recommended for routine use in younger individuals due to uncertainties over the duration of immunity [349]. The specific concern was that protection against mumps in young children might be fleeting, resulting in an increased incidence of postpubertal susceptibility. A series of progressively stronger statements from the ACIP on mumps control followed [350, 351], with routine childhood immunization recommended in 1977. In 1989 the ACIP recommended that all children receive two doses of measles vaccine [352]. Because measles vaccine is generally given to children in the United States as MMR, the effect of implementation of this recommendation has been that most children receive two doses of mumps vaccine.

In general, people who can be considered immune to mumps (1) have documentation of vaccination with live mumps virus vaccine on or after their first birthday, (2) have laboratory evidence of mumps immunity, (3) have documentation of physician-diagnosed mumps, or (4) were born before 1957. During mumps outbreaks, vaccination with mumps-containing vaccine should be considered for people born before 1957.

In 2009 the ACIP revised their recommendations for evidence of mumps immunity for healthcare personnel to include documented administration of two doses of vaccine containing live mumps vaccine or laboratory evidence of immunity or laboratory confirmation of disease. For unvaccinated personnel born before 1957 who lack laboratory evidence of mumps immunity or laboratory confirmation of mumps, healthcare facilities should consider vaccinating with two doses of mumps-containing vaccine at the appropriate interval [353]. There is no increased risk associated with vaccinating immune persons; thus, those who are unsure of their histories of mumps disease or vaccination should be vaccinated.

Adverse effects of vaccination with the Jeryl Lynn vaccine, such as parotitis and low-grade fever, are infrequent and minor. Mild allergic reactions of short duration such as rash, pruritus, and purpura occur infrequently. Postvaccination aseptic meningitis has been reported in association with several mumps vaccine strains used outside the United States, but not for the Jeryl Lynn strain [354].

Mumps vaccine is contraindicated for pregnant women because of a theoretical risk to the fetus. Mumps vaccine virus has been shown to infect the placenta, but there is no evidence that it causes congenital malformations in humans [355]. Inadvertent vaccination during pregnancy should not be considered an indication for termination of pregnancy. Because live mumps vaccine is produced in chick embryo cell culture, persons with a history of anaphylactic reactions to eggs should be vaccinated with caution according to established protocols [356]; however, egg allergy is not a contraindication for vaccination. The vaccine also contains trace amounts of neomycin; thus, persons with anaphylactic reactions to neomycin should not receive the vaccine. Mumps vaccine should not be administered to immunodeficient persons; however, when vaccination against measles is indicated for symptomatic HIV-infected children, MMR vaccine can be used. Children who are infected with HIV but are asymptomatic should be vaccinated.

The benefit-to-cost ratio for mumps vaccination is high, with an estimated savings of $7–$14 per dollar spent on mumps vaccination programs [357, 358]. Zhou and colleagues calculated a savings of over $1.5 billion annually by vaccinating against mumps in the United States, equating to a saving of $3,614 per case prevented per year based on 2001 data [359].

School immunization laws beginning during the late 1970s have had a dramatic effect on outbreaks and the overall incidence of mumps. In 1977 when the ACIP recommended that children routinely be vaccinated against mumps [350], only five states had immunization laws. By 1993, this number steadily increased to 43, including the District of Columbia (DC). Presently, all states but Iowa require vaccination with mumps-containing vaccines for entrance into primary school [360]. The dramatic reduction in reported cases of mumps in the United States since 1991 reflects increasing implementation of school immunization laws requiring two doses of MMR.

In 1985, states without immunization laws had twice the incidence rates as states with comprehensive laws requiring proof of immunity against mumps [100]. During the resurgence of mumps in 1986, the reported incidence of mumps in 15 states without any requirement for mumps vaccination was 12-fold higher than that observed in states that had a comprehensive requirement [100]. An outbreak in New Jersey in 1983, in which students in the sixth grade were nearly seven times more likely to develop mumps than those in lower grades, reflected the partial school law that covered students from kindergarten through grade 5 [361, 362].

Although children can be exempted from immunization laws for various reasons, including religious, personal, or moral beliefs, the end effect of immunization laws is that presently nearly all children entering kindergarten have received at least one dose of mumps-containing vaccine. By 1996, ≥90 % of 19–35-month-old children had received at least one dose of MMR [363–365]. For the 2011–2012 school year in the United States, median coverage with two doses of MMR among kindergartners was 94.8 % based on

47 reporting states and the DC [126]. Among 49 reporting states and the DC, exemption rates ranged from <0.1 to 7.0 %, with a median rate of 1.5 % [126]. Based on the 2011 National Immunization Survey (NIS), among children 19–35 months of age (born January 2008–May 2010), coverage with one or more doses of MMR vaccine was estimated at 91.6 % [366], which is above the national *Healthy People 2020* target of 90 %.

Over 250 million doses of mumps-containing vaccine have been distributed in the United States since licensure in 1967. During this time, the incidence of mumps has been reduced by 99.8 %, with only 363 cases reported in 2011 compared to over 200,000 annually in the prevaccine era.

10 Unresolved Problems

Despite the remarkable success of mumps vaccination programs in reducing disease incidence, sporadic and sometimes large mumps outbreaks continue to occur, even in settings of high two-dose vaccine coverage. In 2006, the United States experienced its largest mumps outbreak in 19 years, with 6,584 cases reported, representing a 20-fold increase in the disease incidence in recent years. Disease incidence returned to the baseline the following year but then spiked again in 2009–2010. Whereas outbreaks in previous decades predominantly affected young, often unvaccinated children in primary and secondary schools, more recent outbreaks involve young adults on college and university campuses, the overwhelming majority of whom have a history of mumps vaccination [66, 132, 138, 367–373]. Thus, rather than a failure to vaccinate, contemporary outbreaks appear to be a consequence of vaccine failure, with the preponderance of evidence pointing toward waning immunity [81, 83–85, 135, 137–140]. Indeed, vaccine effectiveness during recent outbreaks is significantly lower than that reported under controlled clinical trials. In contrast to the prelicensure studies demonstrating a single dose of vaccine to be ≥95 % effective in preventing mumps [333, 334], more recent data from outbreak investigations have estimated vaccine efficacy to be less than 70 %, even for two doses of vaccine [84, 138, 369, 374]. These data support the growing body of evidence of waning immunity, i.e., two doses of MMR do not confer long-term protection against mumps. To some extent, waning immunity may be a consequence of the remarkable success of mumps vaccination programs themselves, i.e., as vaccine coverage increases, opportunities for virus transmission (which in the past served to periodically boost the vaccine-induced immune response) decreases. If indeed immunity is waning in older vaccinated cohorts, then adding a booster vaccination should be considered. The benefits of doing so are highlighted by the experience with military recruits who were spared involvement in the mumps resurgence in the United States in 2006, despite belonging to the same age group and residing in barracks across the United States, paralleling the high density, close-contact environment of university campuses. The likely reason for sparing of the military may lie with the military's practice in 1991 of routine administration of MMR to recruits without regard to prior vaccination status [6, 165]. This was later changed by some branches to targeted MMR vaccination of those lacking documentation of two doses of MMR or lacking serologic evidence of immunity to measles, mumps, or rubella.

While it is clear that the neutralizing antibody is required for protection [327, 375, 376], the amount required is not known. Neutralizing antibody titers in the range of 1:4–1:8 were found to be associated with protection in studies conducted during the prevaccine era [51, 98, 376, 377]; however, it does not appear that vaccine-induced antibody of this concentration is adequate [378]. More work is needed in this area to firmly establish a correlate of protection, if one exists. Particular attention should be given to evaluating the role of cellular immunity in disease prevention. While the presence or absence of mumps virus-specific cell-mediated responses correlates with the presence or absence of virus-specific antibody, the magnitudes of the two types of immune responses do not correlate [379, 380], and, in some instances, cell-mediated immune responses have been detected in seronegative persons [381, 382]. The precise role of cell-mediated immune responses in protection needs to be investigated.

In this era of routine use of more sophisticated epidemiological tools, such as viral gene amplification and nucleotide sequencing, it has been observed that virus isolates invariably cluster into genotype groupings distinct from those of the vaccine strains used, leading to the speculation by some that the recent rash of mumps outbreaks are due to the evolution of virus strains capable of escaping vaccine-induced immunity. While it was demonstrated that serum from recent vaccinees possessed strong neutralizing activity against a wide array of mumps virus strains [37], in light of the evidence of waning immunity, the persistence of such broad virus-neutralizing activity is not known and needs to be established.

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Paramyxoviruses: Parainfluenza Viruses

25

Janet A. Englund and Anne Moscona

1 Introduction

Acute respiratory infection is now the leading cause of mortality in young children under 5 years of age, accounting for nearly one-fifth (20 %) of childhood deaths worldwide and killing two to three million children each year [1]. Human parainfluenza viruses (HPIVs) types 1, 2, and 3, human metapneumovirus [2], and respiratory syncytial virus (RSV) cause the majority of childhood lower respiratory tract disease in the United States. The parainfluenza viruses types 1 and 3 belong to the *Respirovirus* genus within the *Paramyxovirinae* subfamily of the *Paramyxoviridae* family of negative-stranded RNA viruses, while parainfluenza virus type 2 belongs to the *Rubulavirus* genus.

Human parainfluenza viruses (HPIVs) cause lower respiratory tract diseases including bronchitis, bronchiolitis, and pneumonia in infants, children, and immunocompromised individuals [3] and are responsible for up to 30–40 % of all acute respiratory tract infections in infants and children. In adults, the impact of these respiratory viruses may be serious as well. For example, in adult hematopoietic stem cell transplant patients, respiratory viruses cause about two-thirds of the respiratory illnesses, with high mortality [4, 5]. HPIV3 accounts for the vast majority of HPIV infections following transplantation, causing pneumonia with a 35 % acute mortality rate and a 75 % mortality rate at 6 months [4, 6]. Parainfluenza virus type 1 (HPIV1) is the principal cause of croup (laryngotracheobronchitis) in children. Parainfluenza virus type 2 (HPIV2) resembles HPIV1 in its clinical manifestations, but serious illnesses occur less frequently. Infections with parainfluenza virus type 4 (HPIV4) are detected less frequently.

With the use of sensitive molecular detection methods, the frequency and importance of HPIV infections in the community and hospital settings are becoming more appreciated. A recent survey study estimated that HPIV accounted for 7 % of all hospitalizations for fever and/or acute respiratory illness in children under 5 years in the United States or 23,000 hospitalizations every year in the United States that can be attributed to HPIV [7]. Half of these result from HPIV3 infection, with most of the remainder caused by HPIV1. The effective use of corticosteroids and nebulized epinephrine for HPIV1-associated croup has decreased the need for hospitalization for croup, and this development helps explain the decreased fraction of severe cases caused by HPIV1 [8].

Despite the impact of these viruses on illness and hospitalization of young infants worldwide, no specific treatment or prevention strategies are yet available, with the exception of the symptomatic benefit derived from corticosteroids for HPIV1-associated croup [9]. Development of antiviral drugs and vaccines for these viruses has been hindered by gaps in fundamental knowledge about the pathogens and about the human immune response to respiratory viruses. Recent discoveries provide a basis for optimism that effective treatments and vaccines may be available in the near future.

2 Historical Background

Chanock [10] first reported the isolation of a virus from human sources in Cincinnati in 1956 from children with croup, a virus originally designated as the "croup-associated" (CA) virus. Two additional strains were identified by their ability to adsorb guinea pig erythrocytes onto infected rhesus monkey kidney cells in culture in 1958 [11]. These two viruses, first designated "HA-1" and "HA-2," for hemadsorption 1 and 2, shared many biological properties with CA virus while being antigenically distinct, and they

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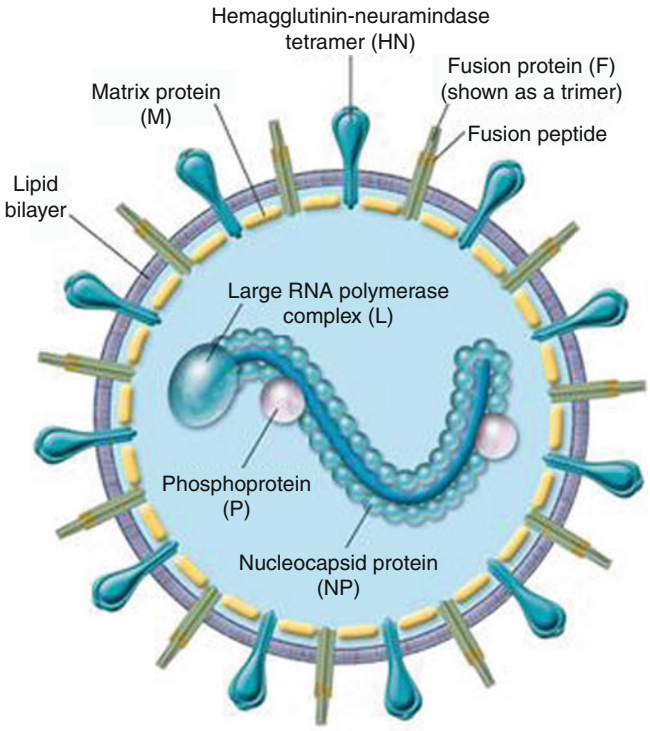

Fig. 25.1 A schematic diagram of the parainfluenza virion. *L* large RNA polymerase protein, *M* matrix protein, *NP* nucleocapsid protein, *P* phosphoprotein [26]

were later reclassified as HPIVs: HPIV1 (HA-2), HPIV2 (CA), and HPIV3 (HA-1) [12]. HPIV4 was first isolated in 1960 [13], with two subtypes of this virus now recognized: HPIV4a and HPIV4b. During the same period, these viruses were compared with isolates obtained from animals. The Sendai virus [14] recovered from rodents was found to share antigens with HPIV1 and is classified as a subtype of this strain [15, 16]. In 1959, a hemadsorbing virus [17] antigenically similar to HPIV3 virus [18] was recovered from cattle with "shipping fever." A simian virus, SV5 [19], has been shown to be related to HPIV2 virus [20, 21] and is now called HPIV5.

3 Biological Characteristics

HPIVs are RNA-containing viruses with a spike-covered lipoprotein envelope [22]. Figure 25.1 shows a schematic diagram of a representative virus. The genome consists of single-stranded, non-segmented, negative-sense ribonucleic acid. The spikes are formed by oligomers of two glycoproteins, the hemagglutinin-neuraminidase (HN) and the fusion protein (F). The tetrameric HN is responsible for the receptor-binding (hemagglutinating) and receptor-cleaving (neuraminidase) activities [23], as well as for activating the membrane fusion process during entry. The trimeric glycoprotein (F) directly mediates fusion of virus and cell membranes during viral entry. The mature form of the F glycoprotein is generated by enzyme cleavage, which results in two disulfide-linked subunits, F_1 and F_2 [24], and requires an activation step in order to mediate fusion; activation is provided by the HN upon receptor engagement [25–28]. The viruses are relatively antigenically stable; although subgroups of the specific types may have recognizable antigenic differences at certain epitopes, these differences are stable and nonprogressive. Conserved epitopes—particularly on the fusion proteins—generate antibodies that neutralize across these subgroups [29, 30]. HPIV3 viruses remain infectious in an aerosol for periods greater than 1 h [31]. While there are no reported systematic studies of the stability of other HPIVs in aerosol, HPIV1 has been isolated from air samples collected near infected children [32].

A biological feature characteristic of the HPIVs is their ability to replicate in the respiratory epithelium without deeper invasion or immediate host cell death [26]. In vitro models of HPIV3 infection in the human airway epithelium indicate that infection occurs exclusively in ciliated epithelial cells and utilizes sialic acid residues for initiating infection [33]. The virus buds from the host cell membrane without lysing the cell, permitting continued release of infectious particles from a single cell [22]. HPIV3 virus may infect the mucosa of infants in the presence of maternally derived circulating antibodies [34–37] and also frequently reinfects older children despite circulating antibodies [38]. In fact, multiple reinfection is the hallmark of these viruses. Excretion of virus may be prolonged up to 1 month or longer, even with the second or third infection [39–41]. Immunocompromised patients may shed HPIV for many months, often with no or minimal intermittent symptoms [42]. Epidemiologic evidence suggests that reinfected children are infectious for their contacts [38]. The incubation period is approximately 3–6 days, and the virus spreads rapidly to a high percentage of persons in closed populations, indicating a high degree of infectiousness. In studies carried out in the 1950s, adult volunteers with preexisting antibody were infected with as little as 1,500 median tissue culture infectious doses ($TCID_{50}$) and more than half of the volunteers infected with HPIV developed signs and symptoms of an upper respiratory illness [43].

4 Diagnostic and Laboratory Methods

4.1 Culture

Classical methods of HPIV isolation in tissue culture have been utilized in most epidemiologic studies. However, direct identification of HPIV from cell culture usually requires fur-

ther evaluation because many viruses do not cause a direct cytopathic effect. Infection is therefore detected by a secondary step, for example, HPIV-type-specific labeled monoclonal or polyclonal antibodies [35, 44] or specific molecular detection using amplification strategies. The epidemiology of HPIV4 has been obscured due to difficulties in viral detection; HPIV4 does not cause cytopathic effect in many cell lines and detection of this virus was frequently missed using standard culture techniques.

4.2 Molecular Identification

Molecular methods have been used to detect virus and to examine questions of antigenic diversity among the HPIVs [29, 45–50]. Batteries of monoclonal antibodies directed toward epitopes of the surface glycoproteins were used for antigenic characterization, and gene sequencing later defined these epitopes. Reverse transcriptase-polymerase chain reaction (RT-PCR)-based sequencing assays have been adapted to epidemiologic studies of nosocomial infections [51–58]. The development of more sensitive, specific, and rapid diagnostic assays for respiratory viruses is critical for two major reasons. Diagnosis is becoming increasingly important to clinical management of patients and nosocomial disease in the health-care setting, and there is an urgent need for evaluation of the global distribution of disease.

The significant progress in applying molecular biology advances to respiratory virus diagnosis has led to several new clinically useful strategies [59–67]. For the practitioner, guidelines and clear data about the situations in which specific kinds of assays may be appropriate are needed. In the clinical setting, rapid identification of HPIV has been most frequently performed in clinical specimens using antigen detection with commercially available kits for rapid screening that have sensitivities and specificities of 80–90 %. Antigen detection has been the most widely used type of rapid test for HPIV until the early 2010s. These tests are performed directly in NP secretions, generally using fluorescent-conjugated antibody (DFA) and less frequently employing an enzyme-linked immunosorbent assay [60]. Most recently, molecular detection assays using multiplex quantitative reverse transcription-PCR-enzyme hybridization have been optimized to identify a panel of respiratory viruses and differentiate between HPIV1, HPIV2, and HPIV3 [68–70]. Several FDA-licensed molecular-based diagnostic multiplex RT-PCR assays for the detection of HPIV and other clinically important respiratory viruses are available, and the sensitivity, specificity, and positive and negative predictive values of these methods are excellent. Relatively simple RT-PCR kits such as those produced by Luminex Technology, Idaho Technology, or 3 M are currently being used in many medical center settings; sensitive and specific detection of HPIV and other respiratory viruses can be reliably reported to the clinician within 1 h [71]. Nonetheless, as for all rapid diagnostic assays available at the present time, confirmation by other means may still be important, especially in the face of a negative result in an ill child. Simple, effective assay kits (whether antigen or nucleic acid based) should allow for simple screening of children and are likely to be used more commonly in the future as antiviral therapies become available. Identification of the etiologic agent, even if no specific therapy is available, is key to containing respiratory virus outbreaks and avoiding transmission to vulnerable individuals.

The MassTag polymerase chain reaction method provides a paradigm for new detection strategies for early recognition and containment of a wide range of respiratory pathogens and exemplifies new molecular techniques that are sensitive and specific and potentially adaptable to diverse laboratory settings. Briese et al. have described the development of a MassTag polymerase chain reaction (PCR) for differential diagnosis of respiratory disease [72]. In this multiplex assay, the pathogen gene targets are coded by a library of 64 distinct mass tags. The microbial RNA or DNA is amplified by multiplex RT-PCR using up to 64 primers. Each primer is labeled with a different molecular weight tag, attached to the primer by a photo-cleavable link. After amplification, the mass tags are released from the amplified material by UV irradiation, the identity of the tag determined by mass spectrometry, and the identity of the organism determined by the presence of its two specific tags, one from each primer. The technology has been successfully applied to respiratory disease [72], where multiplex primer sets were designed to identify up to 22 respiratory pathogens in a single reaction. Such technology could be of use in the public health setting for identification of outbreaks and global surveillance.

4.3 Serology

Serological studies have been the main source of data delineating the importance of the parainfluenza viruses as etiologic agents of respiratory disease. Such studies have demonstrated the ubiquity of viral infection with HPIV and the similarity of age at acquisition of antibody throughout the world [73–80]. Many community studies have utilized both serological and viral isolation data but have relied most heavily on serological data to determine the frequency of infection within the population [81–86]. Vaccine studies also rely on serology as indicators of vaccine infectivity [74]. However, the use of newer, more sensitive technologies such as RT-PCR techniques and MassTag polymerase chain reaction method for surveillance in the future will be important.

5 Epidemiology

5.1 Morbidity and Mortality Data

Official morbidity reports do not include acute respiratory infections; furthermore, laboratory diagnosis is required to establish the diagnosis of an HPIV infection. Morbidity data, therefore, are based on prospective research studies of respiratory disease conducted around the world. Standardized techniques have been employed to provide some estimate of the impact of HPIV as causes of acute respiratory disease in diverse populations, with older studies relying on some combination of viral culture and serology and more recent studies mainly relying on molecular detection. Most studies have been cross-sectional studies of the etiology of either illnesses of hospitalized patients or epidemics in closed populations and provide a limited view of the total worldwide morbidity resulting from these viruses. Prospective investigations of the etiology of acute respiratory disease conducted over extended periods of time in large populations merit special mention because of the unique perspective provided. Seven studies carried out in the latter half of the twentieth century in Washington, DC [34, 87]; Chapel Hill, North Carolina [36, 88, 89]; Tecumseh, Michigan [86, 90]; Seattle, Washington [82]; Houston, Texas [39, 91, 92]; Tucson, Arizona [93, 94]; and Great Britain [95, 96] differed in methods of patient selection, types of illnesses surveyed, and ages of subjects, but as a group, they documented respiratory illness over an extended time period in widely diverse geographic environments and socioeconomic groups.

Other studies have focused on the occurrence of respiratory illness in specialized population groups. Children in hospital or outpatient settings have been studied extensively both in North America and Great Britain [7, 35, 97–104]. These patients include those with underlying conditions that put them at high risk for serious consequences of respiratory infections [45, 105–107]. Studies of uncomplicated respiratory disease in institutionalized children [38, 108–110] day-care groups [41, 111] and school children [112] have contributed information about the spectrum of disease caused by these agents. Additional family studies addressing the importance of HPIVs have been described using more classical techniques [113, 114]. The use of PCR detection in children in the family and day-care setting [111, 115] has contributed to the epidemiology by documenting the presence of prolonged shedding, multiple infections and reinfections, and asymptomatic disease. The role of HPIV has also been studied specifically in children with bronchiolitis [116], asthmatic children [117, 118], patients with chronic bronchitis [103], newborn nurseries [119], and children with hematologic malignancies [120]. HPIVs, especially HPIV3, are a serious problem for children with immune system compromise, including stem cell transplant (HSCT) patients. Known risks for increased mortality from HPIV3 after transplantation include graft-versus-host disease, steroid use, the presence of copathogens [121–123], and infection with HPIV3 within the first 100 days after transplant [123]. The lack of any effective therapy is especially troubling in these vulnerable groups (addressed in detail below).

The role of HPIVs as etiologic agents of respiratory illnesses in adults has also been investigated [81, 85, 124–129]. These studies have reported sampling of patients with acute respiratory disease in hospitals, military services, university student health services, and industrial medical facilities, generally relying on culture and serology. More recently, respiratory disease in hospitalized patients with underlying respiratory conditions has been prospectively evaluated utilizing a combination of culture, serology, and RT-PCR techniques [130, 131]. In the past, challenge experiments with HPIV have been conducted in volunteer studies in the United States [132–135] and Great Britain [43, 135, 136].

Evidence of infections with paramyxoviruses has been demonstrated throughout the world [77–79, 137], including tropical climates [80, 138–140] and isolated communities [75, 141], with the notable exception of the absence of HPIV1 and HPIV2 antibody in children in very remote Indian tribes in South America [142]. In developing countries, HPIVs are second only to RSV as the most commonly identified respiratory pathogens in children [138, 140, 143–150]. As in Europe and North America, HPIV3 is the most common HPIV isolated from severely ill infants, but types 1 and 2 have also been found [146, 150, 151].

Limited mortality data are available and consist chiefly of case reports [152–154], fatal pneumonia in severely immunocompromised patients [106, 121, 155–157], and case series in children with hematologic malignancy or HSCT [9]. Most deaths caused by HPIV are likely related to HPIV3 virus infections in young infants, but even in this age group, the etiology of fatal cases is not documented sufficiently to allow a reasonable estimate of the mortality rate in the general population. This problem is magnified in developing countries, where mortality attributed to acute respiratory disease is many times greater than in Europe or North America [1, 158]. Although the exact impact of HPIV in more recent studies in Africa has had varying results [159, 160], available data suggest that HPIV infections contribute to excess mortality [143, 145, 148, 149].

5.2 Incidence and Prevalence Data

5.2.1 Serological Surveys

HPIVs infect most persons during childhood. Serological surveys show that 90–100 % of children have antibodies to HPIV3 by 5 years of age [75, 80, 84]. Antibodies to HPIV1 and HPIV2 are not as universal as antibodies to HPIV3 or RSV, although 74 % of children over 5 years of age have

Table 25.1 Frequency of infection with parainfluenza virus type 3 (para 3) among children studied from birth, Houston Family Study, 1975–1980

Age (months)	Number of child-years	Number with infection with HPIV3			
		Primary	Reinfection	Total (rate)[a]	LRD[b] (rate)
0–12	121	75	6	81 (66.9)	14 (11.6)
13–24	90	30	31	61 (67.8)	9 (10.0)
25–36	63	2	21	23 (36.5)	2 (3.2)
37–48	39	0	13	13 (33.3)	1 (2.6)
49–60	24	0	4	4 (16.7)	0
Totals	337	107	75	182 (54.0)	26 (7.7)

[a]Per 100 child-years
[b]Lower respiratory tract disease

been shown to possess antibody against HPIV1 and 59 % against HPIV2 [161]. Similar data have been obtained in serological surveys in a wide variety of geographic areas [75–77, 80, 84, 140, 141] but not in remote South American tribes [142]. Serological studies of natives of Tristan da Cunha who moved to the United Kingdom demonstrated the rapidity with which an isolated population acquires antibodies to these viruses when they move to a heavily populated area [162].

5.2.2 Association of HPIVs with Illnesses

HPIV viruses were isolated from about 3 % of persons of all ages with acute respiratory disease presenting for medical care in Houston over a 5-year period [92]. During this time, 30 % of infections were type 1 and 10 % were type 2. Over 40 % of persons with HPIV1 and HPIV2 infections occurred among persons over 5 years of age, an older population than in those infected with HPIV3. Studies of lower respiratory disease have shown the highest isolation rates of HPIV1 and HPIV2 viruses to be in children between 4 months and 5 years of age [34, 36]. The rate for lower respiratory disease associated with HPIV1 virus infection in the Chapel Hill pediatric practice was 17 per 1,000 children per year for children under 4 years of age [36, 88, 89]. Infants followed in the Tucson study had lower rates in the first 6 months of life, but thereafter had rates comparable to those reported from Chapel Hill [94]. Lower respiratory disease after 2 years of age was relatively uncommon [37, 128]. Rates of HPIV infection, diagnosed by PCR methods, were demonstrated in 12 % of day-care attendees with respiratory illness, but only a minority (4/37, 11 %) were due to types 1 and 2 [52, 111] (Table 25.1).

Few studies of open populations have had sufficiently sized population denominators to allow calculation of attack rates. In Chapel Hill, studies of children with lower respiratory disease presenting to a pediatric practice from 1964 to 1975 showed that 15 children per 1,000 per year were infected with HPIV3 each year for the first 3 years of life [36, 88, 89]. The rates for lower respiratory disease in Tucson infants were similar in the first 6 months but fell below those in Chapel Hill during the last half of the first year [94].

In Houston, 121 children were followed from birth for infection with HPIV3 [92] (Table 25.1). About two-thirds were infected during each of the first 2 years of life. The risk of illness was at least 30 per 100 children per year. Most lower respiratory tract illnesses accompanied primary infection with a risk of 13 per 100 child-years. Over 90 % were infected at least once by 24 months of age, and almost 40 % had been infected twice. Similarly, 21 of 62 (34 %) infants in Chapel Hill followed longitudinally through the first 2 years of life developed respiratory illnesses associated with HPIV3 virus infection and 6 (9.7 per 100 children) had lower respiratory tract involvement. Four of fifteen were reinfected during the second year, yet 66 % of the total had neutralizing antibodies at the end of 2 years, indicating that mild or inapparent infections occurred at a rate similar to that of children presenting with illness. These two studies with intensive follow-up show that lower respiratory tract involvement due to HPIV3 is common in toddlers.

In contrast, seroepidemiological studies in school-age children [112] and young adults [81, 85, 126] have revealed a relatively low rate of HPIV seroconversions, although infections by these agents are well documented in adults. Prospective studies in long-term care facilities have shown relatively similar rates of HPIV1, HPIV2, and HPIV3 infection determined by prospective serological surveys compared to rates of infection with RSV, HMPV, and influenza [130].

HPIV3 virus infections have been detected in all studies of hospitalized children with acute lower respiratory disease [34, 87, 101, 104, 163], and this virus is now recognized to be second only to RSV as a cause of hospitalization for bronchiolitis and pneumonia in infants [7, 35–37, 89, 95]. Henrickson et al. assessed the disease burden in Wisconsin over 2 years (1996–1998) using multiplex PCR detection. The authors estimated that approximately 545,000 hospitalizations of children under 18 years old for lower respiratory tract disease occurred each year; the most common viruses detected each year in hospitalized children were RSV (A and B, 117,000), HPIVs (HPIV1 and HPIV2, 48, 000; HPIV3, 18,000), and influenza (A and B, 39,000). The HPIVs were detected much more frequently in immunocompromised children than in previously healthy children (33 % vs. 16 %).

A recent New Vaccine Surveillance Network study enrolled children younger than 5 years of age in the United States who were hospitalized with febrile or acute respiratory illnesses in the 2000s and found that HPIV accounted for almost 7 % of hospitalizations for fever and/or acute respiratory illness (ARI) in these young children. The authors estimated that HPIV3 is responsible for half of the 23,000 HPIV hospitalizations yearly [7]. In this study, nasal turbinate/throat swabs were tested for the pediatric respiratory viruses HPIV, RSV, and influenza virus, with culture and reverse transcription-polymerase chain reaction. Over the course of 4 years (2000–2004), 2,798 children were enrolled, and 191 HPIVs were identified from 189 children (6.8 % of enrolled: 73 HPIV type 1, 23 HPIV type 2, and 95 HPIV type 3), compared with 521 RSV and 159 influenza viruses. Mean HPIV hospitalization rates were 3.01, 1.73, 1.53, 0.39, and 1.02 per 1,000 children per year for ages 0–5, 6–11, 12–23, 24–59, and 0–59 months, respectively. The investigators concluded that the pediatric HPIV inpatient burden is substantial [7].

5.2.3 HPIV4 Virus Infections

HPIV4 virus has not been reported as frequently as the other HPIVs, but serological surveys indicate that infection may be common [164–166]. Most of these infections are considered to be asymptomatic, but isolation of the virus has likely been missed in studies relying on culture techniques [164]. More recent studies in hospitalized children [167] or studies of children attending day care [52] have documented variations in infection over time, with rates of symptomatic disease similar to that and sometimes exceeding that of other HPIV types.

5.3 Epidemic Behavior

HPIV can be isolated in any month of the year in both temperate [34, 89] and tropical climates [138–140]. Although HPIV3 circulation peaks generally in the spring, HPIV2 generally peaks in the fall, and HPIV1 seems to peak in the fall of only odd-numbered years [168] (Table 25.2).

5.3.1 HPIV1 and HPIV2

The longest continuous observation of the epidemic behavior of HPIV1 has been carried out at the Children's Hospital, Washington, District of Columbia. From 1957 until 1961, the virus was endemic [34]. Beginning in 1962–1974, sharp epidemics of HPIV1 virus occurred every 2 years in the autumn of even-numbered years [34, 87]. A similar pattern was noted in Great Britain between 1962 and 1977 [95, 96]. Epidemics of HPIV1 virus occurring in the fall of even-numbered years after 1962 have been described in other reports as well [89, 97–99, 101]. After 1970, a 3-year lapse occurred before resumption of biannual epidemics [169]. Since 1973, HPIV1 epidemics have occurred in the autumn of odd-numbered years in Houston, accompanied by HPIV2 [92]. In other longitudinal studies, including those in Tecumseh [86], HPIV1 virus occurred in the endemic pattern until 1971 but thereafter seen in autumn and early winter [90]. The evidence suggests that HPIV1 has varied patterns of occurrence but the predominant pattern in temperate zones is biannual epidemics in the fall [168].

HPIV2 appears to be nearly as ubiquitous as HPIV1 based on serological surveys, but is not associated as frequently with severe clinical disease. Because many laboratories have depended on the detection of viruses from more seriously ill or hospitalized patients with lower respiratory disease to document the presence of the virus in the community, less information on the occurrence of HPIV2 is available; this is changing with the availability of rapid molecular diagnostic methods for respiratory viruses. In the past surveillance studies of young children with minor respiratory illness, HPIV2 has had a distinct epidemic pattern with high attack rates in small, defined populations [41, 109, 110]. HPIV2 appears to occur in a sporadic epidemic pattern, often disappearing from the community for fairly long periods of time. Several studies in the United States have noted HPIV2 epidemics to occur in the fall of the odd-numbered years [87, 89, 90, 92, 170]; however, in Great Britain [95] and in the first Tecumseh study [86], HPIV2 occurred in well-defined but somewhat erratic epidemics, and in the Seattle surveillance studies [82], it was rarely identified.

5.3.2 HPIV3

Infections with HPIV3 virus have usually been described as endemic in nature, occurring throughout the year [87, 89]. Small outbreaks have been noted, but no predictable periodicity in their occurrence was noted until after a sharp outbreak occurred in Houston in 1977 (Fig. 25.2) [92]. Since that time, HPIV3 infections have tended to occur in the

Table 25.2 Clinical and epidemiologic manifestations of various HPIV types

Human serotype	Major clinical syndrome	Peak age (months)	Sex predominance	Periodicity[a]
HPIV1	Croup	6–24	Male	Epidemic (fall)
HPIV2	Croup	6–24	Male	Epidemic (fall)
HPIV3	Pneumonia and bronchiolitis	0–6	None	Endemic
HPIV4	URI	Unknown	Unknown	Endemic

[a]Peak season in parentheses

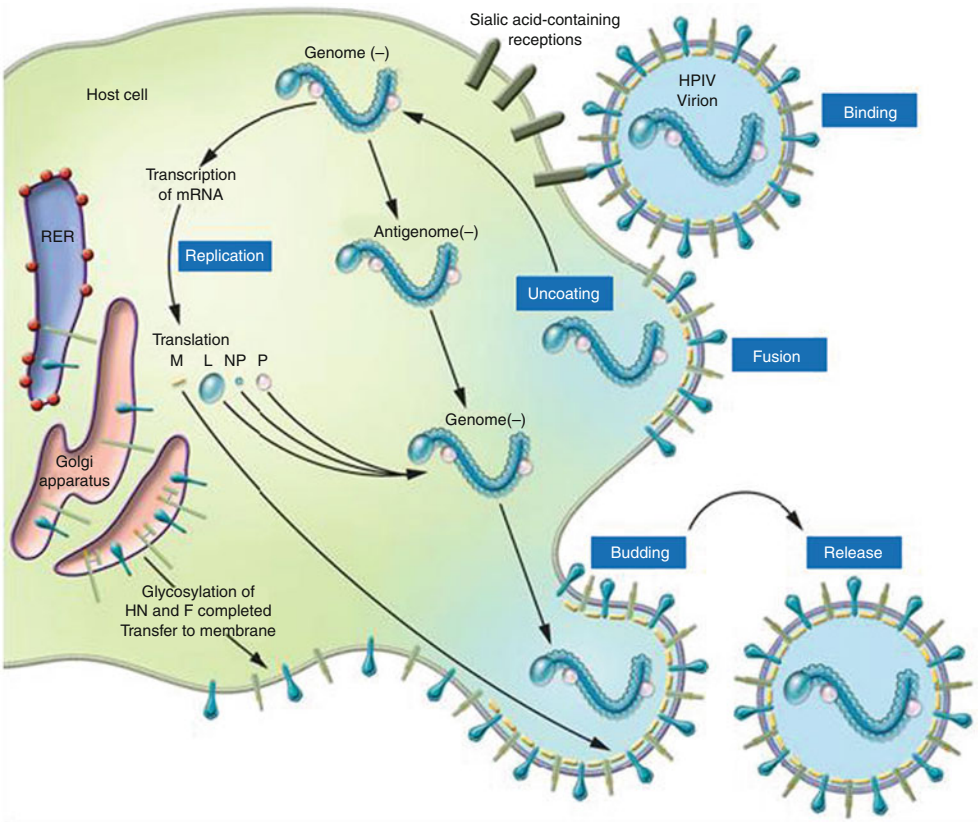

Fig. 25.2 Schematic illustration of the parainfluenza virus life cycle. RER, rough endoplasmic reticulum [26]

spring or during the months when HPIV1 and HPIV2 were not prevalent [168]. Although other major respiratory viruses generally cause relatively discrete epidemics, outbreaks of HPIV3 have occurred concurrently with outbreaks of other viruses [89].

5.3.3 HPIV4

HPIV4 has been assumed to cause relatively mild disease and generally has not been included in rapid antigen detection panels or diagnostic tests offered in pediatric centers. The addition of PCR techniques has increased detection rates of this virus. Recently, HPIV4 infection was specifically investigated in 225 young children attending day care in Fort Lewis, WA, using RT-PCR techniques [52]. HPIV was detected in 87/523 (17 %) of illnesses, with HPIV4 present in 10 % of these HPIV illnesses, the second most common type following HPIV3 (85 % of HPIV-positive illnesses). Marked differences in the proportion of HPIV4 infections between the early and later years of the 3-year study period were seen (22 % the first 2 years vs. 2 % the last year). No differences in the demographic profiles of children infected with HPIV4 versus other HPIV types were detected, including age, sex, race, or duration of time spent in day care, and no seasonal pattern was apparent for HPIV4 infection. Only one of 9 HPIV4 infections was associated with croup; the remaining cases all had uncomplicated upper respiratory tract infections. HPIV4 shedding was documented for up to 16 days, with a median of 12 days. In addition, HPIV4 was detected in 4/127 (3 %) asymptomatic enrollment swabs, with no asymptomatic shedding documented for either HPIV1 or HPIV2, and 3/127 (2 %) for HPIV3.

Other recent studies using molecular techniques to detect HPIV4 have been primarily based on hospitalized children, with a potential bias toward children with more severe illness. The proportion of HPIV4 as a cause of HPIV infection in these hospital studies ranges from 1.2 to 9.1 %, with a median of 3.5 % [58, 167, 171, 172], compared to the 10 % rate documented in the day-care study. The relatively higher rate of HPIV4 infection in the outpatient study suggests that HPIV4 may not be as likely to cause severe infection.

5.4 Geographic Distribution

HPIVs are found throughout the world and cause illness in young children wherever they have been identified. There is remarkable similarity of serological and isolation or PCR data obtained from tropical [80, 138, 173], temperate [38, 78, 84], and arctic [141, 174] climates. However, antibody to HPIV1 was found in very few Tiriyo Indians in South America and none under age 20; in the Xikrin tribe, there was no antibody in persons under age 17 years. HPIV2 antibody was present in Xikrin of all ages

but almost entirely absent in other tribes. This suggests that fresh introductions of virus are needed in very remote tribes in which the population base is too small to sustain the infection [142].

5.5 Age Distribution

5.5.1 HPIV1 and HPIV2

Data from studies of lower respiratory disease show few cases of severe disease in infants under 4 months of age [35, 36, 82, 95, 104, 138], with the most severe disease documented in premature infants or in newborn intensive care units [175, 176]. After 4 months of age, the number of cases of croup and other lower respiratory diseases increases dramatically [36], until approximately age 6 years. Lower respiratory illnesses in persons infected by HPIV1 and HPIV2 viruses are unusual in adolescents [89] and adults [81, 128], although they have been reported [129, 177].

Studies of milder illness have shown a similar age distribution. Infection and minor clinical illness occur more frequently in younger children than in adolescents and adults [86, 113, 178]. Infections do occur in older persons [81, 85, 86, 90, 95, 114, 126], most of which presumably represent recurrent infections in persons with antibody. Studies conducted in Tecumseh [86, 90] showed serological evidence of frequent infections in adults in age groups likely to include parents of school children. In family studies, infection in adults occurred concurrently with illness in their children, but attack rates in adults were distinctly lower than those in younger family members [113, 178]. Rates of hospitalization for HPIV1 and HPIV2 in adults with lower respiratory tract disease in the United States appear to represent only 0.2 and 2.5 % of adults hospitalized for lower respiratory tract disease [177].

5.5.2 HPIV3

Initial infections with HPIV3 virus occur early in life. Among children followed from birth in the Houston Family Study, 62 % were infected during the first year of life, and by the end of the second year, 92 % had been infected at least once and 36 % had one or more reinfections [92]. Similar to RSV infections, HPIV3 virus infections often occur in the very first months of life, despite the fact that these infants may still possess circulating antibodies derived from their mothers [34–37, 95]. Infants born with the highest liters of maternal antibody to HPIV3 may be spared during the first months of life. The risk of lower respiratory disease is greater for primary infection during the second year than for primary infection during the first year [92]. In studies of lower respiratory disease in the Chapel Hill pediatric group practice, the age-specific attack rate for HPIV3 disease paralleled that of RSV [36]. The average annual attack rate ranged from almost 15 to 7 per 1,000 children per year for children under 3 years of age [88].

After the first 3 years of life, the incidence of lower respiratory illnesses associated with HPIV3 infections falls off considerably, although studies indicate that reinfections are common in older children and adults. These infections are not generally associated with evidence of lower tract involvement [38, 81, 113, 125, 126]. Studies of HPIV3 infection in adults hospitalized with lower respiratory tract infection indicate an infection rate of approximately 3 % based on serology alone [177]. In a prospective study in adults with underlying respiratory disease, and using diagnostic techniques based on culture, PCR, and serology, HPIV3 was the most common respiratory virus after influenza detected in adults over the age of 45 [83]. HPIV infections are being increasingly recognized, like RSV, as an etiology of serious respiratory disease in elderly adults. Like RSV, HPIV3 causes outbreaks in geriatric facilities and can cause severe disease in older individuals [179–181].

5.6 Gender Differences in Infection

Boys have been long recognized to have a greater frequency of croup [182]. The Chapel Hill pediatric practice studies demonstrated infection rates for HPIV1 virus of 1.8 lower respiratory illnesses per 100 boys compared to 1.1 per 100 girls [36]. This sex difference disappeared after age 6 years. A similar predominance of serious illness in young males has been noted in other studies of lower respiratory disease [183]. Rates of infection in young boys and girls seem to be the same, but clinical manifestations of infection are more severe in boys [89]. Insufficient data are available to analyze HPIV2. HPIV3 caused identical rates of lower respiratory illness in males and females for children observed in Chapel Hill [89] and in the Fort Lewis day-care study [111]; however, in the Houston longitudinal study, boys were more likely to have lower respiratory tract involvement with primary infection [92].

5.7 Special Settings

Nosocomial infections with respiratory viruses occur readily if susceptible individuals are not isolated from those with HPIV shedding or respiratory disease. HPIV infections are detected commonly in studies of hospital-acquired infections in children on pediatric wards [163, 184–186] and neonatal units [119, 175, 176, 187].

Increasing reports of outbreaks and severe disease associated with HPIVs are being reported in immunocompromised hosts [45, 105, 106, 121, 155–157, 188–190].

Hematopoietic stem cell marrow transplant units and hematology/oncology wards are particularly susceptible to nosocomial infections due to cohorts of vulnerable patients who may shed viruses for prolonged periods of time, even asymptomatically [9, 42, 54, 191, 192]. Bronchoalveolar lavage fluid from these patients may contain high viral loads [193], and patients may be hospitalized for prolonged periods with increased exposure to viruses and other pathogens. Lung transplant recipients are also at high risk for serious morbidity and mortality from HPIVs [106], and outbreaks in lung transplant centers have been reported [194]. Symptom-based isolation, institution of good handwashing, and viral surveillance of patients with sensitive and rapid methods of detecting infection are necessary to institute control measures and recognize potential candidates for experimental antiviral therapy.

6 Mechanisms and Route of Transmission

Transmission of HPIV is by direct person-to-person contact or large-droplet spread. These viruses do not persist long in the environment, but HPIV1 has been recovered from air samples collected in the vicinity of infected patients [32]; from 1 to 10 % of HPIV3 virus particles in aerosol may be viable after 1 h [31]. Adult volunteers who have had prior natural infection have been reinfected experimentally by inoculation of the upper airway with a coarse aerosol or nasal drops or both [43, 132]. The high rate of infection early in life coupled with the high frequency of reinfection suggests that the virus spreads readily, that reinfected persons may be infectious, and that a relatively small inoculum may result in infection.

Outbreaks of HPIV1 and HPIV3 in symptomatic and asymptomatic healthy young adults stationed at the Amundsen-Scott South Pole Station in 1978 after 10 and 29 weeks of total isolation present an interesting addition to the story of HPIV shedding and viral transmission [195, 196]. Both HPIV1 and HPIV3 were recovered from frozen throat swabs of personnel overwintering at this station, and serological responses during midwinter outbreaks of HPIV infection when no new personnel had arrived for over 8 weeks suggest that persistent infection accompanied by prolonged shedding of HPIV occurs in healthy persons and may lead to outbreaks of respiratory illness.

There is little evidence of animal reservoirs for human disease and no evidence of vector spread. Sendai virus is a rodent strain of HPIV1 virus, but there is no evidence that it is related to disease in humans [22]. HPIV5 (formerly called SV5), a simian virus related to HPIV2 virus, has been reported to cause human infections but only on very rare occasions [20, 197]. Bovine HPIV3 virus is one of the agents commonly associated with an economically important disease of cattle usually called "shipping fever" [198], but there is no evidence of spread between cattle and man.

7 Pathogenesis and Immunity

7.1 Pathogenesis

The viral surface glycoproteins play a key role in the first steps of infection by HPIV. Infection of host cells is initiated by attachment of the virus to the host cell through interaction of the receptor-binding glycoprotein, hemagglutinin-neuraminidase (HN) with a sialic acid-containing receptor molecule on the host cell surface, as diagrammed in the schematic viral life cycle shown in Fig. 25.2. Upon interaction with receptor, the receptor-binding glycoprotein triggers or activates the viral fusion protein (F) to its fusion-ready state, promoting fusion of the virus into the cell and initiation of infection [25]. The fusion glycoprotein F is synthesized as a single polypeptide chain that forms a trimer before being cleaved by host cell proteases to yield a membrane-distal and membrane-anchored subunit. The new N-terminal region of the membrane-anchored subunit, termed the fusion peptide, contains the hydrophobic residues that insert into target membranes during fusion at neutral pH (reviewed in [199]). Infection also may result in fusion between cells, which involves the interaction of receptor-binding proteins and fusion proteins expressed on the surface of an infected cell with the membrane of an adjacent uninfected cell. The role of the HPIV HN molecule in promotion of fusion during viral entry has been elucidated in recent years [25, 27, 200–202]. Unlike influenza viruses, in which separate surface glycoproteins are responsible for the neuraminidase (NA) and receptor-binding (HA) activities, the HPIV HN is a dual-function molecule, carrying out both receptor binding during entry and receptor cleavage during budding and release of virus. The third function of HN, once receptor engaged, is to trigger or activate F to undergo its final conformational alteration that permits fusion [25]. One bifunctional active site on HN possesses both binding and neuraminidase activities, while a recently identified second site on HN possesses binding and F-protein-triggering activities [27, 203]. Finally, recent evidence suggests that HN interacts with F prior to engaging receptor and exerts a stabilizing effect on F that prevents F from being activated before it is in a suitable position with respect to the target cell [204].

After fusion of the viral envelope with the plasma membrane of the cell, the viral genetic material is released into the cytoplasm, as shown in Fig. 25.2. The nucleocapsid contains the genome RNA in tight association with the viral NP protein, and this RNA/protein complex is the template for both transcription and genome RNA replication. The

first step in the viral growth process, termed primary transcription to denote transcription directly from the infecting nucleocapsid template, initiates at the 3' end of the genome. Each of the mRNAs is transcribed and present in infected cells, at a level correlated to its relative location on the negative-sense viral genome. For five of the six genes of the HPIVs, transcription generally leads to generation of a single mRNA species that encodes a single protein molecule. However, the P gene of HPIVs encodes a number of proteins, and in several members of the family, more than one mRNA is synthesized and the mRNA also encodes a number of smaller protein products in an alternate reading frame from the P protein. These "C" proteins, as they are commonly called, are a nested set of carboxy-coterminal proteins, with each protein encoded in the same reading frame but differing by its start site on the mRNA. The functions of the C proteins are known to include key roles in evasion of the host immune response [205].

Unlike transcription, replication of the viral RNA genome depends on ongoing protein synthesis and therefore is linked to prior transcription of the viral genome. The second stage of genome RNA replication occurs when the newly replicated and encapsidated full-length plus-stranded RNA (antigenome)-containing nucleocapsids are copied into negative-stranded RNA-containing progeny nucleocapsids identical to the nucleocapsid that is packaged into progeny viral particles. These progeny viral nucleocapsids are templates for transcription.

After replication, as shown in Fig. 25.2, new viruses are formed by budding of newly replicated and assembled nucleocapsids containing the viral RNA genome along with the P and L proteins through areas of the plasma membrane that contain the viral surface glycoproteins and the M (matrix) protein. In polarized epithelial cells, the viruses bud from the apical surface of the cell. It is thought that the non-glycosylated M protein binds to the nucleocapsid, and also likely interacts with the cytoplasmic tails of the transmembrane glycoproteins, and thereby mediates the alignment of the nucleocapsid and the areas of the plasma membrane containing viral glycoproteins in preparation for budding [206–211]. It is likely that entry, fusion, and budding involve the participation of the cellular actin cytoskeleton as well as microtubule function [212–217]. Host cellular proteins that are involved in cell mobility may be important for these steps; one possible component of the entry/fusion mechanisms involves interaction between paramyxovirus F proteins and Rho A [215, 216].

Host cells have developed a variety of defense mechanisms in the face of viral infection. In some of these, an interferon (IFN)-induced antiviral state is created; some cell types undergo apoptosis, which limits viral replication. Many viruses have been found to possess strategies for evading these host immune responses, particularly the innate immune response that plays a key role in controlling virus infection [218]. For the paramyxoviruses including the HPIVs, evasion of this response is mediated by the C and V proteins. The V proteins are involved in a wide range of mechanisms for evading the immune response, including prevention of apoptosis [219, 220], cell cycle alterations [221], inhibition of double-stranded RNA signaling [219, 222], and prevention of IFN biosynthesis [219, 220, 222]. The strategies whereby different V proteins inhibit STAT proteins have been found to be highly diverse. For example, HPIV type 2 V protein targets STAT2 for degradation [223, 224], while mumps virus V protein can target both STAT1 [225] and STAT3 [226] and can also interact with cellular RACK1, potentially modifying IFN receptor activity [227].

7.2 Immunity

HPIVs replicate in the epithelium of the upper respiratory tract and spread to the lower respiratory tract within 3 days. The disease manifestations result from inflammatory obstruction of the airway. Epithelial cells of the small airways may become infected with resultant necrosis and inflammatory infiltrates. The interplay between virus-induced cell damage, beneficial immune responses, and inflammatory responses that contribute to disease for HPIV has not been well studied as it has for RSV; however, it is likely that in many cases, disease severity is increased and the pathology of clinical disease is actually caused by the inflammatory response rather than by the cytopathic effects of the virus. This concept is highlighted by the fact that virus titers in the infected host are generally waning by the time disease symptoms become apparent [228] and that virus titer does not correlate with the severity of lower respiratory disease. The pathologic changes that are present in children that have died with parainfluenza infection suggest exaggerated inflammation [152, 229] rather than simply tissue destruction by virus.

HPIV infections can induce both humoral and cellular immune responses in infected humans, including innate responses, local and systemic IgG and IgG responses, and T-cell responses. HPIV primary infection does not confer permanent immunity; however, although reinfection occurs, immunity is usually sufficient to restrict virus replication from the lower respiratory tract and prevent severe disease. Mucosal IgA levels correlate with protection from replication of HPIVs in humans [134, 135] and seem to provide the best correlate for protection, at least in adults. Cell-mediated immunity figures importantly in prevention of disease: for example, HPIV3 infection of T-cell-deficient infants can cause a fatal giant-cell pneumonia [134, 135], and in bone marrow transplant recipients, HPIV pneumonia has a 30 % mortality [106]. However, neutralizing antibodies against the HN and F proteins of HPIV are critical for long-term protection [230].

8 Patterns of Host Response

8.1 Clinical Manifestations

Primary infections with HPIVs are usually symptomatic, with clinical manifestations ranging from an afebrile upper respiratory illness to severe, life-threatening lower respiratory disease. The most characteristic and clinically important syndrome associated with infections with HPIV1 and HPIV2 is croup or laryngotracheobronchitis. HPIV1 was isolated from 20 % of patients with croup in studies in the Chapel Hill pediatric practice [36, 169], but HPIV2 virus is much less frequently associated with croup than is HPIV1 [34, 89, 169]. Studies in ambulatory populations suggest that most initial infections with HPIV1 virus result in febrile upper respiratory illness, whereas initial infections with HPIV2 virus result in less severe upper respiratory illness with many illnesses presenting without fever [41, 109]. Reinfection with HPIV results in similar respiratory symptoms from those caused by other viruses [92, 113]. The clinical manifestations of infections with HPIV viruses may differ in developing countries [147], with more cases of lower respiratory disease noted, although differences in surveillance methods could account for some of these features. The association of HPIV with croup, well described in developed countries, does not appear to be as common in developing countries [138, 146, 151, 231], where HPIV1 and HPIV2 infections are typically associated with pneumonia and tracheobronchitis [138, 146, 151, 231] (Table 25.2).

The clinical manifestations of infections with HPIV3 are varied. In the Chapel Hill studies, the clinical diagnosis varied with age [89]. Infants under 1 year of age were likely to present with bronchiolitis or pneumonia, whereas children from 6 to 18 months might often have croup. Older children were usually diagnosed as having tracheobronchitis. In general, no consistent clinical presentation of HPIV3 virus infection was found. The severe manifestations of HPIV3 infection, usually pneumonia, in developing countries are similar to those in developed countries. Figure 25.3 shows representative radiographic evidence of the pulmonary disease associated with HPIV3 in an immunocompromised child.

Prospective studies of children indicate that primary infection with HPIV3 is usually symptomatic but often mild [41, 92]. In children followed for the first 2 years of life, about one-third of primary infections involved the lower respiratory tract, but only 5 % of primary infections resulted in lower respiratory illnesses for which families sought medical. Frequency of reinfection—both symptomatic and asymptomatic—decreased with age. Reinfection was symptomatic among young children at the Washington, DC, children's home, Junior Village, only 20 % of the time [38], but this is likely higher than that in the family setting.

Evidence that HPIV infections may predispose to bacterial infections is accumulating [232–235]. Both otitis media and bacterial tracheitis in children have been associated with these infections [236], with otitis media documented in about 1/3 of children diagnosed with acute onset of HPIV infection [237], rates similar to that of otitis media following influenza or rhinovirus infection. An outbreak of invasive pneumococcal disease in a chimpanzee colony followed infections with HPIV3 [238]. Animals with HPIV3 upper respiratory illness were 5.7 times more likely to develop invasive pneumococcal infection despite the fact that most of the animals had received pneumococcal vaccine.

8.2 Clinical Syndromes

8.2.1 Pediatric Disease

Clinical diagnosis of the etiology of sporadic episodes of acute respiratory disease is difficult because many viral respiratory diseases have similar signs and symptoms. Infections with HPIV may be suspected when characteristic clinical manifestations occur in known epidemiologic patterns. The determination of HPIV outbreaks in the community is best ascertained using sensitive laboratory methods such as RT-PCR (see Sect. 4.2).

In developing countries, HPIV1 and HPIV2 infections appear to be less consistently associated with croup. In an HPIV1 epidemic in Trinidad, pneumonia was the most common lower respiratory tract diagnosis [138]. In such a setting, the clinical disease pattern will be less useful in identifying the occurrence of an HPIV1 epidemic. HPIV3 virus infections are much less predictable, but this etiology should be considered for infants less than 6 months of age with bronchiolitis or pneumonia occurring at times other than during RSV epidemics. Although medically attended lower respiratory illnesses associated with HPIV3 virus have had an even sex distribution [36], longitudinal studies have found the incidence of both croup and lower tract involvement more common in boys [92].

8.2.2 Disease in the Immunocompromised Host

The severe impact of HPIV infection in immunocompromised patients was first recognized in children with underlying immunodeficiencies, in whom giant-cell pneumonia has been documented at autopsy [156, 239]. Persistent respiratory tract disease and shedding have been observed in these children (Fig. 25.3) as well as HIV-infected patients [240]. In adult hematopoietic stem cell transplant patients, respiratory viruses cause about two-thirds of the respiratory illnesses, with high mortality [5]. HPIV3 accounts for up to

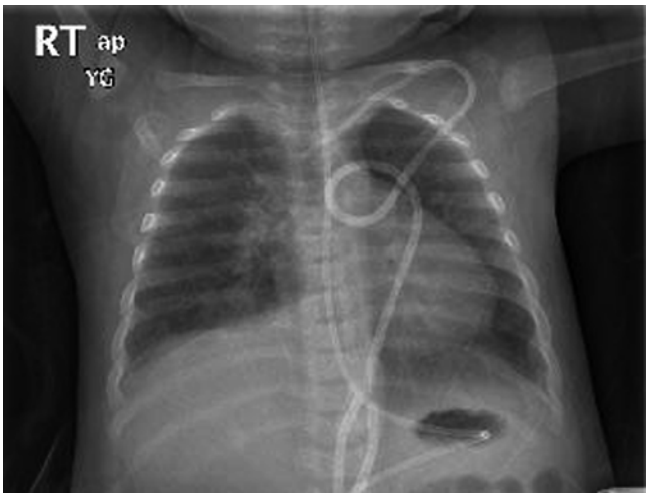

Fig. 25.3 Chest radiograph of an 8-month-old child with severe combined immunodeficiency infected with HPIV2 obtained 3 weeks following a hematopoietic stem cell transplant. The child was infected with HPIV2 pretransplant but unable to clear the virus due to the immunodeficiency and did well clinically until 3 weeks posttransplant, when he had respiratory distress requiring oxygen support. Chest radiograph is remarkable for right perihilar opacities associated with mild perihilar peribronchial cuffing, suggestive of viral lower tract infection. The child was treated with the experimental drug DAS181 (Ansun Biopharma, San Diego, CA), under an emergency IND, cleared the HPIV, and was discharged home

90 % of these cases [121], causing pneumonia (Fig. 25.4) with a 35 % acute mortality rate and a 75 % mortality rate at 6 months posttransplant [121]. High rates of pneumonia have been described in patients undergoing stem cell transplantation [106]; solid organ transplantation with kidney, heart, or lung [194, 241, 242]; and patients with hematologic malignancies undergoing chemotherapy, particularly induction chemotherapy [243]. The largest reports of HPIV infection have been reported in hematologic stem cell transplant recipients [121], where HPIV infection was diagnosed in 253/3,577 transplant recipients (7.1 %), with 78 % of the infections community acquired. In this study based on patients hospitalized in the 1990s, diagnosis was confirmed mainly using direct immunofluorescence testing and culture; rates of HPIV infection using more sensitive methods at the same institution in the early 2000s were noted to be higher—up to 13 % during the first year posttransplant [42]. Most infections in the transplant patients begin with typical symptoms of an upper respiratory tract infection with a low-grade fever, although asymptomatic infections or intermittent asymptomatic shedding have been noted [42]. Sinusitis may be present in up to 40 % of patients [243]. Progression from upper to respiratory tract disease is variable but common, ranging in various reports from 18 to 77 %. Risk of progression in stem cell transplant recipients is associated with steroid use and lymphopenia and may be less with nonmyeloablative conditioning [9, 121, 243]. Radiographic findings can vary from mild focal infiltrates to diffuse interstitial and alveolar interstitial infiltrates (Figs. 25.3 and 25.4) [106]. Risk of death following the development of lower tract disease can range up to 45 % [244]. Dissemination of disease to the pancreas, brain, pericardium, and myocardium has been reported [156, 245], but the most common serious adverse sequelae associated with HPIV infection in stem cell transplant patients and lung transplant recipients are significant declines in pulmonary function posttransplant [194, 246], even in stem cell transplant recipients who had only upper respiratory tract HPIV infection.

9 Control and Prevention Based on Epidemiologic Data

9.1 Vaccines

The development of a vaccine for the HPIVs, as for RSV, has been difficult because of the need to induce an immune response in young infants, who have both an immature immune system and maternal antibodies that may interfere with the development of an adequate immune response. An inactivated HPIV1, HPIV2, and HPIV3 vaccine used in infants in the late 1960s was immunogenic but did not offer protection from infection [247, 248]. Since that time, it has been challenging to identify vaccine viruses that are adequately attenuated for the vulnerable seronegative infant, yet also immunogenic. The development of reverse genetic systems has allowed for specific engineering of attenuated yet immunogenic vaccine viruses. Experimental vaccines are now under evaluation, with a reasonable expectation that a vaccine for HPIV3, and perhaps also HPIV1, will be feasible [230].

Two different strategies of interest are currently under active development by NIAID and industrial partners for HPIV3 vaccines: candidates based on live-attenuated bovine parainfluenza type 3 (BPIV3) strains and those based on a cold-adapted live-attenuated strain. Studies of both bovine-derived vaccines and live-attenuated human HPIV3 vaccines derived from well-characterized passages of human-derived vaccine strains have shown good evidence of safety, infectivity, and immunogenicity in children and infants [249–257]. In addition, new live chimeric candidate vaccines based on a chimeric construct expressing the HPIV3 F (fusion) and HN (hemagglutinin-neuraminidase) proteins and the RSV F proteins from the bovine HPIV3 viral genome have been utilized in human trials in seropositive and seronegative children and young infants [258, 259], suggesting that a combined HPIV3/RSV vaccine could be feasible [252, 260]. Recombinant HPIV3 vaccines have now passed phase

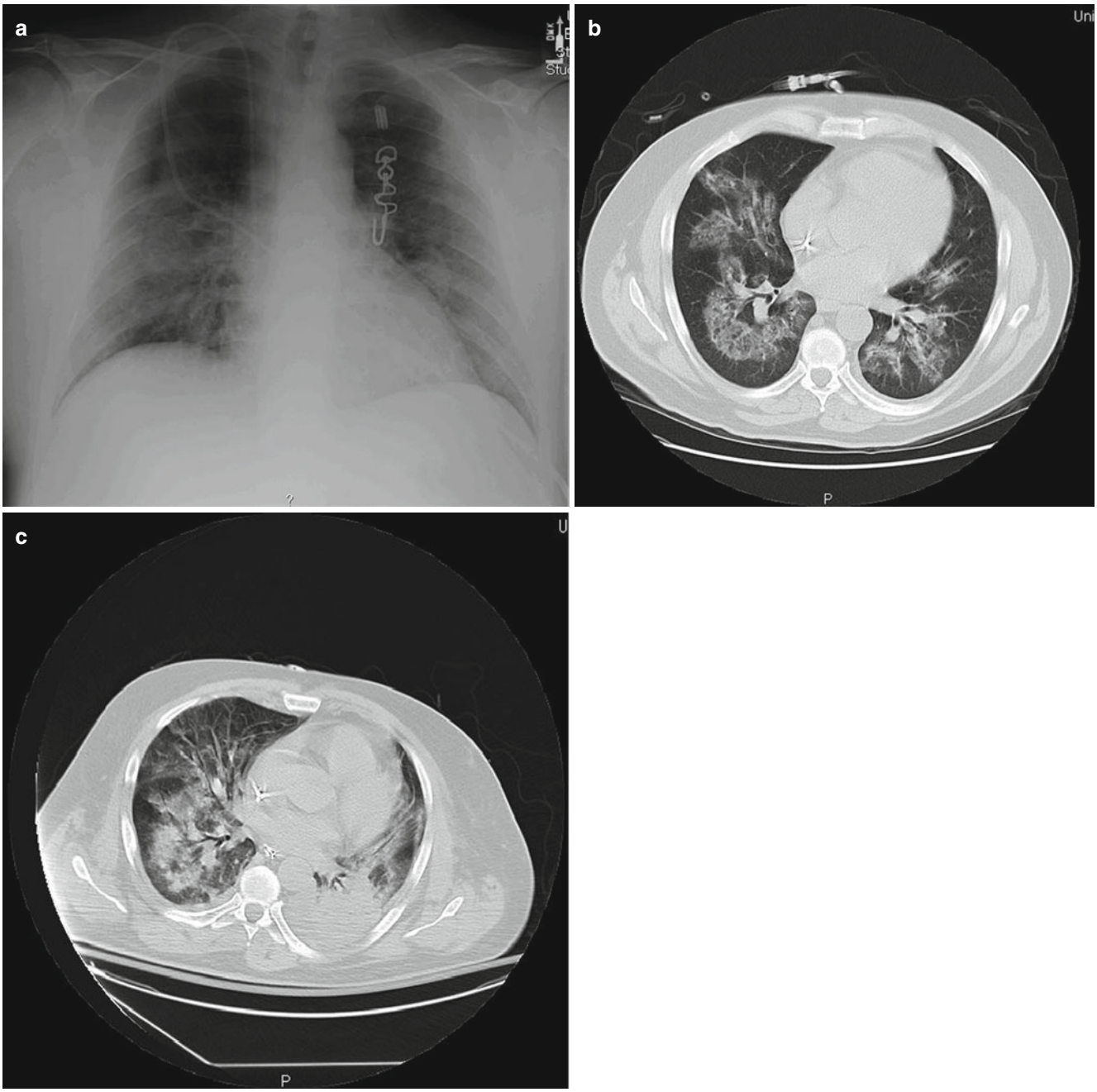

Fig. 25.4 Chest radiographic (**a**) and computed tomography findings (**b**, **c**) of a neutropenic adult male hematopoietic stem cell transplant recipient with HPIV3 infection diagnosed on day 11 posttransplant. No other pathogens other than HPIV3 were identified despite two bronchoalveolar lavage procedures and PCR testing for multiple bacterial, fungal, and viral pathogens. His history was significant for sinusitis pretransplant, with fever and upper respiratory tract symptoms documented beginning on day 7 posttransplant. He required intubation on day 18 posttransplant and, despite treatment with ribavirin, IVIG, and steroids, died soon afterward. (**a**) Chest radiograph, day 11 posttransplant. (**b**) Computed tomography of chest, day 11 post stem cell transplant. (**c**) Pulmonary consolidations and pleural effusions shortly before death, day 18 posttransplant

I evaluation and are undergoing testing in proof-of-concept trials [230].

The approach farthest along for HPIV1 vaccines is an engineered live-attenuated virus, with a series of attenuating mutations in several genes. In addition, a murine HPIV1 (Sendai virus) is being evaluated in a similar strategy to the bovine virus strategy for HPIV3. Approaches to HPIV2 also include specifically attenuated engineered viruses. Attenuated HPIV1 and HPIV2 vaccines are both at the pediatric phase I stage of clinical trials [230].

9.2 Antivirals

Therapy for the treatment of HPIV infections has relied on nonspecific symptomatic or anti-inflammatory treatments. For example, symptomatic benefit for the treatment of moderate to severe HPIV1-associated croup has been demonstrated with short courses of glucocorticoids, such as dexamethasone, shown to provide clinical and economical benefits, although ineffective for HPIV2- or HPIV3-associated respiratory disease [261, 262]. There is no specific antiviral therapy available for the treatment of HPIV infections. While ribavirin has been discussed as a potential HPIV antiviral based on in vitro data, it has no proven benefit, although there are anecdotal reports of benefit and lack of benefit in nonrandomized case series [121, 263–265]. Ribavirin therapy is not recommended [266] as published reports do not suggest a link between ribavirin use in cases of HPIV infection and improved outcome.

The potential benefit of antiviral treatment, especially for very young infants and immunocompromised hosts, is increasingly recognized [26, 244]. Several features of the viral life cycle make HPIV vulnerable to attack. HPIVs enter their target cell by binding to a receptor molecule and then fusing their viral envelope with the cell membrane to enter the cytoplasm. Binding, fusion, and each step in cell entry are critical stages which could be targeted to prevent HPIV infection and disease [26, 267, 268]. One potential strategy for HPIV and other sialic-binding viruses targets the removal of lung epithelial sialic acid receptor for HPIV, thereby preventing viral entry. A new recombinant sialidase, first developed as an antiviral agent for influenza, functions by cleaving sialic acids from the host cell surface, thereby inactivating the host cell receptor recognized by HPIV [269]. Reports of successful use of this agent in several adult and pediatric transplant recipients under compassionate use have been reported [270, 271, 272]. The molecule is an engineered neuraminidase molecule (DAS 181; Fludase) [268] and has not entered clinical trials at this time for HPIV but shows promise in proof-of-concept treatment of immunocompromised humans [273], and trials in specific populations are underway.

The HN molecule, in addition to binding to receptors, contains neuraminidase (receptor-cleaving) activity and cleaves the sialic acid moieties of cellular receptors, allowing new virions to be released from the host cell surface and infection to spread. While neuraminidase inhibition is unlikely to be as effective for parainfluenza viruses as it has been for influenza virus as an antiviral strategy [26, 274, 275], specific inhibition of this activity could prevent virion entry into additional uninfected cells [276].

The fusion protein undergoes a series of structural transitions as it mediates viral entry, and each of these steps is a potential target for antiviral development. F must form a transient intermediate to draw the viral and cell membranes together, and a conserved coiled-coil motif required for this step can be specifically targeted by fusion-inhibitory peptides that bind to specific sequences on the F protein. Such peptides inhibit viral fusion and entry in a dominant-negative manner by binding to F's transient intermediate, preventing F from advancing to the next step in fusion. Recently, it has been shown that membrane targeting of fusion-inhibitory peptides by use of a lipid tag allows efficient inhibition of the transient intermediate of fusion and also increases serum half-life and tissue biodistribution of the peptides, making them promising candidates for development as HPIV antivirals [277–279].

Finally, it is possible to subvert the normal process whereby HN activates F and cause HN to activate the F protein's fusion mechanism before it reaches the target host cell, thus incapacitating F before it can mediate viral entry; compounds are under development that will act by this mechanism [280]. As with other rapidly evolving RNA viruses (influenza, HIV, etc.), it may be important to use several antiviral agents simultaneously to attack different viral targets, to avoid the inevitable development of resistance to single antivirals. It will therefore be critical to develop several antiviral strategies in parallel.

10 Unresolved Problems

The clinical spectrum of illness at a young age, the failure of serum antibody to adequately protect against disease, and the ubiquity of infections make the HPIVs serious and challenging foes. Better understanding of the pathogenesis of disease and of the protective immune response will facilitate the development of effective antivirals and vaccines. The determinants of protective immunity need to be defined for natural infection, so that the natural response can be duplicated by active immunization. The use of attenuated live virus vaccines and particularly live virus vaccines built on the backbone of HPIV requires further investigation as a potentially beneficial and economical approach to immunization of young children. In addition, immunization of women of childbearing age should be considered as a means of protecting infants by passively acquired antibody during the first months of life.

As the number of immunocompromised patients increases due to advances in transplantation and antiviral HIV therapy and new methods of monoclonal antibody immunosuppression and treatment for a wide variety of diseases, the impact of respiratory viruses in general and HPIV in particular is becoming more well known. The need for safe and effective antivirals for the prophylaxis and treatment of HPIV infections in immunocompromised patients should be a high priority, and the need to avoid resistance by potentially using

several simultaneous antiviral strategies is important to any antiviral approach designed to combat RNA viruses.

A major unsolved problem is the substantial excess mortality from acute respiratory disease in developing countries. Acute respiratory disease remains one of the leading causes of death in children under 5 years of age in the developing world; available information suggests that HPIVs contribute significantly to this problem. As conjugate vaccines are becoming more widespread in developing countries to protect against pneumococcal and *Haemophilus influenzae* type B disease and measles vaccine uptake increases, the rates and causes of morbidity and mortality due to respiratory diseases in these young children require further investigation. Studies of the factors contributing to the excess mortality are urgently needed, and methods need to be explored that are adaptable to delivery in developing nations for preventing serious respiratory disease.

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Suggested Reading

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Paramyxoviruses: Respiratory Syncytial Virus and Human Metapneumovirus

26

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1 Human Respiratory Syncytial Virus

1.1 Introduction

Human respiratory syncytial virus (RSV) and human metapneumovirus (MPV) are members of the family *Paramyxoviridae* of the *Mononegavirales* order, comprising the nonsegmented negative-strand RNA viruses. *Paramyxoviridae* has two subfamilies: *Paramyxovirinae*, which includes the parainfluenza viruses 1–4 and measles and mumps viruses, and *Pneumovirinae*, which includes RSV and MPV. *Pneumovirinae* has two genera: *Pneumovirus*, which includes human RSV, bovine respiratory syncytial virus, and pneumonia virus of mice, and *Metapneumovirus*, which includes human MPV and avian metapneumovirus, sometimes called avian pneumovirus.

1.2 Historical Background

RSV was isolated first in 1956 from an ill chimpanzee in a laboratory setting that had an illness similar to the common cold. A virus causing a similar cytopathic effect in cultured cells was recovered from infants with respiratory illness shortly after, and studies of human antibodies in the serum of infants and children indicated that infection was common early in life [1, 2]. Now it is known that RSV is the most common viral agent of serious pediatric respiratory tract disease worldwide. In most areas of the world, RSV is the most common cause of pneumonia and bronchiolitis in infants less than 1 year of age. RSV causes severe disease in young infants, but disease is not restricted to the early life period. The virus can cause severe lower respiratory tract illness in large numbers of elderly subjects and also in subjects who are severely immunocompromised such as hematopoietic stem cell transplant recipients [3–7].

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1.3 Epidemiologic Analysis

1.3.1 Mortality Data

Mortality in infants and children caused by RSV is uncommon in developed countries with modern critical care units. Estimates of the mortality rate are about 0.3 % of hospitalized children or less. Mortality has been dropping over the last several decades, and by the late 1990s the estimated number of deaths in the USA was several hundred children a year or less [8, 9]. Large epidemiologic studies report that the US mortality in children may be only about 100 cases a year. Interestingly, while many providers think of RSV infection as principally a pediatric illness, there are over 17,000 deaths per year in the elderly, making them the highest risk population for death [9]. In less developed countries, however, infant deaths due to RSV infection may be more common.

Infants with underlying illness are at highest risk among young children for morbidity and mortality from RSV infection. Infants with chronic lung disease requiring supplemental oxygen following treatment for prematurity, due to bronchopulmonary dysplasia, are perhaps at the highest risk for prolonged, severe, or fatal illness due to RSV [10]. Infants with severe congenital pulmonary or cardiac disease have been reported to be at risk of death in 3–4 % of cases when hospitalized, although this rate is likely decreasing in the USA [11]. Both children and adults with primary immunodeficiency or medically induced immunosuppression are at

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high risk of mortality due to RSV infection. The most severely immunocompromised, and thus those at highest risk of mortality, are hematopoietic stem cell transplant patients of any age [12]. In some settings, the mortality rate of RSV infection in hematopoietic stem cell transplant patients with severe immunosuppression verges on 100 % [12].

1.3.2 Morbidity Data

Hospitalization rates of infants for RSV disease vary with the setting, probably due to variations in exposure, genetics, socioeconomic, and other risk factors and due to the local practice style of medical providers. Many developed countries report hospitalization rates of about 1 in 100–200 infected infants during the first year of life [13, 14]. Studies of RSV disease in developed countries suggest that of those infants hospitalized, about 9 % require mechanical ventilation [15, 16]. There are certain populations at extraordinarily high risk of hospitalization with RSV, for example, Alaskan native infants younger than 1 year have been reported to have a hospitalization rate of 53–249 per 1,000 infants [16]. Low socioeconomic status is a risk factor for higher rate of hospitalization in most areas.

RSV also is one of the most common viral causes of serious lower respiratory tract illness in the elderly, especially in institutionalized subjects [17]. Exacerbations of chronic obstructive pulmonary disease (COPD) are frequently associated with RSV infection [18, 19]. The elderly do not exhibit a remarkably diminished level of antibodies to RSV [20]. Decreased levels of T cell memory in the elderly and specifically in patients with (COPD) may contribute to the increased susceptibility to RSV infection in these populations [21]. Many think of influenza virus as the principal viral respiratory pathogen in this population, but in a hospitalized cohort, influenza A virus and RSV infection resulted in similar mortality, lengths of stay, and rates of use of intensive care [17]. RSV infection accounted for over 10 % of hospitalizations for pneumonia.

1.3.3 Serological Surveys

Seroepidemiology studies suggest that virtually all children are infected in the first 2 years of life, and early infection is especially common in infants attending group day care. Serological methods are helpful in epidemiology and vaccine studies, but serologies are not often used for diagnosis in clinical settings. Because of the transfer of maternal RSV-specific antibodies across the placenta, and the high prevalence of early infection, it is unusual to find infants who are RSV seronegative. In older children and adults, a fourfold rise in serum antibodies is often used as evidence of RSV infection, but asymptomatic infections in which viral shedding is low in titer often are not accompanied by serum antibody rises. Serological tests in infants are even less sensitive, because young infants may not exhibit a large or durable rise in antibodies. Neutralizing antibody tests are considered the best functional marker of infection, but sensitivity is much higher in antigen binding assays using individually purified RSV F or G proteins [22].

1.3.4 Laboratory Methods

Isolation of the virus in cell culture provides a definitive test for diagnosis of active infection. Various methods of obtaining respiratory virus secretions for testing have been compared. Most studies suggested that aspiration or gentle flushing of nasal secretions using a solution like saline is best, though some types of nasal swab have given reasonable results. The virus is more thermolabile than most, and thus samples should be transported on wet ice to the diagnostic laboratory and processed rapidly. Prolonged times of transport to remote reference laboratories reduce the effectiveness of isolation. After inoculation onto susceptible cell culture substrates, highly trained staff can recognize cytopathology in the cell monolayers by visual inspection and conventional bright-field microscopy after about 3–7 days of incubation. Detection may be more efficient when using shell vial cultures and immunofluorescence [23]. Various cell lines have been used for RSV detection, such as HEp-2 epithelial cells, MRC-5 fibroblasts, and rhesus monkey kidney cells, but the R-Mix commercial mix of human and mink lung cells may perform better for detection of RSV [24].

Culture is expensive and requires highly trained staff and therefore is not usually available at the point of care, which is often an outpatient clinic or emergency department. Therefore, rapid diagnostic methods were developed for the detection of viral proteins or RNA in respiratory secretions. RSV antigen tests mostly rely on direct immunofluorescent assays (DFA) on exfoliated cells in secretions or enzyme immunoassay (EIA). Nucleic acid detection assays based on RT-PCR are now widely available for RSV, often in a multiplexed panel for detection of multiple respiratory virus pathogens. These tests are typically more sensitive than any of the virus isolation or protein-based detection assays discussed above. Enhanced sensitivity is especially helpful when testing adults, who often shed virus in very low titers. Positive RT-PCR tests need to be interpreted in the context of the clinical scenario, since the tests can remain positive for prolonged periods of time after infection, well beyond the period during which infectious virus can be isolated. Since children may experience symptomatic respiratory infections every few weeks during the winter, caution must be used in interpretation of positive PCR tests, especially when multiple viruses are detected simultaneously in a sample. Some instances of multiple PCR test positivity likely represent residual RNA from a previous virus infection and a second RNA type representing live virus from the active current infection.

RSV typically is propagated in monolayer cell cultures of continuous cell lines of human epithelial cell origin, such as HEp-2 cells. Monkey kidney cells of various types are also

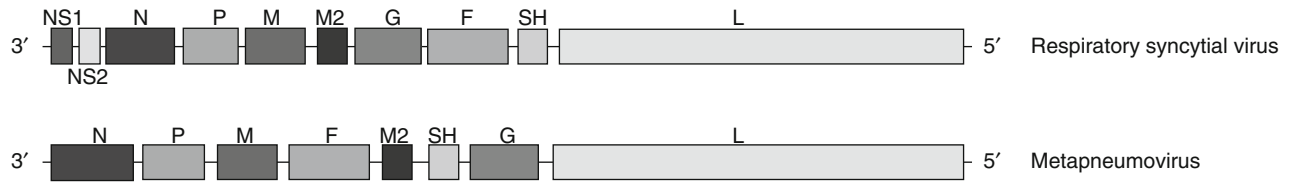

Fig. 26.1 Schematic of the genomic organization of respiratory syncytial virus and human metapneumovirus. Gene segments drawn roughly to scale

used commonly for propagation in the laboratory. In fact, the virus replicates to some extent in most cell lines of mammalian origin. In non-polarized epithelial cells, the virus often causes a typical cytopathic effect in which multinucleated giant cells form due to cell-cell fusion, termed cell syncytia. This in vitro effect is the origin of the virus name, although it is not clear that RSV causes syncytia in vivo. In polarized epithelial cells in culture, the virus assembles and buds from the apical surface of cells, mimicking to some extent the budding of virus into the lumen of the airway.

1.4 Biological Characteristics

The virus has a negative-sense single-stranded RNA genome with 10 genes encoding 11 proteins. Figure 26.1 compares the genomes of RSV and MPV, which are similar in many respects.

The replication proteins are common to both of the viruses, as are the matrix (M) protein and the surface fusion (F) and glycosylated attachment (G) glycoproteins. The gene order differs slightly, and RSV possesses two nonstructural (NS) genes NS1 and NS2 that are not present in MPV. The functions of these genes are not fully defined, but involve interactions with the host response machinery, especially interferons. The presence of these host response-modifying genes may explain in part why RSV appears to cause severe disease more commonly than MPV. Many of the gene sequences exhibit some clear global sequence relatedness; however, the extent of the relatedness of many of the sequences of corresponding proteins is relatively low. Based on sequence homology, it is not expected that there is a significant amount of immunologic cross-reactivity in responses to the two viruses.

The RSV virion buds from airway epithelial cells, incorporating host cell membrane as the lipid bilayer that forms the envelope of the particle. Since the virus is enveloped, chemicals that disrupt lipid bilayers (detergents) inactivate the virus, leading to the strong recommendations for healthcare provider hand washing following patient contact. The genome is a single strand of RNA, which forms a helical complex with the nucleoprotein (N). The final nucleocapsid structure likely is formed by the complete set of replication proteins, which also include the phosphoprotein (P) and the large RNA-dependent RNA polymerase (L). It is suspected that the M protein helps the particle to form by bridging the nucleocapsid and the lipid envelope with its incorporated surface proteins. The surface of the particle incorporates three integral membrane surface glycoproteins, the highly glycosylated RSV G protein suspected to be the attachment protein, the fusion protein F, and the small hydrophobic protein (SH). RSV F (a Type I integral membrane protein) and RSV G (a Type II integral membrane protein) form oligomers on the surface of the particles, which appear like small spikes with globular heads when seen in electron microscopic (EM) images by negative stain. The morphology of particles in EM images or in fluorescent microscopy images labeled by conjugated antibodies suggests that the virion particles are filamentous. However, spherical particles, filaments, and more pleomorphic forms have been observed; therefore, it is uncertain what the morphology of infectious particles in vivo during natural infection is.

The F protein is critical for entry into cells, by breaching the barrier of the cell lipid bilayer. It is thought that binding of virion particles to susceptible cells at physiologic pH triggers a conformational change of the F protein from a metastable pre-fusion state [25] to an altered post-fusion conformation [26, 27] in which the hydrophobic fusion peptide in the protein is exposed and extended to insert into the host cell membrane. This membrane insertion event accomplishes a fusion of the viral and host membranes, allowing delivery of the nucleocapsid to the cytoplasm where viral replication occurs. This event is termed "fusion from without" when the particle enters a cell. An alternative fusion event ("fusion from within") occurs when newly expressed F protein on the surface of infected cells causes fusion of an infected cell to an adjacent cell in culture causing "syncytia." It is not certain whether this latter form of fusion (cell-cell fusion) occurs during natural infection and contributes to pathogenesis or if the formation of syncytia is a tissue culture phenomenon only.

1.5 Descriptive Epidemiology

Although there are many animal forms of RSV, there is no known animal reservoir of human RSV; close contact with infected humans is the only source of RSV infection.

1.5.1 Incidence and Prevalence Data

Early prospective studies showed that approximately half of infants in the USA are infected during their first year of life; most were infected after two winter epidemics [28]. About a quarter of infants exhibit signs and symptoms of lower respiratory tract disease (wheezing and/or pneumonia) during a primary RSV infection [14, 28–31]. RSV is the most common virus associated with hospitalization for respiratory illness and in fact is one of the most common of all causes of infant hospitalization. For example, RSV caused 13 hospitalizations per 1,000 US children younger than 1 year in one large study [32]. During winter RSV epidemics, over 75 % of the children hospitalized for acute lower respiratory tract infection are infected with RSV [33, 34]. The rate of very severe disease in hospitalized infants is high, with about 2–5 % of hospitalized infants requiring mechanical ventilation for respiratory failure [35].

1.5.2 Risk of Infection and Reinfection

Although primary infection of infants is probably most efficient, RSV can infect subjects of any age [28, 36, 37]. Some adult infections are asymptomatic, and most are limited symptoms related to infection of the upper respiratory tract, such as the common cold [28, 36, 38]. Since otherwise healthy adults all possess measurable RSV serum antibodies and RSV-specific T cells, it is clear that prior exposure and induction of immune responses may not prevent infection. However, disease severity is usually reduced after one or several infections early in life, thus immune effectors such as serum neutralizing antibodies do prevent severe disease during reinfections. Unlike influenza virus, RSV does not exhibit a progressive antigenic drift. Although RSV antigenic diversity is observed in field strains, diversity of the antigenic proteins is not required for RSV to cause reinfection [39–41]. Most experts believe that serum neutralizing antibodies protect against severe lower respiratory tract disease but do not result in sterilizing immunity against infection at the respiratory mucosa. Thus, healthy adults show signs and symptoms of the common cold in about half of cases of natural or experimental infections, even though they have experienced numerous previous RSV infections [39].

There is a single serotype of RSV, but two antigenic subgroups of RSV, with about 25 % antigenic relatedness, have been defined using immune sera; the subgroups are designated A and B. Antigenic dimorphism for RSV had been noted in an early study [42] and subsequently was delineated using MAbs, which identified extensive differences in the G protein and less extensive differences in F and other proteins [43]. The two subgroups exhibit a three- to fourfold reciprocal difference in neutralization by polyclonal convalescent serum. Analysis of glycoprotein-specific responses in experimental rodent models or human infants by enzyme-linked immunosorbent assay (ELISA) with purified F and G glycoproteins showed that the F proteins are 50 % related antigenically and the G proteins are 1–7 % related [44]. Consistent with this level of antigenic relatedness, F protein expressed by a vector immunization was equally protective in small animals against challenge with the homologous or heterologous subgroup virus, whereas the G protein was 13-fold less effective against the heterologous subgroup virus [45]. Thus, the F protein is responsible for most of the observed cross-subgroup neutralization and protection. In some communities a pattern of infection with viruses of alternating subgroups has been described, but this is not a universal phenomenon. RSV subgroup B virus is more difficult to isolate and propagate in culture, so subgroup B viruses are less commonly associated with severe disease in some studies. However, clearly viruses of both subgroups can cause severe disease leading to hospitalization [46–50].

1.5.3 Risk of Serious Lower Respiratory Infection (LRI) During Infancy

Infants exhibit the highest risk of severe lower respiratory tract disease. This elevated risk is explained by a myriad of physiologic, immunologic, and other factors. First, the highest point of resistance in the airways is the bronchioles, and the resistance of airways is inversely proportional to the airway radius to the fourth power (resistance ~ $1/\text{radius}^4$, from Poiseuille's law). The bronchioles of infants are narrow, and inflammation and secretion in the bronchioles leads to turbulent airflow, and further reduction of the airway lumen size. These physical factors lead to the clinical signs of wheezing (a sign of outflow obstruction) and air retention. Increased respiratory rate can compensate to some extent for respiratory compromise, but prolonged tachypnea can lead to fatigue and eventual respiratory failure. Also, during primary infection infants do not possess active immunity to infection, which allow this efficient virus to replicate in the airway to titers as high as 10^7 infectious particles per mL of secretion. Mothers pass RSV antibodies to infants across the placenta during the third trimester, but premature infants do not obtain maternal antibodies prior to 32 weeks' gestation. Also, the airways of premature infants are smaller than those of term infants. Another factor leading to respiratory difficulty is dehydration. Obstruction of the nasal passages with thick secretions can impede the feeding of infants, who are obligate nose breathers.

1.5.4 Role of RSV Infections in Adults

RSV also is an important cause of serious infection in elderly adults; in fact RSV appears to cause substantially more fatalities in elderly adults than in children [17, 51]. As above, a large study of the US population showed that RSV was associated with approximately 17,000 all-cause deaths per year among persons of all ages in the USA [9], with most of those deaths in the elderly.

1.5.5 Role of RSV in Infections with Underlying Cardiopulmonary and Other Medical Diseases

Virtually any serious medical or genetic condition is associated with some increased risk of severe diseases [32]. Certain particular categories of subjects are at highest risk for severe RSV disease, including infants with congenital heart or chronic lung disease, immunodeficient subjects of any age and the elderly [9, 10, 17, 33, 52–57]. These latter subjects are thought to have reduced competency of RSV-specific T cells. Prematurity increases risk to a small extent, but more importantly chronic lung diseases are important factors [10, 33, 56, 58–62]. Infants who are born prematurely and then suffer persistent chronic lung disease, especially those needing oxygen supplementation, are at very high risk of hospitalization with RSV. Children with cystic fibrosis are at high risk of severe disease [63, 64]. In children younger than 2 years with cystic fibrosis, the consequence of RSV infection may be prolonged respiratory morbidity [65]. Children with congenital heart disease also are at increased risk [33, 56, 58, 62, 66, 67].

1.5.6 Role of RSV Infections in Patients with Immunodeficiency

RSV is a common cause of severe respiratory disease in immunocompromised patients, including lower respiratory disease [68]. Mortality rates in some populations of immunocompromised patients verges on 50 % [7, 69]. Infants with congenital severe combined immunodeficiency are at special risk [57, 70], but any acquired immunosuppressive condition such as cancer or transplantation puts patients at risk, especially when T cell function is compromised [68, 71, 72]. RSV infection can cause severe lung disease in recipients of lung transplants, sometimes with a long-term outcome of obliterative bronchiolitis [73–75]. Children with HIV infection shed RSV for extended periods, but disease is not especially severe in HIV-infected children prior to onset of AIDS [76–78]. Interestingly, although most immunocompromised subjects appear to be at risk because of T cell problems, infants with phagocytic cell defects including those with interferon-γ receptor deficiency or chronic granulomatous disease also are at risk of severe RSV disease.

1.5.7 Role of RSV in Nosocomial Infection

Transmission of RSV in the hospital setting can lead to serious disease, especially in critical care units with neonates or other high-risk infants [79–86]. Nosocomial outbreaks in inpatient transplantation facilities are sometimes severe and unit outbreaks can be difficult to terminate because transplant patients can shed RSV for many months [57, 58, 69, 84, 87, 88]. Theoretically, transmission in inpatient healthcare settings should be preventable through strict compliance with infection control practices, especially hand washing and contact precautions, which are universally recommended for RSV patients. A high level of compliance with precautions is difficult to achieve in busy care settings but is needed to prevent transmission by healthcare providers. The use of the prophylactic monoclonal antibody palivizumab has been studied to interrupt an outbreak in a neonatal intensive care unit setting [89], but currently palivizumab use is not recommended for this purpose.

1.5.8 Role of RSV in Otitis Media

Bacterial otitis media is a common complication of RSV upper respiratory tract infection. In fact, RSV infection is probably the most common precipitating factor associated with otitis media. RSV antigens and nucleic acids have been reported in middle ear fluids [90, 91]. The disease is predominantly due, however, to Eustachian tube dysfunction, resulting in bacterial stasis in the middle ear and subsequent otitis media.

1.5.9 Epidemic Behavior

RSV regularly occurs in annual epidemics. The US National Respiratory and Enteric Virus Surveillance System (NREVSS) considers that the RSV season starts when the first of two consecutive weeks during which the mean percentage of specimens testing positive for RSV antigen is ≥10 %. The RSV season is considered to have ended in a community when the mean percentage of positive specimens is ≤10 % in reference laboratories for two consecutive weeks.

1.5.10 Geographic Distribution

RSV infection occurs in infants and adults worldwide, in yearly epidemics. The virus has been isolated in every area of the world in which surveillance studies have been conducted. The principal season varies depending on the climate and region, but infection is ubiquitous. Virtually all children in the world are infected within the first few years of life.

1.5.11 Temporal Distribution

Epidemics occur in the winter and early spring in the USA. Onset of the annual epidemic varies by region of the country and by year but typically begins in the USA in October or November and lasts through late spring. Within a region, the timing of RSV season changes slightly each year. Florida often has the earliest onset of RSV epidemic and the longest lasting season in the USA. Near the equator, infections may be more common during rainy season.

1.5.12 Occurrence in Different Settings

During community outbreaks of RSV, the venues with the highest level of young infants and children exhibit the highest rates of transmission of virus, especially large families and day-care settings with large numbers of children per

room. Hospital infection control practices must be used to prevent RSV spread, using measures including careful hand hygiene, contact precautions for patient isolation, gowns and gloves [92, 93], and, when direct coughing occurs, facemasks and goggles [94, 95]. RSV rapid diagnostic testing can be used in hospital infection control practice to identify RSV-infected patients during the admission process [96, 97]. RSV is shed for prolonged periods. It has been reported that 92–100 % of hospitalized children are still shedding infectious virus after 7 days [98, 99].

1.5.13 Environmental Risk Factors

Exposure to tobacco smoke and poor nutrition increase the incidence of disease [100–102]. Low socioeconomic status increases the risk of severe disease for uncertain reasons. Lower income populations exhibit a five- to tenfold increased risk of hospitalization in many studies [14, 32, 103].

1.5.14 Other Factors

Breast-feeding may confer some protective benefit against RSV disease, but the extent of the benefit of breast-feeding has been controversial. The results of epidemiologic studies on benefit have been conflicting, although recent studies suggest that breast-feeding may have a strong protective effect only in girls [104]. The sex of the infant modulates the severity of RSV infection for additional reasons that are not fully understood. Even though the same high proportion of both male and female infants become infected, males have a higher incidence of RSV lower respiratory tract disease than girls [30, 32, 105–108]. Ethnic and genetic factors appear to play a role. Alaska and other Native American children are at increased risk of severe respiratory disease during RSV infection [109].

1.6 Mechanisms and Routes of Transmission

Direct contact with secretions from an infected human, usually by fomites (contaminated objects, including hands), allows transmission of virus. In some cases, infected subjects may inoculate contacts at short distance by coughing via large-particle droplets. However, the virus is not spread efficiently by small-particle aerosols [110, 111]. The nasal and conjunctival mucus membranes are probably the most common portals of entry [112]. RSV is one of the most infectious viruses spread by contact, and transmission is very efficient even among subjects who possess partial immunity due to prior infection. Spread among family members and day-care contacts is especially common. The infectious doses for humans are probably only a few infectious particles per mL of respiratory secretions, but infants routinely shed at least a millionfold higher concentration of virus during the peak of illness. The incubation period is not known definitely but is about 3–6 days and likely varies according to the intensity of exposure and the amount of virus in the inoculum. The period of viral shedding is many days; infants can shed infectious virus for weeks [98, 99]. RSV can survive on hard surfaces for greater than 24 h.

1.7 Pathogenesis and Immunity

Infection occurs by inoculation of the nasal or conjunctival mucosa, often by self-inoculation with infected secretions from a close contact. Adult volunteers were infected experimentally if virus was inoculated onto the conjunctival sac or into the nose, but not following introduction into the mouth [112]. The incubation period from time of inoculation to onset of illness for RSV is likely about 4–5 days [113]. Virus replication initiates in the nasopharynx and rapidly can reach concentrations of over a million particles per mL in the upper airway in children. Adults, who are partially immune, typically shed much lower amounts of virus. Virus spreads quickly to the lower respiratory tract, often causing symptoms within days of onset of upper respiratory symptoms. Virus may spread from cell to cell, but also it is likely that small aspirations of upper respiratory secretions with virus inoculate the lower tract.

Higher titers of virus in respiratory secretions usually are associated with increased severity of disease, in prospective studies of natural infection [114] or of clinical vaccine trials [115]. RSV infection is an acute infection and virus shedding usually resolves within days to weeks. RT-PCR tests for virus nucleic acid may remain positive for prolonged periods. Some animal studies suggest that a negligible amount of infectious virus may persist in airways far after apparent resolution of shedding, as evidenced by the recovery of low amounts of infectious virus during immunosuppression several months after infection [116].

The virus infects respiratory epithelial cells in the lung and airway. It is not clear whether RSV also replicates productively in macrophages and dendritic cells in the airway or not. RSV protein antigens have been detected in circulating mononuclear cells [117], and viral genomic RNA and mRNA have been detected by RT-PCR in blood cells [118], but this probably represents cells from the airway recirculating, as infectious virus in the blood (viremia) is not detected. RSV replicates in the cells at the luminal surface of the respiratory epithelium and virus both enters and is shed from the apical surface of infected epithelial cells [119, 120]. Studies of polarized, differentiated respiratory epithelial cells in vitro show that RSV infection preferentially infects ciliated cells at the luminal face [121], but it is not clear if infection is restricted to such cells in humans. Histopathology studies of infected humans are very limited, but show RSV antigens in

the superficial cells of the airway in a patchy distribution, with antigen-positive cells and debris in the airway lumen.

Pathology caused by RSV infection during infant bronchiolitis includes necrosis and proliferation of the bronchiolar epithelium and destruction of ciliated epithelial cells [120, 122]. There is an influx of a large variety of immune cell types including neutrophils, lymphocytes, and macrophages. The respiratory tissues become edematous. Mucous secretion, sloughing of dead cell debris, and the influx of apparent inflammatory cells obstruct the lumen of the narrow bronchioles and alveoli. The small diameter of infant bronchioles is easily obstructed in the presence of dead cells and edema of the airway tissues [123]. Virus-infected cells can be identified in the epithelium of the bronchi, bronchioles, and alveoli [120]. It is surprising, given the extent of disease, that RSV antigen staining is usually patchy or focal, and even in some cases of fatal RSV bronchiolitis, antigen is present only in small amounts [119, 120]. The number of cases that have been collected is limited, however, and there is virtually no histopathology from milder cases.

RSV upper respiratory infection is complicated frequently by otitis media caused by bacteria. It is unusual to observe frank bacterial pneumonia or sepsis as a complication of RSV infection, in contrast to some other respiratory viruses like influenza. Although some clinicians may empirically use antibiotics during infant pneumonia, there is no evidence that antibiotic therapy alters the course of RSV bronchiolitis or pneumonia. Antimicrobial therapy should not be used in most cases of RSV bronchiolitis or pneumonia because of the lack of benefit and risk of selection of antibiotic-resistant colonizing organisms. Nevertheless, there is some suggestion that bacterial-viral interactions may affect the overall rate of disease in the population. It is interesting that annual RSV and influenza virus epidemics correlate directly with the time of peak incidence of invasive pneumococcal disease in many population studies [124]. A double-blind, randomized, placebo-controlled trial of pneumococcal conjugate vaccine showed a vaccine-attributable reduction in rates of childhood viral pneumonia requiring hospitalization, caused by any of seven respiratory viruses, including RSV [125].

The immune mechanisms responsible for resolution of infection and protection against reinfection by RSV are not fully defined.

Antibodies. Most experts agree that high levels of serum neutralizing antibodies are associated with relative protection against severe lower respiratory tract disease in otherwise healthy subjects. This idea is supported strongly by the observation that prophylaxis of high-risk infants with a neutralizing monoclonal antibody prevents about half of hospitalizations in that group of patients. Many population studies suggest that infants born with high levels of transplacental RSV-neutralizing maternal antibodies develop milder illness or illness at an older age than infants with low maternal antibody levels [15]. Most infants and children in whom maternal antibodies have declined to a low level make their own serum and secretory antibodies to both the F and G surface glycoproteins in response to RSV infection [126]. Antibody responses in neonates are particularly low in quality and magnitude due to immunologic immaturity and the suppressive effect of passively acquired maternal antibodies [126, 127]. Antibody-mediated immune suppression by passive antibodies primarily affects humoral rather than cell-mediated immunity [128, 129]. High levels of serum antibodies do not appear to provide solid immunity against disease in the upper respiratory tract. Mucosal secretory IgA appears to contribute to local protection against reinfection in the airway, although potent protective IgA responses are likely relatively short lived. In human infants, the decrease in virus shedding in nasal secretions was associated with the appearance of RSV-specific IgA antibodies [130].

T Cells. T cells clearly play a major role in resolution of active infection. RSV-specific T cells with cytolytic activity, thought to be CD8+ T cells, have been detected in peripheral blood mononuclear cells from infants with RSV disease [131]. Immunodeficient children, especially those with T cell defects, often fail to clear RSV infection and can shed virus for many months [57]. Adults with leukemia or hematopoietic stem cell transplant also have a very high incidence of prolonged RSV infection leading to severe disease and sometimes death.

1.8 Patterns of Host Response

1.8.1 Symptoms

RSV infection usually causes upper respiratory tract symptoms during primary infection in otherwise healthy term infants; asymptomatic primary infection is not common. There is often profuse rhinorrhea, and the upper tract disease is very often complicated by otitis media. Symptoms of lower respiratory tract involvement occur in about a third of primary cases [28]. The principal diagnoses are bronchiolitis (manifested by tachypnea and wheezing) and pneumonia. These entities are probably not discrete processes but more likely represent a continuum of disease involving increasing tissue distribution. The typical illness starts with nasal congestion, followed in a few days by cough. The infection is sometimes associated with fever, which is usually low grade. After several days of upper tract symptoms, infants may wheeze. Many infants suffer mild wheezing that resolves, but some cases progress with tachypnea, diffuse inspiratory crackles, and expiratory wheezes. Most children recover in 1–2 weeks with supportive care and observation. If expiratory obstruction becomes severe, however, hyper-expansion of the chest occurs due to air retention, and the compliant nature of the infant chest wall leads to intercostal and

subcostal retractions during tachypnea. With prolonged tachypnea, fatigue may occur with poor oxygenation and CO_2 retention (measured by pulse oximeter or arterial blood gas measurement), markers of respiratory failure. Intubation and mechanical ventilation is used in this setting to manage the respiratory failure.

Infection during the first day or weeks of life may just be characterized by temperature instability or fever, irritability, and lethargy even in the absence of overt respiratory signs or symptoms. In very young infants, especially those born prematurely, apneic spells may occur in response to RSV infection. Apnea may be the first reported evidence of infection in some cases, and apneic spells may recur during the acute infection. These events thankfully are usually self-limited and rarely cause neurologic damage. Apneic events are an indication for hospitalization and careful medical supervision with respiratory monitoring. Because of the association with apnea, some have considered whether RSV is associated with sudden infant death syndrome (SIDS). Although RSV has been detected in the lung tissues of some cases of SIDS, there is no statistically significant association between RSV and SIDS. The reported cases likely reflect a temporal association caused by the high prevalence of RSV in this age group, the prolonged pattern of shedding, and the simultaneous peak incidence of both RSV infection and SIDS in winter months [132].

It is not clear whether infection with RSV causes prolonged abnormal pulmonary function during childhood or whether children with underlying predisposition to lower respiratory tract disease of all causes manifest their susceptibility first to RSV because of the young age of RSV infection. Certainly measureable pulmonary function abnormalities are common after RSV lower respiratory tract disease, and these findings may persist for a decade or more [133]. Recurrent wheezing is common during subsequent viral infections after severe RSV bronchiolitis or pneumonia, with an incidence of 10–50 % [134]. A large case-control study of 200 children hospitalized for bronchiolitis or pneumonia in which RSV was the most common cause found that 7 years later there was a strong predisposition in these subjects toward decreased pulmonary function, recurrent cough, wheezing, and bronchitis [135]. Seminal prospective studies by Martinez et al. involved measurement of pulmonary function in infants at birth and then found a strong correlation between prior lower pulmonary function and the development of wheezing during RSV infection [136]. This correlation persisted in children during the first 3 years of life [136]. Even some individuals who do not typically exhibit recurrent wheezing have postexercise or pharmacologically induced bronchial reactivity [137], which may be responsive in part to bronchodilators [134].

Symptomatic upper respiratory tract RSV infections manifested by common cold symptoms are common in otherwise healthy adults, especially in those with frequent exposure to small children [80]. In the elderly, particularly those with underlying medical diseases, severe pneumonia may occur, leading to hospitalization or even death.

1.8.2 Diagnosis

Astute clinicians can often make a presumptive diagnosis of infection based on the clinical signs of wheezing or pneumonia in an infant during a local epidemic. Laboratory testing of nasal or lower airway secretions by antigen test (ELISA) or nucleic acid detection (by RT-PCR) provides rapid diagnosis of the presence of virus in many cases. The gold standard for diagnosis is isolation of the virus in cell culture, but this test is typically only available in referral laboratories because of the need for extensive equipment and a high level of technical expertise.

1.9 Control and Prevention

1.9.1 Treatment

Medical Treatment

Primary treatment is supportive care, which includes oral or intravenous hydration; monitoring of respiratory status, especially of oxygen saturation during tachypnea; use of supplemental oxygen; removal of secretions from the upper airway; and, in the case of respiratory failure, intubation and mechanical ventilation. Advances in support care in pediatric critical care units have caused a major decrease in morbidity and mortality from RSV in the developed world. Infants hospitalized for RSV disease should be monitored for apnea. Investigators have studied nitric oxide [138, 139] mixtures of helium and oxygen [140, 141] and surfactant treatment [142] in clinical experimental studies in the support of infants with severe RSV disease. Nitric oxide treatment does not appear to mediate a bronchodilator effect during RSV infection [139].

Antivirals

Therapy of RSV disease by any antiviral agent is challenging because it is a rapid acute infection, and by the time the onset of disease is recognized, it may be too late to alter the course of disease by reducing viral load. The guanosine (ribonucleic acid) analog ribavirin is a nucleoside inhibitor that inhibits viral RNA synthesis and viral mRNA capping. The drug has in vitro antiviral activity against RSV, and aerosolized ribavirin therapy has been associated with a small but statistically significant increase in oxygen saturation during the acute infection in several small studies. Decreases in mechanical ventilation and duration of RSV-associated hospitalization have not been proven (reviewed in [143]). Ribavirin was approved in 1986 in the USA for treatment of RSV infection [144]. Clinically, the drug usually is administered as a

small-particle aerosol using a tent, mask, or mechanical ventilator, delivered for 6–18 h daily for a period of 3–7 days. The drug now is not recommended for routine use because follow-up studies have not shown a major benefit. The drug may be considered for use in select patients with documented, potentially life-threatening RSV infection. Over a dozen other experimental small molecule inhibitors of RSV fusion to cells have been described and tested in preclinical studies for inhibition of RSV, but none have progressed in development to date. A third approach employs short interfering RNAs (siRNAs), taking advantage of an ancient host cell regulatory system. Single-stranded and double-stranded RNA molecules that exhibit RSV-specific small interfering RNAs have been developed for treatment, which cause RNA interference activity against RSV, destroying the corresponding RSV RNA. These novel compounds have shown promising results in preclinical studies [145] and have been tested in small clinical trials. Human immune globulin with a high titer of RSV antibodies and the RSV monoclonal antibody palivizumab have been tested as therapy of acute RSV disease, but they were not effective for treatment of established disease.

Anti-inflammatories

Anti-inflammatory strategies have been investigated. No benefit of corticosteroid therapy on disease severity or length of hospital stay has been demonstrated, despite studies in over a dozen randomized clinical trials of outpatients or hospitalized infants with RSV bronchiolitis. Since the drug is of no benefit on its own [146] and may prolong virus shedding, it is not recommended. In the future, a possibility might be to combine an effective antiviral treatment with an anti-inflammatory agent [147].

Antibiotics

Intravenous antimicrobial therapy is not appropriate in hospitalized infants with RSV bronchiolitis or pneumonia unless there is clear evidence of secondary bacterial infection. Otitis media occurs very often in infants with RSV bronchiolitis; oral antimicrobial agents can be used for therapy of otitis media if necessary.

Bronchodilators

It is intuitive to think of using beta-adrenergic agents, commonly used for the treatment of asthma, to treat the wheezing associated with RSV infection. These agents are not usually recommended for routine care of first-time wheezing associated with RSV bronchiolitis. Short-term improvements in oxygenation and clinical scores can be achieved by these therapies, but it has not been established that their use results in improvements in duration or severity of illness or disease outcomes. Studies in this area have been conflicting, but systematic reviews of randomized clinical trials of nebulized beta-agonist therapy for treatment of bronchiolitis suggest that they offer little benefit [148, 149]. Alpha-adrenergic receptor stimulation results may decrease interstitial and mucosal edema [150], and use of nebulized epinephrine (with combined alpha- and beta-adrenergic activity) has been studied with conflicting results [151, 152]. Alpha-agonist stimulation of the sympathetic nervous system is expected to reduce capillary leakage by constricting precapillary arterioles, reducing hydrostatic pressure and consequently bronchial mucosal edema [150]. Racemic epinephrine treatment relieves some respiratory distress but does not affect length of stay [153]. The usefulness of such agents in the management of RSV bronchiolitis is not clear.

1.9.2 Prevention and Immunoprophylaxis

The most effective mode of prevention is to avoid contact with infected subjects. In the hospital setting, careful adherence to infection control practices is important for the protection of high-risk patients from RSV infection. Careful hand washing may reduce transmission in family and day-care settings. Pharmacologic intervention is indicated to prevent hospitalization for the highest risk infants, however. Passive RSV immunoprophylaxis with antibodies has proven a costly but relatively effective intervention. Parenteral infusion of RSV-neutralizing antibodies into experimental animals was shown early on to confer substantial resistance in the respiratory tract to a subsequent RSV virus challenge [154]. Significant reductions in RSV-associated hospitalizations and disease severity in high-risk human infants were first accomplished with prophylactic administration of human immunoglobulin with high RSV-neutralizing activity given by the intravenous route (RSV-IVIG; FDA licensed in 1996) [155, 156]. Monthly intravenous infusions during the RSV season reduced the frequency of pediatric hospitalization and duration of stay by approximately 55 % and decreased the number of days spent in intensive care by 97 %. The use of RSV-IVIG was superseded by the use of a monoclonal antibody (MAb) that was developed subsequently that could be given by intramuscular route, and production of the former has been discontinued. Several MAbs were developed for immunoprophylaxis against RSV. The most successful of these was based on murine MAb 1129 [157], which is specific to the F protein and efficiently neutralizes viruses of both RSV subgroups A and B. This MAb was humanized by recombinant methods by transferring its variable regions onto a human IgG1 backbone, resulting in a recombinant antibody now named palivizumab [158]. This MAb is 50–100-fold more effective for in vitro neutralization on a per weight basis than was RSV-IVIG, and thus the total amount of immunoglobulin administered could be reduced to an amount that could be given IM. Palivizumab (trade name Synagis) was licensed in 1998 for RSV prophylaxis of high-risk infants, following studies demonstrating its

safety and efficacy [159–162]. Palivizumab is administered monthly through the RSV season and has been widely used in high-risk patients with prematurity, chronic lung disease, and hemodynamically significant heart disease [163]. More potent derivatives of this recombinant antibody have now been developed [164]; however, the lead candidate from these affinity maturation efforts exhibited increased side effects in a large efficacy study.

1.9.3 Vaccines

Prevention of severe disease probably will best be accomplished by development and use of an effective vaccine. Vaccine development for RSV has proven exceptionally difficult, however. First, young infants are a difficult population to immunize. Obstacles to immunization at this early age include immunologic immaturity and immunosuppression by maternal antibodies, as already noted [165]. Also, severe adverse events occurred in early RSV vaccine trials. A formalin-inactivated RSV vaccine candidate (FI-RSV) was developed and evaluated in infants and children in the 1960s [166, 167]. This vaccine suspension was made by mixing concentrated, inactivated virus with alum adjuvant and was delivered by the intramuscular (IM) route. This inoculation did not protect against infection or disease, but rather during subsequent natural infection vaccinees experienced more frequent and severe disease. Most FI-RSV vaccinees (80 %) required hospitalization during subsequent natural infection, compared to 5 % in the control group. Autopsies of two fatalities showed evidence of RSV replication and pulmonary inflammation [167]. This event put a chilling effect on RSV vaccine development efforts. Therefore, RSV protein vaccines have been problematic for use in infants given their possible potential for disease enhancement, together with the poor immunogenicity in this population.

However, an RSV protein vaccine might be useful in boosting immunity in RSV-experienced older children and adults who are at increased risk of severe RSV disease due to underlying disease or advanced age. Protein vaccines for RSV have been evaluated clinically for use in such RSV-experienced individuals, in whom they appear to be safe. One experimental subunit vaccine consisted of purified F protein (PFP) isolated from RSV-infected cell culture. This purified protein vaccine candidate was evaluated in adults, in older children with and without underlying medical diseases, and in the elderly [168]. The PFP vaccine candidate was well tolerated and moderately immunogenic in these settings. A large multicenter study in children 1–12 years of age with cystic fibrosis did not provide evidence of significant protection against RSV infection [169]. PFP also has been evaluated for maternal immunization in the third trimester of pregnancy. In the single study to date, the increase in antibody titers was only minimal [170]. Maternal immunization studies are being pursued currently with newer non-replicating vaccine candidates such as emulsion vaccines [171] and nanoparticle protein preparations [172, 173].

Live-attenuated vaccines represent an attractive strategy for preventing RSV, since live infection induces a balanced immune response that is not associated with enhanced disease on subsequent natural infection. Many live-attenuated RSV vaccine candidates have been developed over several decades. It has proven difficult to identify a candidate that is satisfactorily attenuated while remaining satisfactorily immunogenic in the youngest infants. Clinical trials of a safe, live-attenuated RSV vaccine for intranasal administration have shown restriction of viral replication in infants following administration of a second dose and have been encouraging [174], and additional attenuated vaccine candidates are being developed [175].

1.10 Unresolved Problems

Despite over 50 years of research on RSV, many challenges and questions remain. There are many unanswered fundamental questions about the biology of the organism and the pathogenesis of disease. Why does reinfection occur throughout life? What are the definitive mechanisms of immunity in humans? Is there a genetic basis for susceptibility to severe disease? Does severe RSV disease cause asthma? What drives the seasonality of RSV?

The mortality in infants has been greatly reduced in the USA through advances in critical care, but little RSV-specific intervention is available. Currently, there is little to offer for therapy except for supportive care. Prophylaxis of high-risk infant with a MAb prevents some hospitalizations but is expensive and is not always effective. There are no licensed vaccines. Given the disaster of early FI-RSV trials, it is not clear that a non-replicating vaccine can be proven safe enough in preclinical models to absolutely assure that enhanced disease will not occur. On the other hand, the explosion of new technologies for generation of recombinant RSV strains, the determination of pre- and post-fusion antigen structures, and new tools for the detailed study of the molecular and genetic basis of human immune responses suggest that much progress will be made in the RSV research field in the coming years.

2 Human Metapneumovirus

2.1 Introduction

Human metapneumovirus (HMPV, MPV) is a paramyxovirus isolated in 2001 from Dutch children with acute respiratory infection (ARI). Epidemiologic studies have since confirmed that MPV is a leading cause of upper and lower

respiratory infection in children and adults worldwide. MPV causes many hospitalizations annually in young children and presumably deaths in developing nations. Children with comorbid conditions including prematurity, immune compromise, and chronic cardiopulmonary disease are at higher risk for severe disease. However, the majority of MPV-associated hospitalizations occur in otherwise healthy infants and children. Conversely, while MPV is associated with severe lower respiratory infection in adults at rates similar to those of influenza virus and RSV, almost all of the MPV infections in this population are in patients with comorbidities. MPV causes predictable annual outbreaks with late winter-early spring predominance in temperate regions. Substantial progress has been made in defining the biology, pathogenesis, and immunology of the virus. Small animal and nonhuman primate models of MPV have been developed and used to elucidate mechanisms of immunopathogenesis and test candidate vaccines. A variety of vaccine approaches against MPV are under study, including recombinant subunit, vectored, and live-attenuated vaccines. Human and murine monoclonal antibodies have been generated that exhibit potent in vivo efficacy in rodent models. A subset of integrins with a binding site for a natural ligand that contains an Arg-Gly-Glu (RGD) motif (RGD-binding integrins) has been identified as receptors that mediate MPV attachment and entry via an RGD motif in the MPV fusion protein. Remarkable scientific progress has been made during the decade since the discovery of MPV.

2.2 Historical Background

MPV was discovered by investigators in the Netherlands who cultivated specimens from children with respiratory infection on a variety of cell types [176]. A hitherto unknown virus produced cytopathic effects (CPE) in tertiary monkey kidney cells, but could not be identified by antibody staining or RT-PCR for common viruses. Electron microscopy of infected cells revealed pleomorphic enveloped particles with surface projections suggesting protein spikes, while biochemical experiments showed that the virus contained a lipid envelope and did not hemagglutinate avian or mammalian red blood cells. Elegant randomly primed RT-PCR experiments yielded multiple fragments of genome, which sequence analysis identified as related to avian metapneumovirus (AMPV).

AMPV (formerly turkey rhinotracheitis virus), identified in 1979, is an important global pathogen of poultry including turkeys and chickens [177]. There are four serotypes of AMPV (A–D), and MPV is most closely related genetically to AMPV-C [178]. Phylogenetic analysis shows that MPV likely diverged from AMPV-C ~200 years ago and thus MPV is of zoonotic origin [179, 180]. However, MPV exhibits extremely restricted or no replication during experimental infection of chickens or turkeys and thus is a true human pathogen [176]. Human poultry workers exhibit serological evidence of asymptomatic infection with AMPV, providing evidence for the feasibility of an original trans-species transmission event [181].

While recently identified, MPV is not truly a new virus. Studies using archived sera collected in the 1950s revealed a 100 % seroprevalence in humans greater than 5 years old [176]. Nasal washes collected prospectively during the 1970s from children with ARI had detectable MPV RNA upon retrospective testing by RT-PCR 25 years later [182]. The specialized cell culture requirements, slow growth, and limited CPE of MPV likely prevented the earlier discovery of this common respiratory pathogen.

2.3 Methods for Epidemiologic Analysis

2.3.1 Sources of Mortality Data

Limited mortality data are available and consist of sporadic case reports, case series, or the identification of fatal cases of MPV infection in research studies. MPV is not a reportable infection and there is no specific ICD-9 diagnostic code. Thus, an accurate estimate of the mortality associated with MPV is not feasible. However, lower respiratory infections are a leading cause of death in children worldwide, primarily in developing nations. MPV is a common cause of severe lower respiratory infection and thus likely accounts for a substantial number of deaths globally.

2.3.2 Sources of Morbidity Data

A substantial body of literature has accumulated in the last decade describing the epidemiology, disease burden, and clinical features of MPV. Many groups have used standard techniques to provide some estimate of the burden of MPV in diverse populations. Most reports have been cross-sectional studies of selected populations, usually based on convenience samples of patients with acute respiratory illness. These studies are thus limited by potential selection bias, narrow time periods, and incomplete demographic or clinical data and often lack controls. Nonetheless, the broad application of these studies to sizable global populations has illuminated the frequency of MPV infection. Many of these studies have focused on special populations, such as patients with asthma, immune compromise, or chronic obstructive pulmonary disease (COPD), and thus offer valuable information about MPV among these persons.

A number of prospective, well-designed studies in adults and children have been published and offer the best estimates of the population-based incidence of MPV infection. Some of these used preexisting prospective data and samples collected prior to the discovery of MPV for retrospective

analysis. Classical methods including active day care and clinic surveillance, as well as newer approaches such as home surveillance with parent-collected swabs, have been used. These studies have been built upon the foundations of seminal longitudinal studies conducted to investigate other viruses including influenza, parainfluenza viruses, and RSV. Taken together, these reports provide a broad survey of MPV epidemiology across diverse geographic environments, socioeconomic populations, and high-risk groups.

2.3.3 Serological Surveys

Seroprevalence studies using diverse methods have been performed in different populations. Most have used enzyme-linked immunosorbent assay (ELISA) techniques against whole virus or purified proteins to detect IgM or IgG. A few have used immunofluorescent detection of MPV-specific antibodies or measured serum virus-neutralizing antibodies. These data have mainly been useful in determining the ubiquity of infection with MPV. Some studies have measured acute and convalescent sera to diagnose MPV infection, while others have attempted to establish a serum neutralizing titer that correlates with susceptibility to infection. Inherent limitations of serological surveys include the potential for cross-reactive antibodies and the lack of standardized reagents for MPV.

2.3.4 Laboratory Methods

Most studies have used RT-PCR to detect MPV due to the difficulty in cultivating the virus. The original isolation of MPV in tertiary monkey kidney cells was possible because the investigator had access to a monkey kidney cell source that was free of the endogenous simian foamy virus (A.D.M.E. Osterhaus, personal communication). Primary monkey kidney cells commercially available in the USA all contain SFV, and even the addition of anti-SFV antisera cannot prevent the growth of this endogenous virus prior to the slow emergence of MPV. Further, the fusion protein of MPV requires cleavage by exogenous trypsin for robust in vitro growth. Trypsin is added by most clinical virology laboratories only to cultures of Madin-Darby canine kidney (MDCK) cells for the isolation of influenza virus; however, MDCK cells are very poorly permissive for MPV even in the presence of trypsin. Finally, primary isolation of MPV often requires one or more passages prior to visible CPE and few laboratories routinely follow this procedure. Unlike RSV, MPV is not particularly labile to freeze-thaw cycles [183] and thus can be retrospectively isolated from PCR-positive specimens. Fluorescent antibody staining of patient specimens or shell vial cultures can facilitate more rapid identification [184–187].

Thus, molecular diagnostic techniques have been used for virtually all studies of MPV epidemiology. A number of sensitive and specific real-time RT-PCR assays have been described [188–195]. Many early assays were based on limited sequence data and subsequently were found to be suboptimal for detecting multiple diverse strains [195]. Both individual and multiplexed RT-PCR assays offer more sensitive detection of MPV than culture; however, multiplex assays sometimes balance decreased sensitivity for a single agent with the convenience of detecting multiple viruses simultaneously [196]. Another limitation of molecular detection for all viruses is the ability to detect low levels of viral nucleic acid in the absence of infectious virus. It has become common to detect more than one virus in a single specimen, and the interpretation of these data is far from clear. Community respiratory viral infections are frequent in childhood, and the likelihood of detecting viral genome prior to the onset of illness or for prolonged periods after illness resolution complicates the assignment of causation to one of several co-detected viruses.

2.4 Biological Characteristics

MPV is an enveloped pleomorphic virus ranging in size from 150 to 600 nm, containing a single-stranded negative-sense RNA genome [178]. Complete genomic sequences of numerous MPV strains have been published [178, 180, 197]. The genome comprises eight separate open reading frames encoding nine distinct proteins (Fig. 26.1). MPV genes are analogous to those of RSV (though NS1 and NS2 are absent in MPV), but the organization of genes differs. AMPV and MPV have been taxonomically classified in the separate *Metapneumovirus* genus based on the gene order. Phylogenetic analysis of MPV genes consistently identifies four genetic clades, two major groups designated A and B, each with two minor groups designated A1, A2, B1, and B2 [180, 198–203]. One group has suggested further sublineages based on partial F sequence diversity [204], but there is no evidence that this further genetic distinction is of any antigenic or immunologic importance.

The two major surface proteins are the fusion (F) and attachment (G), with a third integral membrane short hydrophobic (SH) protein. F is the target of neutralizing antibodies in animal models, F-only vaccines induce protection in animals, and F-specific monoclonal antibodies provide passive protection [205–213]. In contrast, G-specific antibodies do not neutralize virus and G-only vaccines induce neither neutralizing antibodies nor protection [206, 209, 214]. Thus, it appears that MPV is unique among human paramyxoviruses in that the attachment protein does not contribute to protective antibodies. Further, the G protein exhibits a high degree of genetic variability between subgroups, with as low as 29 % amino acid identity between the major A and B subgroups and a minimum 60 % identity within subgroups [202, 215–218]. The selective pressure for this diversity is unclear.

In contrast to G, the F protein is conserved, with a minimum 94 % amino acid identity between A and B subgroups and a minimum 98 % identity within subgroups [179, 202, 203]. Presumably there are functional constraints on the diversity of F, since the mutation rate of MPV is high, similar to other RNA viruses. The major question regarding the diversity between major or minor subgroups is whether it contributes to antigenic variation or escape in human populations.

Cross-neutralization against heterologous virus from the A and B lineages was tested using experimental infection of ferrets [202]. This study found relative neutralization of homologous to heterologous virus ranging from 12 to 96-fold difference, thus providing some evidence for antigenic serotypes. However, subsequent experiments using hamsters, African green monkeys, chimpanzees, and rhesus macaques found that the A and B groups were 64–99 % related antigenically [219]. Infected animals developed neutralizing antibodies that were highly effective against heterologous virus, and previously infected primates were protected against challenge with heterologous virus. Cynomolgus macaques infected with A or B subgroup viruses or with candidate vaccines exhibited only a 6–16-fold difference in neutralizing titer against homologous and heterologous viruses [220, 221]. Taken together, these data show that while MPV F exhibits some antigenic diversity, the virus does not have truly distinct serotypes. The potential implications for human epidemiology are discussed further below.

2.5 Descriptive Epidemiology

2.5.1 Incidence and Prevalence Data

Numerous studies document the fact that MPV infection is ubiquitous and that reinfection is common. Serosurveys testing large sample collections in Canada, China, Croatia, Germany, Israel, Japan, the Netherlands, Taiwan, Thailand, the USA, and Uruguay show that 95–100 % of children have antibodies against MPV by the age of 5 years [222–231]. In many of these studies, 50–75 % of children are seropositive by age 2 years, suggesting that most acquire primary MPV infection early. Most identify a decrease in serum MPV antibody titer from birth to 6–12 months, presumably due to the expected decline of maternally derived antibodies. Studies in Japan and India that compared MPV and RSV titers in the same cohort found that after the expected nadir during early infancy, RSV titers began increasing at an earlier age than MPV [232, 233]. This finding is interesting in light of epidemiologic data suggesting that primary MPV infection peaks between 6 and 12 months of life compared with the peak of RSV at 2–3 months (discussed below). Longitudinal studies in adults and children have documented reinfection by a fourfold rise in serum antibody titer [222, 230, 231, 234–237].

2.5.2 Risk of Infection and Reinfection

The serological data show that MPV infection is nearly ubiquitous during the first years of life. Further, reinfection occurs throughout life. In children, primary MPV infection is associated commonly with lower respiratory illness, while reinfection is associated with upper tract disease [182, 238].

2.5.3 Risk of Serious Lower Respiratory Infection (LRI) During Infancy

Most epidemiologic studies of MPV in children show that the virus is the second leading cause of lower respiratory infection after RSV. The prevalence of MPV in studies of children with LRI is 5–25 %. A 25-year prospective study of otherwise healthy children <5 years old detected MPV in 12 % of children with LRI; several children experienced recurrent infection [182]. A 2-years multicenter study of inpatient and outpatient Japanese children with ARI identified MPV in 57/637 (8.9 %) [239]. A 5-years observational study of otherwise healthy Korean children <5 years old found MPV in 24/515 (4.7 %), similar to the rates for influenza and parainfluenza virus type 3 (PIV-3) [240]. A very large observational study in Queensland, Australia, tested specimens obtained from patients of all ages with LRI from 2001 to 2004; MPV was detected in 707/10,025 (7.1 %). Ninety-two percent of patients with MPV were <5 years old, and MPV was the second most common virus after RSV in these children [241]. A South African group tested specimens from children hospitalized with ARI who were subjects in a prospective pneumococcal vaccine trial; MPV was present in 126/1,409 (8.9 %) and was the most common virus after RSV. The estimated incidence of MPV-associated hospitalization in HIV-negative children was 29/1,000; a number of children had repeat infections [242]. A prospective study of hospitalized children in Hong Kong over a 13-month period found MPV in 32/587 (5.5 %); the estimated incidence of MPV-associated hospitalization was 4.4 per 1,000 children <6 years old [243]. A Chinese group conducted a prospective 2-years study of children hospitalized with ARI and identified MPV in 227/878 (25.9 %), with most <6 years old [244]. A large, population-based, prospective surveillance study conducted in three US cities over 6 years found that the incidence of hospitalization for MPV in children <5 years old was 1 per 1,000, lower than the rate of RSV-associated hospitalization in the same cohort (3/1,000) but similar to the rates for influenza (0.9/1,000) and PIV-3 (0.5/1,000) [245].

2.5.4 Role of MPV Infections in Adults

Respiratory disease is among the leading causes for hospitalization of adults in the USA, and "influenza and pneumonia" ranks among the top 10 causes of deaths annually. Although the data are limited, MPV appears to be associated with a substantial burden of ARI in adults, primarily those with comorbidities. A prospective study in Rochester, NY,

enrolled four cohorts during four winters: healthy adults >65, high-risk adults >65 with comorbidities, healthy adults 19–40 years old, and adults hospitalized for acute cardiopulmonary illness [246, 247]. Overall, MPV infection was detected in 8.5 % of ARI in the cohort. The rate of MPV infection was highest in young adults at 13 %, though many of these were asymptomatic and detected only serologically. Of note, this group had a mean age of 33, was predominantly female, and had daily exposure to children. Of the hospitalized patients, the incidence of MPV annually ranged from 4.4 to 13.2 %. More than 85 % of the hospitalized MPV-infected subjects had underlying conditions, chiefly cardiopulmonary disease or diabetes mellitus. There were six deaths in this study, all with comorbidities; one had concomitant bacteremia with *Streptococcus pneumoniae*. Interestingly, the incidence of MPV infection was similar to the annual average infection rate for RSV (5.5 %) and influenza A (2.4 %) in these cohorts during the same study period.

A prospective, population-based study in Nashville, TN, recruited adults hospitalized for ARI at several county hospitals over three winters [248]. Of 508 subjects, 23 (4.5 %) had MPV, 33 (6.5 %) influenza, and 31 (6.1 %) RSV. Notably, MPV-infected subjects were significantly older than influenza-infected subjects (mean 76 vs. 60 years) and had higher rates of chronic cardiopulmonary disease (78 % vs. 52 %). The overall population-based rates of hospitalization for the three viruses were similar, at 1/1,000 for MPV, 1.5/1,000 RSV, and 1.2/1,000 for influenza. However, for subjects ≥65 years, hospitalization rates were much higher for MPV and RSV at 2.2/1,000 for MPV and 2.5/1,000 for RSV compared to 1.2/1,000 for influenza, likely reflecting the use of influenza vaccine for older adults. A prospective study of community-acquired pneumonia in Canada found MPV in 4 % of hospitalized cases during an 18-month period, all with underlying conditions [249]. A Dutch group detected MPV in 2 % of bronchoalveolar lavage specimens from intensive care unit patients. All were >50 years old with comorbid conditions and 83 % died [250]. Similarly, a retrospective study in North Ireland found MPV in only 0.8 % of residual respiratory specimens from adults, but 33 % of these died [251]. Together, these data show that MPV is an important cause of acute respiratory disease in adults, primarily older adults or those with underlying comorbid conditions.

2.5.5 Role of MPV Infections in Patients with Asthma

MPV is associated with acute asthma exacerbations [252–254]. MPV was detected in 10 of 132 hospitalized Finnish children with acute wheezing [255]. Similarly, MPV was isolated from 7 % of adults hospitalized for acute asthma exacerbation, only one of whom tested positive 3 months later [256]. Premature infants who developed MPV bronchiolitis within the first year of life had decreased lung function at 1 year of age [257]. A prospective, case-control study of children with MPV bronchiolitis during infancy compared to infants with acute gastroenteritis found that MPV infection early in life was significantly associated with a later diagnosis of asthma and recurrent wheezing at 5 years of age [258].

2.5.6 Role of MPV in Infections with Underlying Cardiopulmonary Disease

MPV causes severe disease in children with comorbid conditions such as cardiac and pulmonary disease or prematurity [259–263]. A prospective 1-year study of hospitalized children found that 34 % of patients with MPV had a history of prematurity, chronic lung disease, complex congenital heart disease, or immunodeficiency [264]. Vicente et al. detected MPV by RT-PCR in 6 % of adults >64 years old with acute exacerbations of COPD; none had other pathogens identified by culture or PCR [265]. A Canadian study found that 4 % of hospitalized adults with community-acquired pneumonia or COPD exacerbations tested positive for MPV, all with comorbid conditions; one also had influenza A and *S. pneumoniae* [266]. RSV was present in 9 % and influenza A in 6 % of the cohort. MPV was detected in 12 % of adults hospitalized for COPD exacerbation during one winter in Connecticut, none with other viruses co-detected; RSV was present in 8 % and influenza A in 4 % of the entire cohort [267].

2.5.7 Role of MPV Infections in Patients with Immunodeficiency

Severe and fatal MPV disease can occur among immunocompromised individuals, including solid organ and stem cell transplant recipients, HIV-infected persons, and chemotherapy patients [268–275]. MPV is associated with morbidity and mortality in adults with hematologic malignancies and stem cell transplant; [270, 276] MPV was detected in bronchoalveolar lavage specimens from 5/163 (3 %) episodes of acute respiratory infection in stem cell transplant recipients, and four died [270]. HIV-positive South African children with MPV were significantly more likely to receive a diagnosis of pneumonia and experience longer hospitalization, lower mean oxygen saturation, and bacteremia; further, HIV-positive children were fivefold more likely to be infected with MPV than HIV-negative children [242].

2.5.8 Role of MPV in Nosocomial Infection

MPV has been implicated in several hospital and institutional outbreaks leading to mortality [277, 278]. Kim and colleagues [279] report the transmission of MPV to pediatric hematology-oncology patients during a nosocomial outbreak. The incubation period was between 7 and 9 days. Standard, but not droplet, precautions were used. In laboratory studies, infectious virus persists on metal and nonporous surfaces for up to 8 h [183]. Due to the significant morbidity and mortality of MPV in high-risk children, isolation precautions are important.

2.5.9 Role of MPV in Different Clinical Syndromes

MPV is associated with both upper and lower respiratory tract disease [182, 280–282]. Rhinorrhea and cough are the most frequent symptoms, while hoarseness, laryngitis, sore throat, and croup are less common [238–241, 243, 282]. A large prospective study of children with URI detected MPV in 5 % of patients, similar to RSV, influenza, and PIV but less frequent than adenovirus and rhinovirus. In these children with URI associated with MPV, fever was present in 54 %, coryza in 82 %, cough in 66 %, pharyngitis in 44 %, hoarseness in 8 %, and conjunctivitis in 3 % [280]. MPV is associated with acute otitis media and has been detected in nasal secretions and middle ear fluid [182, 280, 283–285].

Signs and symptoms of LRI with MPV include cough, wheezing, and rhonchi. A large Chinese study of children with acute respiratory infections found that wheezing was more common in children with MPV than with RSV [282]. In that study, children with MPV were diagnosed with pneumonia significantly more than children with RSV, 47 % versus 31 % ($p=0.002$). Conversely, a larger percentage of children with RSV were diagnosed with bronchiolitis compared to MPV, 62 % versus 42 %, respectively ($p<0.001$). Other studies also note the trend toward a higher percentage of children with MPV and pneumonia compared to RSV [182, 238, 245] although this is not always statistically significant [243].

MPV has been associated rarely with neurologic complications, including febrile seizures and altered mental status, but there is no conclusive evidence for direct neural infection. One case report describes a patient who died and had MPV isolated by RT-PCR from brain and lung tissues [286]. MPV was detected by RT-PCR in nasal specimens from 4 of 1,570 persons with encephalitis of unknown etiology [287]. Several other reports describe the detection of MPV in a respiratory specimen from patients with encephalitis [286, 288–290]. Only one case reports detection of MPV in cerebrospinal fluid [291].

2.5.10 Epidemic Behavior

The incidence of viral infection varies between countries and years, but MPV circulates in every year [176, 182, 239–241, 243, 282, 292–296].

2.5.11 Geographic Distribution

Epidemiologic studies have verified the presence of MPV worldwide.

2.5.12 Temporal Distribution

MPV is present during all months in temperate regions, although predominant in late winter-early spring, often following the peak of RSV (Fig. 26.2) [176, 182, 191, 239, 241, 246, 264, 280, 282, 294, 297].

In subtropical climates such as Hong Kong, a spring-summer season similar to RSV occurs [243]. Biannual peaks of seasonality have been described in some European studies [298, 299]. Annual rates of MPV-associated ARI are lower than RSV and comparable to parainfluenza virus types 1–3 combined and influenza [240, 241, 243, 282, 294, 300] although MPV does on occasion surpass RSV in incidence [293].

2.5.13 Occurrence in Different Settings

Multiple outbreaks have been reported in nursing homes and other long-term care facilities (LTCF). MPV was the only pathogen identified in an outbreak of ARI that occurred over a 6-weeks period at a Quebec LTCF, with 6 PCR-confirmed and 96 epidemiologically linked probable cases. There were three deaths among the confirmed and nine deaths among the probable cases [278]. A California study described an outbreak of ARI in 26/148 (18 %) residents and, importantly, 13 staff of a LTCF; MPV was confirmed in 5 residents, 2 of whom were hospitalized, and no other viruses were detected in any case [301].

2.5.14 Environmental Risk Factors

Attendance in out-of-home day care, breast-feeding, and passive smoke exposure are not significantly associated with MPV infection [245]. MPV infection is not associated with lower socioeconomic status.

2.5.15 Other Factors

Viral coinfection has been suggested with MPV; dual infection with RSV and MPV increased the risk of PICU admission compared to RSV alone in one small study [302]. However, this finding was refuted by subsequent larger reports [303–305]. Other studies demonstrate no significant difference in children with coinfections, including adenovirus, bocavirus, coronavirus, influenza virus, parainfluenza viruses, or RSV [240, 241, 282, 293, 306, 307].

MPV has four distinct genetic lineages or subgroups: A1, A2, B2, and B2 [202, 203, 218]. The predominant subtype varies by year and location [241, 280, 293, 297, 308]. In Italy, over a 3-years period, all four subtypes were identified each season but the predominant subtype changed. In 2001–2002, A1 accounted for 59 % of all strains, the following year B1 and B2 were present equally, and in 2003–2004, 72 % of strains were A2 [308]. Similar variation was observed over 20 years in a US study, with multiple subgroups present in most seasons (Fig. 26.3) [280].

It is unclear whether viruses from different subgroups differ in virulence. A study in Spain reported that children with group A infection more frequently had pneumonia and higher disease severity [309], while in Canada, group B was associated with more severe disease in hospitalized patients [261]. Patients with group B strains in a French study were more likely to have abnormal chest radiographs but did not have significant differences in oxygen saturation, hospitalizations, or clinical severity scores [310]. Other studies have found no major distinctions in disease severity [239, 241,

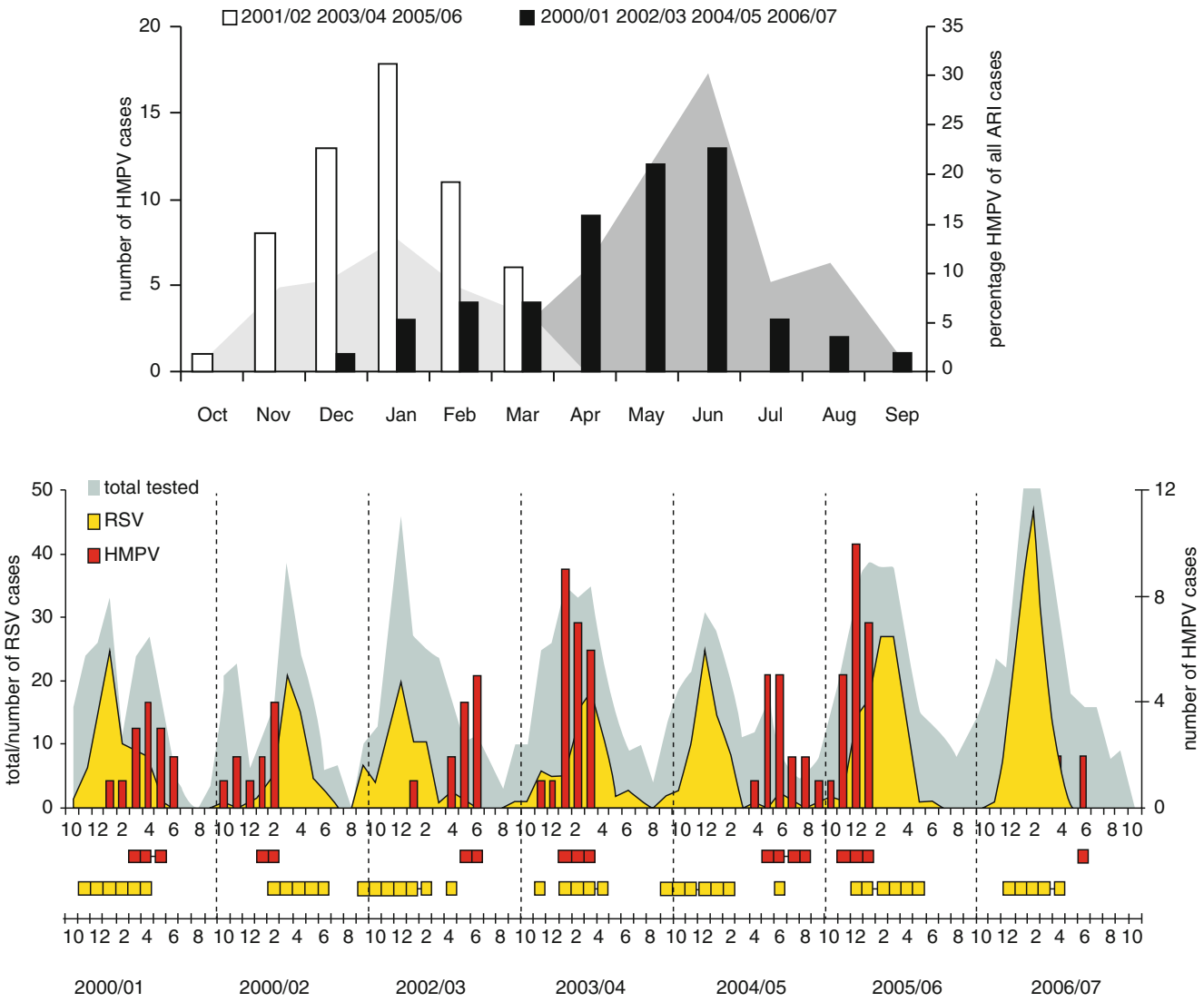

Fig. 26.2 Number of infants admitted to hospital with acute respiratory illness and the seasonality of RSV and MPV in Austria from 2000 to 2007 (*top panel*). The weeks of onset and end of RSV and MPV activity (*bottom panel*) are defined as the first of 2 consecutive weeks where >10 % of specimens test positive by PCR and the last week of >10 % positive preceding 2 consecutive weeks of <10 % positive. Gray indicates total tested; yellow, RSV; red, HMPV (Used with permission from Aberle et al. [298])

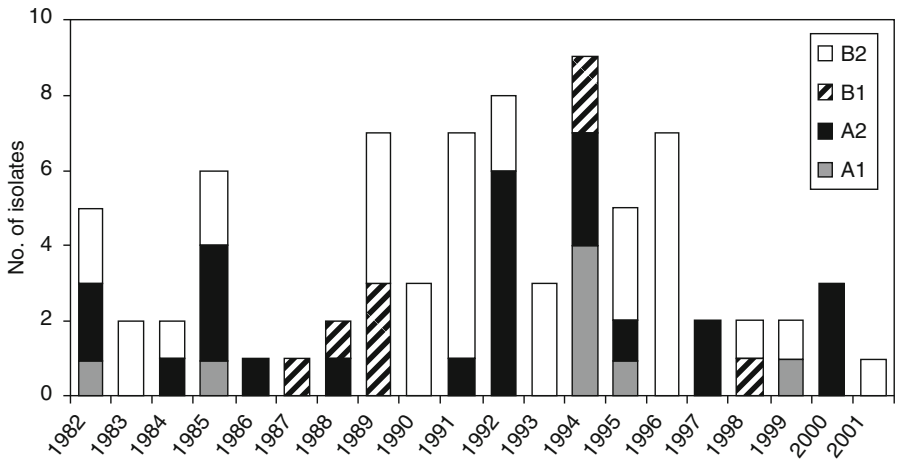

Fig. 26.3 Rates, by year, of each genetic lineage of human metapneumovirus. Data are from specimens collected from children with acute respiratory infection between 1982 and 2001 in the Vanderbilt Vaccine Clinic (Used with permission from Williams et al. [280])

311], laboratory abnormalities [228], or symptoms [240] between subgroups. Group A viruses replicate more efficiently in animal models, suggesting some meaningful biological differences between groups [219, 220].

2.6 Mechanisms and Routes of Transmission

MPV is an enveloped virus and thus inactivated by soap, disinfectants, or alcohols. Spread is thought to occur by direct or close contact with contaminated secretions. Infectious virus can persist at room temperature, especially nonporous surfaces, for up to 8 h [183]. Contact precautions are recommended as for RSV with meticulous hand hygiene. Cohorting of patients and caregivers should be considered during outbreaks in care facilities.

2.7 Pathogenesis and Immunity

Human data are limited; studies in rodents and nonhuman primates reveal mild erosive and inflammatory changes in the mucosa and submucosa of the airways, with viral replication observed in ciliated epithelial cells in the respiratory tract [312–314]. Inflammatory infiltrates with a lymphocytic and monocytic predominance are present in perivascular and peribronchial areas. Sumino and colleagues [315] reviewed the lung pathology of five adults with MPV infection. Histopathology in three patients demonstrated acute, organizing lung injury with diffuse alveolar membrane formation and the presence of smudge cells. The fourth patient had no evidence of lower respiratory tract infection, and the fifth patient had nonspecific acute and chronic inflammation. A similar study in children revealed chronic inflammatory changes of the airways with intra-alveolar macrophages [316]. A major limitation of reports of human pathology is that patients had been mechanically ventilated prior to death, making it difficult to distinguish virus-induced pathology from barotrauma and nonspecific inflammation.

MPV lacks genes present in other paramyxoviruses that inhibit interferon responses; nevertheless, MPV is capable of blocking type I interferon responses by an unknown mechanism [317, 318]. MPV and other respiratory viruses induce pulmonary CD8+ T cells that fail to secrete IFNγ or exhibit cytotoxic degranulation in response to viral peptides; these impaired CD8 T cells resemble exhausted CD8 T cells induced by chronic infections such as HIV and hepatitis C virus [319].

Humans develop neutralizing antibodies to MPV, and passive antibodies alone can protect in animal models. However, immunity wanes over time and likely provides limited cross-protection between subgroups, since reinfections occur in children and adults [246, 248] with genetically different strains [182, 239, 240, 268, 320] as well as strains from the same subgroup [280]. Early protection against reinfection following primary infection was confirmed in macaques; however, when challenged 12 weeks later, virus replication was detectable despite the presence of serum antibodies [220]. Eleven months later, antibody levels had waned still further, and all macaques challenged with heterologous virus and two of three animals reinfected with homologous virus had no evidence of protection [220]. A prospective study in humans noted that baseline MPV antibodies were lower in patients who subsequently became infected versus those who did not become infected [321]. Thus, cross-protection and duration of antibody responses are important issues for vaccine development.

2.8 Patterns of Host Response

2.8.1 Symptoms

MPV causes both upper and lower respiratory tract signs and symptoms clinically indistinguishable from disease associated with RSV and other respiratory viruses [182, 239, 300, 322, 323]. Fever is present in most cases, especially children [239–241, 243, 264, 280, 293]. Transient maculopapular rash has been described in a minority of patients [241, 243, 293], and vomiting or diarrhea are described with low frequency [182, 239, 243]. Laboratory abnormalities are uncommon, although one study identified 27 % of patients with elevated ALT and AST values [293]. White blood cell count and C-reactive protein were not significantly different between RSV and MPV [238, 282].

MPV is rarely detected in asymptomatic persons [182, 191, 324–326], though in otherwise healthy young adults, MPV infection can be subclinical [246]. The duration of shedding in healthy individuals is approximately 7–14 days [239, 327].

2.8.2 Diagnosis

Detection in cell culture requires prolonged incubation and is both insensitive and often impractical. Shell vial culture offers increased sensitivity over traditional culture [184, 328]. IFA has demonstrated a sensitivity of 73 % and specificity of 97 % with RT-PCR as the gold standard [329]. DFA has shown similar results [330]. Commercial antibody kits for immunofluorescent detection of MPV are available. RT-PCR is most commonly used for detection of the virus in epidemiologic studies and is becoming more common in clinical laboratories [176, 320, 331, 332]. Real-time RT-PCR targeting the conserved N gene has high sensitivity for detection of all four subgroups [189, 195].

2.9 Control and Prevention

2.9.1 Treatment

The primary therapy for MPV infections is supportive care, including oral or intravenous hydration, monitoring of respiratory status and oxygen saturation, supplemental oxygen, and mechanical ventilation for frank respiratory failure. There are no licensed antivirals for MPV. Reports of pharmacologic treatment of MPV are limited to severely ill or immunocompromised patients. Ribavirin, an antiviral agent used in severe RSV infection, reduced inflammation and viral replication in mice with MPV infection [333]. Commercial intravenous immunoglobulin (IVIG), ribavirin, and NMSO3 (a sulfated sialyl lipid) effectively inhibited MPV in vitro [334, 335]. Ribavirin, with and without IVIG, has been used in immunocompromised adults [336]. Bonney and colleagues [337] reported successful treatment of an immunocompromised child with MPV using IV ribavirin and IVIG. Subsequently, oral ribavirin and inhaled ribavirin with IVIG have been used [275, 338]. However, no randomized, controlled trials have been conducted, and these data should be regarded as purely anecdotal.

2.9.2 Immunoprophylaxis

Human and murine monoclonal antibodies exhibit therapeutic efficacy in rodent models and thus offer potential for immunoprophylaxis [210, 211, 213].

2.9.3 Vaccines

A number of vaccine approaches for MPV have been investigated. The F protein is conserved between subgroups, immunogenic, and the only target of neutralizing antibodies; in contrast to RSV, the MPV G protein does not induce neutralizing antibodies and is not a protective antigen [206, 209, 214]. A recombinant parainfluenza virus encoding the MPV F protein demonstrated protection against MPV [339], and soluble F protein vaccines reduced viral titers in cotton rats and hamsters [207, 208]. Reverse genetics technology has been developed for MPV and has been used to produce recombinant strains for vaccine development [197, 340]. Viruses lacking the G, M2-1, M2-2, or SH proteins or with point mutations are attenuated and immunogenic in rodent and primate models [197, 221, 341–344].

2.10 Unresolved Problems

The major unresolved problems in MPV epidemiology and research involve understanding mechanisms of disease and developing therapeutic or preventive strategies. Abundant evidence shows that MPV is a significant cause of acute respiratory disease, especially in the very young, older adults, and persons with underlying conditions. As with all mucosal viruses, the short incubation period of MPV combined with the transient nature of mucosal IgA means that reinfection is possible throughout life. However, serum IgG appears to offer protection against severe lower respiratory involvement, and thus a vaccine would likely ameliorate the most severe cases. The best candidate vaccine is not yet clear. Further, MPV has as yet unidentified mechanisms to subvert host immunity. Elucidation of these pathways might help guide vaccine development and uncover novel host targets for immunomodulation.

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Parvoviruses

27

Kevin E. Brown

1 General Introduction

Parvum is Latin for small, and *Parvoviridae* are among the smallest known DNA-containing viruses that infect mammalian cells. The virions are nonenveloped particles about 22 nm in diameter with icosahedral symmetry. The *Parvoviridae* are divided into two subfamilies, *Parvovirinae* and *Densovirinae*, on the basis of their ability to infect vertebrate or invertebrate cells, respectively. The *Parvovirinae* are currently further subdivided into eight genera on the basis of their transcription map, their ability to replicate efficiently either autonomously or with helper virus, and their sequence homology. The five genera are *Protoparvovirus* (formerly *Parvovirus*), *Dependoparvovirus*, *Erythroparvovirus, Bocaparvovirus, Amdoparvovirus, Aveparvovirus, Copiparvovirus and Tetraparvovirus* [1].

At least four different parvoviruses are known to infect humans. Parvovirus B19 (B19V) is the best characterized and is classified as the type member of the *Erythroparvovirus* genus. The other human viruses are the human adeno-associated viruses (*Dependoparvoviruses*), human bocaparvoviruses (*Bocaparvovirus),* and human Parv4, a member of the newly recognised *Tetreparvovirus* genus [2–4].

The human dependoparvoviruses are nonpathogenic and as such are being utilized as vectors for gene therapy [5]. They will not be discussed further in this chapter.

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2 Parvovirus B19 (B19V)

Parvovirus B19 (B19V) was discovered in 1975 [6] and, until the discovery of the human bocaviruses, was the only human pathogenic parvovirus. Unlike many virus infections, the clinical manifestations of infection with parvovirus B19 vary widely with the immunologic and hematologic status of the host. In individuals with underlying hemolytic disorders, B19V is the primary cause of aplastic crisis. In immunocompromised patients, persistent parvovirus B19 viremia may manifest as pure red cell aplasia and chronic anemia, and in the fetus, where the immune response is immature infection, it may lead to fetal death in utero or hydrops fetalis. The major disease manifestation in normal immunocompetent individuals is erythema infectiosum (EI), also called fifth disease or "slapped-cheek" disease. Generally, this is an innocuous rash disease of childhood, but in adults, it may also be associated with an acute symmetrical polyarthropathy, which can mimic acute rheumatoid arthritis.

2.1 Historical Background

2.1.1 The Virus

Parvovirus B19 was discovered by Yvonne Cossart and coworkers [6] in England. They were evaluating tests for hepatitis B surface antigen (HBsAg) using panels of serum samples. One serum (coded 19 in panel B) gave anomalous results, positive in counterimmune electrophoresis (CIE) with human antisera but negative in the more specific radioimmunoassay (RIA) and reverse passive hemagglutination (RPHA) tests that used hyperimmune animal sera. When the precipitin line from the CIE was excised, electron microscopy (EM) showed the presence of 23-nm particles resembling parvovirus. There was no reactivity with antisera to adeno-associated viruses or to rat parvovirus, and the virus was originally labeled "serum parvovirus-like particle" (SPLV). Approximately 30 % of adults had antibody to the new virus detectable by CIE.

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The first clinically significant illness associated with B19V infection was hypoplastic crisis in patients with sickle-cell anemia [7]. Sera from such patients contained B19V antigen, detectable by CIE or EM at the time of crisis, and convalescent sera lacked virus but showed evidence of antibody seroconversion.

In 1985, the virus was officially recognized as a member of the *Parvoviridae*, and the International Committee on Taxonomy of Viruses (ICTV) recommended the name B19V to prevent confusion with other viruses (i.e., human papillomavirus). It is classified as a member of the *Erythrovirus* genus with the name parvovirus B19 (official abbreviation, B19V) [1].

2.1.2 Transient Aplastic Crisis

Transient aplastic crisis (TAC) was the first clinical illness associated with B19V infection. The term "aplastic crisis" was coined by Owren [8] to describe the abrupt onset of severe anemia with absent reticulocytes in patients with hereditary spherocytosis. (Hemolytic crises in hereditary spherocytosis patients are associated with increased bone marrow turnover and reticulocyte production.) Also, in contrast to hemolytic crises, aplastic crisis occurred as a single episode in the patient's life. In TAC cases, there was a common history of a preceding prodromal illness and the occurrence of epidemics in large kindreds of hereditary spherocytosis suggested an infectious etiology.

When stored sera from (over 800) children admitted to a London hospital were examined for B19V antigen by CIE, a precipitin line was found in a child with sickle-cell disease suffering a hypoplastic crisis. Five other patients presenting with similar symptoms were also investigated, and all had evidence of recent infection with B19V (either antigenemia or seroconversion). All were Jamaican immigrants with sickle-cell disease presenting with aplastic crisis. There was a reduced hematocrit and deficient red cell production in their bone marrow [7]. Retrospective studies of sera from Jamaican sickle-cell patients showed that 86 % of TACs were associated with recent parvovirus infection [9].

2.1.3 Erythema Infectiosum

This exanthematous rash illness of childhood was probably first described by Robert Willan in 1799 and subsequently illustrated in his 1808 textbook [10]. The disease was rediscovered in Germany, and in 1899, Sticker gave it the name erythema infectiosum. Six years later, Cheinisse classified it as the "fifth rash disease" of the six classical exanthemata of childhood [11]. Subsequently, many outbreaks of fifth disease were documented in the medical literature but the cause remained a mystery. Often the epidemiologic data suggested "a common source exposure to a highly effective transmitter," and an atypical rubella virus or echovirus was thought to be responsible [12]. However, neither virus could be reproducibly isolated from fifth disease patients.

In 1983, following an outbreak of fifth disease in London, England, 31 of 31 children or adolescents who had been affected had anti-B19V-specific immunoglobulin M (IgM) antibody in their serum detectable by RIA [13]. Similar results were obtained in other epidemics of fifth disease worldwide, and parvovirus B19 is now known to be the etiologic agent for EI [14–16].

2.2 Methodology Involved in Epidemiologic Analysis

2.2.1 Sources of Mortality Data

Parvovirus B19 is a common infection and death due to B19V must be rare. However, life-threatening B19V infection can occur in patients with underlying hemolytic disease. In one study in Jamaica, of 308 children with homozygous sickle-cell disease followed from birth to 15 years of age, 114 (37 %) became infected with B19V and four deaths were attributable to the infection [17]. The prevalence of B19V as a cause of chronic anemia and its contribution to excess mortality in immunocompromised patients is still unknown, although deaths have been reported [18–21]. Case reports of myocarditis following parvovirus B19 infection have also been described [22–24]. Finally, it has also been suggested that B19V infection contributes to the mortality of malaria in tropical countries [25–28].

2.2.2 Sources of Morbidity Data

Parvovirus B19 infection is not a notifiable disease in any country, and official morbidity reports in the United States do not include EI or parvovirus B19 infection. Morbidity data are therefore based on studies of outbreaks of EI (with and without serological confirmation) or on case reports of confirmed infections. In England, laboratory-confirmed parvovirus B19 infections are reported to the Public Health England. The majority of B19V infections are not reported since laboratory investigation is rarely performed for fifth disease. Similarly, there are no formal reporting systems or for documenting the incidence of parvovirus B19 infections in pregnancy.

2.2.3 Serological Surveys

Serological surveys were originally performed using the relatively insensitive CIE [6, 29, 30], now superseded by more sensitive enzyme immunoassays (ELISA). Due to the inability to grow B19V in standard cell culture systems, early serology assays were based on the use of synthetic peptides [29] or fusion proteins in *Escherichia coli* [31] as antigen. However, the epitopes presented by these products do not accurately reproduce the epitopes of the native capsids, and the sensitivity and specificity were disappointing. The expression of B19V capsid proteins as virion-like particles

using transfected B19V genome into CHO cells [32], COS-7 cells [33], or the use of yeast [34] or baculovirus [35, 36] expression systems appears to have overcome these problems, with results based on these antigens showing good correlation with assays based on native virus. The antigens are not only relatively easy to mass produce; they are also noninfectious and therefore without hazard to laboratory workers. They are widely used in seroprevalence studies.

2.2.4 Laboratory Methods

Virus Isolation and Detection

In the absence of suitable methods for isolating virus from clinical specimens, documenting the presence of virus relies on the detection of viral DNA by molecular biology techniques. B19V DNA can be detected in serum at the time of transient aplastic crisis by hybridization or more commonly by quantitative polymerase chain reaction (PCR). Due to the exquisite sensitivity of PCR, B19V DNA can then remain detectable for months or even years at low levels even following complete recovery, and thus quantitative PCR is required to distinguish recent infection from previous infection with the virus.

In situ hybridization can be used to identify B19V DNA within specific cells. Assays for B19V antigen based on monoclonal antibodies are relatively insensitive ($<10^6$ virus particles/ml). Electron microscopy (EM) can be used to detect B19V in serum with the same sensitivity as antigen assays. Within cells, EM cannot always distinguish intracellular virus from ribosomes.

Serological Assays for B19V-Specific Seroprevalence

ELISA-based assays using virus-like particles are both sensitive and specific. Although parvovirus B19V IgG can be detected by both capture assay and indirect assay, an indirect format is generally used for seroprevalence studies. Antibody to virus is usually present by the seventh day of illness (aplastic crisis) or day after the onset of rash, and probably is lifelong thereafter, although some waning of antibody has been suggested [37].

2.3 Biological Characteristics of B19V

2.3.1 Physical Properties

Parvovirus B19 has the typical features of a member of the *Parvoviridae*. On EM, the particles are nonenveloped, 15–28 nm in diameter, and show icosahedral symmetry. Often both "empty" and "full" capsids are visible. Mature infectious parvovirus particles have a molecular weight of 5.6×10^6, a buoyant density in cesium chloride gradients of 1.41 g/ml, and a sedimentation coefficient of 110S.

The DNA in infectious particles made up 19–37 % of the total mass of the capsid. The genome size is extremely limited, consisting of a single strand of DNA of 5,596 nucleotides. The genome has two large open reading frames (ORF) with the left ORF encoding the nonstructural protein and the right ORF encoding the two capsid proteins, VP1 and VP2, by alternative splicing. Parvovirus B19 particles do not appear to contain lipids or carbohydrates. The virion itself has phospholipase activity [38, 39], which is probably critical for viral entry into cells.

As a consequence of their lack of an envelope and limited DNA content, parvoviruses are extremely stable to physical inactivation. Depending on the environment, heat treatment at 60 °C does not inactivate the virus [40, 41], and in clinical studies heat-treated clotting factors (56 °C for 60 min or even, 80 °C for 72 h) did not prevent transmission [42]. B19V is resistant to lipid solvent treatment (ether, chloroform) but can be inactivated by formalin, β-propiolactone, and oxidizing agents. Gamma irradiation will also inactivate B19V, with 1.4 mR producing a 10 $\log_{10}$ reduction in infectivity [43].

2.3.2 Morphology

The parvovirus B19 virion is an icosahedron consisting of 60 copies of the capsid proteins. Most of the capsid protein is VP2, with 5 % or less of the larger VP1 protein [32]. VP2 capsid proteins self-assemble in the absence of B19V DNA, and in these systems, protein expression leads to formation of recombinant virus-like particles. VP1 is not required for capsid formation [35, 44].

The atomic structure of both infectious B19V and B19V VP2 empty capsids has now been resolved to <0.75 nm [45–47]. The virion surface has a major depression encompassing the fivefold axis, similar to the canyon structure found in RNA-containing icosahedral viruses. In B19V capsids, there is also a hollow cylindrical structure about the fivefold axes that appears to penetrate to the inside of the virion. The structural distribution of VP1 in the B19V capsid structure is still unknown. VP1 capsomers alone will not self-assemble to form icosahedral capsids; rather, they form smaller and irregular structures. In native virions, the VP1 unique region appears to be on the outside of the capsid, and it has been proposed that it may extend through the fivefold axis cylinder to the outside of the virion [48].

2.3.3 B19V Strain Variation

Parvovirus B19 is now recognized to have three different genotypes, with ~10 % variability at the DNA level between genotypes [49–51]. Most of the B19V identified is genotype 1 [49], the original B19V genotype, which is distributed worldwide. Genotype 3 seems to be the predominant B19V genotype in Ghana, representing more than 90 % of the sequences identified [52]. Genotype 2 has been primarily identified in tissues of older patients (born before 1973), suggesting that it may have circulated more frequently prior to the 1970s [53]. However, blood samples or donations

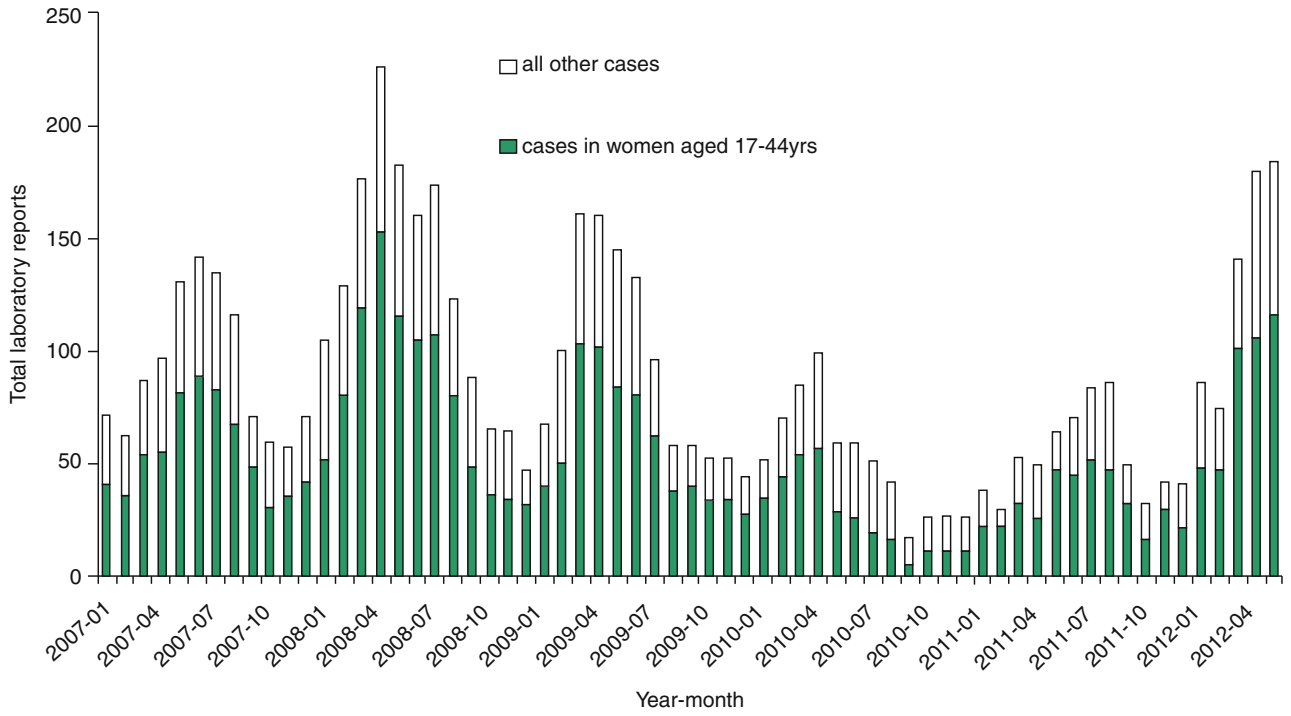

Fig. 27.1 Laboratory-confirmed cases of parvovirus B19 infection 2007–2012 in England and Wales, showing the seasonal variability (© Crown copyright. Reproduced with permission of Public Health England [83])

containing high titer genotype 2 are occasionally identified [54, 55]. The observation of both genotype 2 and 3 sequences in blood and tissues from many different parts of the world [56–58] indicates a more widespread distribution than originally assumed.

Despite the differences in the DNA sequences, the capsid protein sequence is conserved between the different genotypes, and there is evidence for both serological and cross-neutralization [59]. There is no evidence that the different genotypes show any differences in virological or disease characteristics [60, 61].

2.4 Descriptive Epidemiology

2.4.1 Prevalence and Incidence

Parvovirus B19 is a common infection in humans, and although there is some variation in different countries [62], by age 15, approximately 50 % of children have detectable IgG [63]. Infection also occurs in adult life, and more than 80 % of the elderly have detectable antibody [64]. Significantly higher seroprevalence is found in some parts of Africa [65] and Papua New Guinea [66], with >80 % of 10-year-olds having detectable antibody. In contrast, some parts of Asia [67–69] and some isolated communities in other parts of the world have a much lower seroprevalence [70, 71].

The prevalence of parvovirus B19 DNA in blood samples depends on the sensitivity of the assay used. When a relatively insensitive assay that would detect high titer viremia (indicating acute infection) was used between 1:20,000 and 1:40,000 units of blood during epidemic seasons were positive [72]. With more sensitive PCR assays then ~1 % of all donations have low-level parvovirus B19 DNA detectable [73].

Due to the risk of adverse outcome in pregnancy, a number of studies have attempted to calculate the incidence of infection in women of childbearing age [74–77]. This varies between countries but is estimated for women with no known exposure to be between 0.6 % [63] and 2.4 % [78], with most estimates around 1 % [63, 79, 80].

2.4.2 Epidemic Behavior and Contagiousness

Infections in temperate climates are more common in late winter, spring, and early summer months [81–83]. Rates of infection may also increase every 3–5 years (Fig. 27.1), and this is reflected by corresponding increases in the major clinical manifestations of B19V infection, TACs, and EI [84].

The virus can be readily transmitted by close contact. In studies in Europe, the highest force of infection (FOI) is in children aged 7–9, with an overall R_0 (basic reproduction number) of between 1.5 and 3 [85]. The secondary attack (seroconversion) rate has been calculated in various settings. In one study the secondary attack rate from symptomatic TAC or EI patients to susceptible (IgG negative) household contacts was approximately 50 % [84]. In school outbreaks, serological studies are generally not available, but 10–60 % of students may develop a rash disease consistent with B19V

infection [75–77]. The highest secondary attack rates and also seroprevalence and annual seroconversion rates even in the absence of known outbreaks of infection are observed among workers such as day-care providers and school personnel who have close contact with affected children [64, 86]. In one modeling study using seroprevalence data from five European countries, the highest risks of transmission were within households and schools, and where there was close physical contact on a daily basis and for at least 1 h [85].

Although nosocomial transmission in hospital situations has been described [87–89], especially in patients with persistent disease, this is unusual, and health-care workers do not have a significantly higher seroprevalence than age-matched controls [64]. However, patients with TAC or persistent infection should be considered infectious and appropriate precautions taken to limit transmission.

2.5 Mechanism and Routes of Transmission

Parvovirus B19 DNA has been found in the respiratory secretions of patients at the time of viremia [16, 84] suggesting that B19V infection is generally spread by a respiratory route of transmission. In contrast to other respiratory viruses, however, no site of replication for B19V has been found in the nasopharynx. There is little evidence of virus excretion in feces or urine [90].

The virus can be found in serum, and infection also can be transmitted by blood and blood products. Although ~1 % of blood donations have low-level B19V DNA detectable [73], reports of transmission of B19V infection by individual units of blood or platelets are rare. In a linked donor-recipient study, transmission of infection only occurred with components that contained B19V DNA at concentrations greater than 10^6 IU/mL (estimated to occur in 1:6,000 donations) [91].

Without prior screening for parvovirus B19, pooled products could contain high concentrations of parvovirus B19. Parvoviruses, including B19V, are very heat resistant and at high virus concentration can withstand the usual heat treatment (80 °C for 72 h) used to destroy infectivity. In addition, solvent–detergent methods, which only inactivate lipid-enveloped viruses, are ineffective. Parvovirus B19 infection has been transmitted by steam- or dry-heated factor VIII or IX [42, 92] and by solvent–detergent-treated factor VIII [92]. Hemophiliacs who received heat-treated factor VIII alone had lower prevalence of B19V antibody and lower rates of seroconversion compared to those receiving nonheat-treated factor [93].

Recommendations in Europe and America now require all plasma pools for fractionation to be screened for high levels of parvovirus DNA to try to minimize the transmission of parvovirus B19 by blood products.

2.6 Pathogenesis and Immunity

2.6.1 Pathogenesis

Parvovirus B19, like all autonomous parvoviruses, is dependent on mitotically active cells for its own replication. Parvovirus B19 also has a very narrow target cell range and can only be efficiently propagated in human erythroid progenitor cells. For erythroid progenitors from bone marrow, susceptibility to parvovirus B19 increases with differentiation; the pluripotent stem cell appears to be spared and the main target cells are CD36 positive erythroid colony-forming cells (CFU-E) and erythroblasts [94, 95]. In erythroid progenitors, the virus is cytotoxic, producing a cytopathic effect with characteristic light [96] and electron microscopic [97, 98] changes. Infected cultures are characterized by the presence of giant pronormoblasts or "lantern cells," which are early erythroid cells, 25–32 μm in diameter, with cytoplasmic vacuolization, immature chromatin, and large eosinophilic nuclear inclusion bodies. On EM, virus particles are seen in the nucleus and lining cytoplasmic membranes of infected erythroid progenitors, and infected cells show marginated chromatin, pseudopod formation, and cytoplasmic vacuolation, typical of cells undergoing apoptosis [99]. The light microscopic findings are also seen in the bone marrow of infected patients [100].

The basis of the erythroid specificity of parvovirus B19 may be explained by the tissue distribution of the virus cellular receptor, globoside, also known as blood group P antigen [101]. P antigen is found on erythroblasts and megakaryocytes. (It is also present on endothelial cells, which may be targets of viral infection involved in the pathogenesis of transplacental transmission, possibly vasculitis and the rash of fifth disease, and on fetal myocardial cells [102].) Rare individuals who do not have P antigen on their cells are resistant to B19V infection, and their bone marrow cannot be infected with B19V in vitro [103].However, erythroid specificity may also be modulated by specific erythroid transcription factors [104], and other cofactors including a hypoxic environment [105, 106].

Studies in normal volunteers have shown that B19V infection leads to an acute but self-limited (4–8 days) cessation of red cell production and a corresponding decline in hemoglobin level [90, 107]. In patients with normal erythroid turnover, this short interruption of red cell production does not lead to anemia; but in patients with high red cell turnover, due to hemolysis, blood loss, and so forth, the interruption can precipitate an "aplastic crisis." The crisis resolves as virus is "cleared" from the bone marrow by the immune response. In patients who are immunocompromised, the infection may persist and produce chronic pure red cell aplasia.

The infected fetus may suffer severe effects because red blood cell turnover is high and the immune response deficient. During the second trimester, there is a great increase in red cell

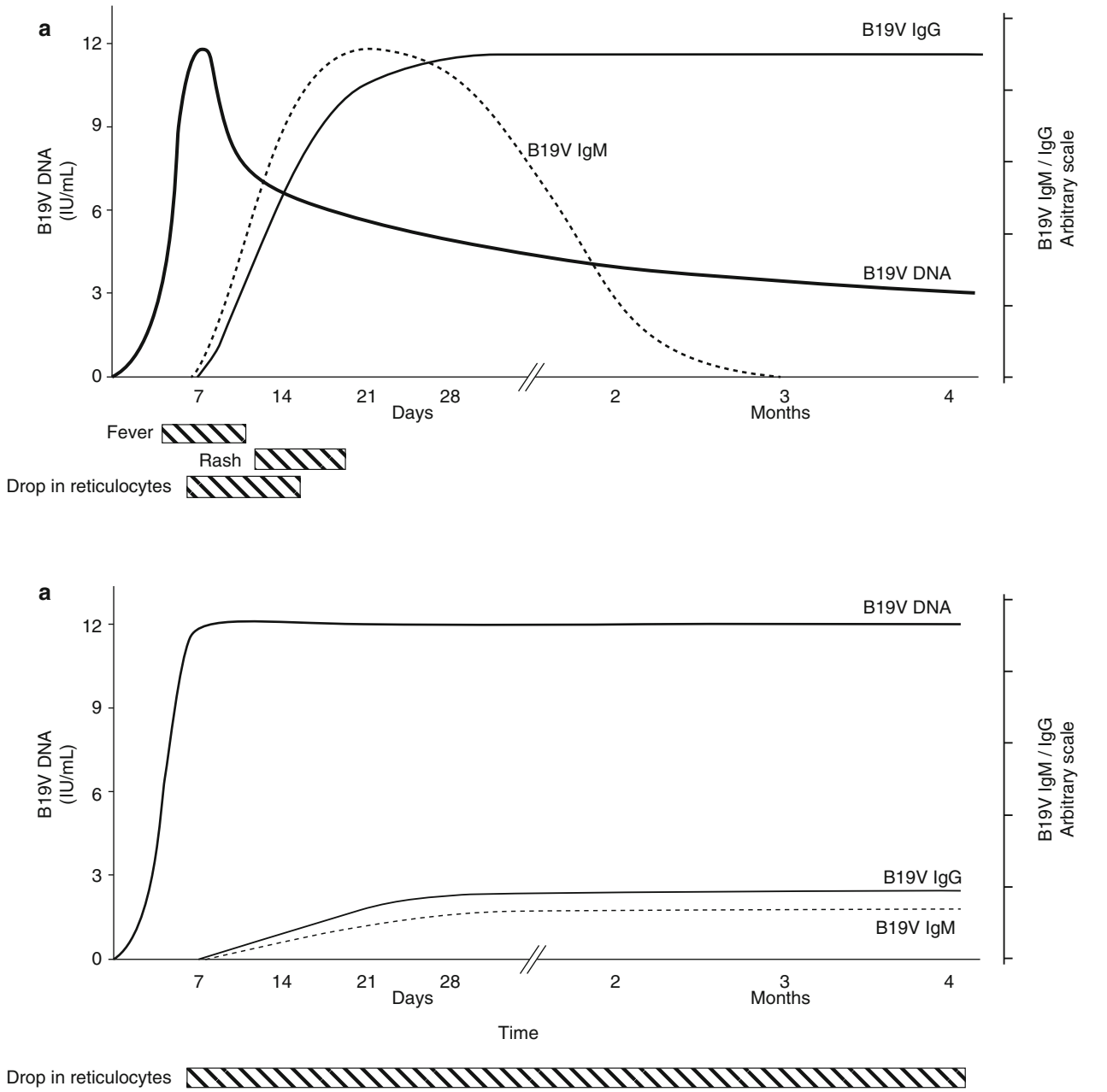

Fig. 27.2 Schematic diagram of the clinical and virological events following B19 parvovirus infection. (**a**) Virology and immune response in immunocompetent individual. (**b**) Virology and immune response in immunosuppressed patient prior to treatment with intravenous immunoglobulin (Data from Anderson et al. [90], Potter et al. [107] and the case presented by Kurtman et al. [18])

mass. Parvovirus particles can be detected by EM within the hematopoietic tissues of liver and thymus [108], and B19V DNA and capsid antigen have been detected in the myocardium of infected fetuses [109]. In addition, there is evidence that the fetus may develop myocarditis [110, 111], compounding the severe anemia and secondary cardiac failure. By the third trimester, a more effective fetal immune response to the virus may account for the decrease in fetal loss.

The pathogenesis of the rash in EI and polyarthropathy is almost certainly immune complex mediated. In volunteer studies, the rash and joint symptoms appeared when viremia was no longer detectable and at the time of development of a detectable immune response [90]. Similar findings have been reported in chronically infected individuals treated with immunoglobulin therapy [112].

2.6.2 Immune Response to B19V Infection

Both virus-specific IgM and IgG antibodies are made following experimental [90] and natural [113] B19V infection (Fig. 27.2). Following intranasal inoculation of volunteers,

virus can first be detected at days 5–6 and levels peak at days 8–9. IgM antibody to virus appears about 10–12 days after experimental inoculation; IgG antibody appears in normal volunteers about 2 weeks after inoculation.

There is a similar time course in natural infections. In patients with TAC, 10^8–10^{14} genome copies/ml of virus DNA may circulate [114, 115]. IgM antibody may be present in patients with TAC at the time of reticulocyte nadir and during the subsequent 10 days; IgG may not be present at the time of reticulocyte depression but appears rapidly with recovery. High levels of B19V DNA are not detectable in patients with clinical fifth disease (the manifestations are secondary to immune complex formation), although B19V DNA can still be detected by PCR [116].

IgM antibody may be found in serum samples for several months after exposure [117]. IgG persists for life and levels rise with reexposure [90]. Transient aplastic crisis due to parvovirus B19 infection does not occur more than once in the life of a sickle-cell patient [17]. Measurable IgA antibodies specific to B19V may play a role in protection against infection by the nasopharyngeal route [118].

In immunocompetent individuals, the early antibody response is to the major capsid protein VP2, but as the immune response matures, reactivity to the minor capsid protein, VP1, dominates [119, 120]. Sera from patients with persistent B19V infection typically have antibody to VP2 but not to VP1 [121]. Recovery from infection correlates with the appearance of circulating specific antivirus antibody.

The importance of the anti-VP1 response in developing neutralizing antibody has been confirmed in animal experiments using recombinant capsid. Rabbits immunized with capsids containing only VP2 produced a strong antibody response, but the sera had low neutralization titers. In contrast, rabbits immunized with capsids containing VP1 produced antibody with neutralizing titers comparable to those produced in humans following acute B19V infection [122, 123].

Persistent B19V infection is the result of failure to produce effective neutralizing antibodies by the immunosuppressed host. Perhaps because of the limited number of epitopes presented to the immune system by parvovirus B19, the congenital immunodeficiency states associated with persistent infection may be clinically subtle, with susceptibility largely restricted to parvovirus, although multiple immune system defects are apparent once directed testing of T- and B-cell function is performed. Administration of commercial immunoglobulins can cure or ameliorate persistent parvovirus infection in immunodeficient patients [112].

The role of the cellular immune response in limiting parvovirus B19 infection has been studied less intensively. Using a combination of recombinant capsids and peptides from the nonstructural and capsid proteins, it is now clear that B19V infection induces both a profound CD8 [124, 125] and CD4 [126, 127] response, both of which are required for viral control. Perhaps surprisingly, prolonged activation of the CD8 response is seen even after apparent acute infection and resolution of symptoms [124], presumably in keeping with the low levels of B19V DNA that are detectable in serum and tissues for months or even years following infection [128, 129].

2.7 Patterns of Host Response

2.7.1 Asymptomatic Infection

Most people with B19V specific antibody have no recollection of any specific symptoms. In one epidemiologic study of a school outbreak, B19V caused asymptomatic infection in approximately 25 % of adults [77]. In household contacts of patients with aplastic crises or EI due to B19V, 32 % (17/52) reported no symptoms [84]. There were more asymptomatic infections in those with darker skin (69 %) compared to whites (17 %), but the numbers studied were small and the clinical presentation of the index infection was different in the two groups (TAC for the black contacts and EI for the white contacts). Parvovirus B19 may go unrecognized in those with darker skin color who do not have an underlying hemolytic anemia, since the rash is particularly difficult to see in these circumstances.

2.7.2 Erythema Infectiosum

As indicated earlier, EI, otherwise known as fifth disease or slapped-cheek disease, is the major manifestation of B19V infection and was well characterized clinically before the discovery of B19V [130, 131]. The nonspecific prodromal illness often goes unrecognized and may be associated with symptoms of fever, coryza, headache, and mild gastrointestinal symptoms, such as nausea and diarrhea. Two to five days later, the classic slapped-cheek rash appears a fiery red eruption on the cheek, accompanied by relative circumoral pallor. One to four days after the slapped-cheek rash, the second-stage rash may appear—an erythematous maculopapular exanthem on the trunk and limbs. As this eruption fades, it takes on a typical lacy appearance. There may be great variation in the dermatological appearance, and rarely it may present as papulo-purpuric glove and sock syndrome (PPGSS) [132–136]. The classic slapped-cheek rash is more common in children than adults, and the second-stage eruption may vary from a very faint erythema that is easily missed to a florid exanthema and may be transient or recurrent over 1–3 weeks. The rash may be accompanied by pruritus, which can be the dominant symptom and may be especially prominent on the soles of the feet [77, 137].

2.7.3 Polyarthropathy Syndrome

In children, B19V infection is usually mild and of short duration. However, in adults and especially in women, there may be arthropathy in approximately 50 % of patients [77]. The

joints can be painful, often with accompanying swelling and stiffness. The distribution is usually symmetrical; mainly the small joints of hands and feet are involved. Joint symptoms last 1–3 weeks, although in 20 % of affected women, arthralgia or frank arthritis may persist or recur for more than 2 months, even to 2 years. In the absence of a history of rash, the symptoms may be mistaken for acute rheumatoid arthritis, especially as B19V infection can be associated with transient rheumatoid factor production [138–140]. In one study of patients attending an "early synovitis" clinic in England, 19 of 153 (12 %) had evidence of recent infection with B19V [140]. Parvovirus B19 infection should be considered as part of the differential diagnosis in any patient presenting with acute arthritis.

It has been postulated that B19V is involved in the initiation and perpetuation of rheumatoid arthritis leading to joint lesions [141], but these results have not been reproducible by other groups. In contrast, parvoviral B19V DNA is frequently found in synovial tissue of patients with rheumatoid arthritis, chronic arthropathy, and control subjects. In one carefully performed controlled study, although B19V DNA was detected in synovial tissue of 28 % of individuals with chronic arthritis, it was also found in 48 % of nonarthropathy controls [142], indicating that PCR-detectable DNA may persist in synovial tissues for months or years. In addition, in one study with long-term follow-up, none of the 54 patients with B19V-associated arthralgia reported persistence of joint swelling or restricted motion, and no evidence of inflammatory joint disease was found [143]. Therefore, it seems unlikely that B19V plays a role in classic erosive rheumatoid arthritis. The association of B19V and juvenile rheumatic disease is more convincing [144], but whether it is the cause of the disease or one of many potential triggers is less clear.

2.7.4 Aplastic Crisis

Transient aplastic crisis is the abrupt cessation of erythropoiesis characterized by reticulocytopenia, absent erythroid precursors in the bone marrow, and precipitous worsening of anemia. TAC was the first clinical illness associated with B19V infection [7]. TAC due to B19V has been described in a wide range of patients with underlying hemolytic disorders, such as hereditary spherocytosis, thalassemia, and red cell enzymopathies such as pyruvate kinase deficiency and autoimmune hemolytic anemia [145]. TAC can also occur under conditions of erythroid "stress," such as hemorrhage, iron deficiency anemia, and following kidney, bone marrow, or liver transplantation [146–149]. Acute anemia has been described in hematologically normal persons [150], and a drop in red cell count (and reticulocytes) was seen in healthy volunteers [90].

Although suffering from an ultimately self-limiting disease, patients with aplastic crisis can be severely ill. Symptoms may include dyspnea, lassitude, and even confusion due to the worsening anemia. Congestive heart failure and severe bone marrow necrosis may develop [151, 152] and the illness can be fatal [17]. Aplastic crisis can be the first presentation of an underlying hemolytic disease in a well-compensated patient [153].

TAC and B19V infection in hematologically normal patients are often associated with changes in the other blood lineages. There may be varying degrees of neutropenia, thrombocytopenia, and transient pancytopenia [145]. Some cases of idiopathic thrombocytopenia purpura [154] and Henoch–Schönlein purpura [155, 156] have been linked to parvovirus B19 infection. Less often, agranulocytosis has been described following B19V infection [157, 158].

Community-acquired aplastic crisis is almost always due to parvovirus B19 [159] and should be the presumptive diagnosis in any patient with anemia due to abrupt cessation of erythropoiesis as documented by reduced reticulocytes and bone marrow appearance. In contrast to patients with EI, TAC patients are often viremic at the time of presentation, with concentrations of virus as high as 10^{14} genome copies/ml, and the diagnosis is readily made by detection of B19V DNA in the serum. As B19V DNA levels fall in serum, B19V-specific IgM becomes detectable.

TAC is readily treated by blood transfusion. It is a unique event in the patient's life, and following the acute infection immunity is lifelong.

2.7.5 Infection During Pregnancy and Congenital Infection

B19V infection in pregnancy may be associated with miscarriage or nonimmune hydrops fetalis [160]. Although nonimmune hydrops fetalis (NIHF) is rare (1 per 3,000 births) and in approximately 18 % of cases the etiology is unknown, 7 % can be ascribed to an infectious cause, generally parvovirus B19 [161]. In one study of 63 fetal deaths due to NIHF, 8 were due to parvovirus B19 infection [162]. In cases where pathological studies were undertaken, the fetuses showed evidence of leukoerythroblastic reaction in the liver and large, pale cells with eosinophilic inclusion bodies and peripheral condensation or margination of the nuclear chromatin. Parvovirus B19 DNA could be detected by DNA hybridization [163] and in situ hybridization [164], and parvovirus particles could be seen by electron microscopy [108].

Even in the absence of treatment, an adverse fetal outcome is not typical after maternal B19V infection. In a prospective British study of more than 400 women with serologically confirmed B19V during pregnancy, the excess rate of fetal loss was confined to the first 20 weeks of pregnancy and averaged only 9 % [165]. No abnormalities were found at birth in the surviving infants, even when there was evidence of intrauterine infection by the presence of B19V IgM in the umbilical cord blood, and there were no long-term sequelae attributable to B19 infection in the 129

children observed for more than 7 years. The findings in studies in other countries were similar [166–170].

Many cases of parvovirus B19-induced hydrops fetalis are now treated with intrauterine blood transfusion. In one study of follow-up of 20 children, no long-term sequelae were observed [171]. However, in a second study of 24 transfused infants, 5/16 infants that were followed had delayed psychomotor development [172]. Neither study included controls, and it is not clear whether the developmental abnormalities were a direct effect of the virus or due to the treatment intervention. Even in the absence of intrauterine transfusion rare case reports of congenital ocular and neurological abnormalities after maternal B19V infection have been reported.

Rare cases of congenital anemia after a history of maternal B19V exposure have been reported [173]. In these cases, the virus load is generally low and the anemia does not respond to immunoglobulin therapy. The B19V infection may mimic Diamond–Blackfan anemia [174, 175], and the role of in utero B19V infection in inducing constitutional bone marrow failure such as that in Diamond–Blackfan anemia is still not clear.

2.7.6 Chronic Bone Marrow Failure

Persistent B19 infection resulting in pure red cell aplasia has been reported in patients with a wide variety of immunosuppressive conditions, ranging from congenital immunodeficiency, acquired immunodeficiency syndrome (AIDS), and lymphoproliferative disorders to transplantation [176]. The stereotypical presentation is with persistent anemia rather than the immune-mediated symptoms of rash or arthropathy. The patients have absent or low levels of B19V-specific antibody and persistent or recurrent parvoviremia as detected by high levels (>10^9 IU/mL) of B19V DNA in the serum. Bone marrow examination often shows the presence of scattered giant pronormoblasts. Often there is a pure red cell aplasia, but other lineages may also be affected.

The prevalence of B19-induced anemia in human immunodeficiency virus (HIV)-seropositive patients is probably higher than recognized. In one early study of 50 patients with AIDS, no patients with B19V viremia were identified. In a larger cohort study, B19V DNA was found in only 1 of 191 (0.5 %) HIV-seropositive men who have sex with men. However, B19V DNA was found in 5 of 30 (17 %) of the transfusion-dependent HIV seropositives, and when a hematocrit of less than 20 was used as a criterion, 4 of 13 (31 %) had detectable B19V DNA [177]. In contrast to the earlier studies, the marrow morphology need not be suggestive of pure red cell aplasia, and giant pronormoblasts may not be present.

Administration of immunoglobulin can be beneficial and ameliorative even if not curative [178]. Temporary cessation of maintenance chemotherapy also led to resolution of the anemia, and in two cases, reinstitution did not lead to recurrence [179, 180] suggesting that decreasing the level of immunosuppression may allow the host to produce antibody and resolve the virus infection.

Virus-associated hemophagocytic syndrome (VAHS) is characterized by histiocytic hyperplasia, marked hemophagocytosis, and cytopenia in association with a systemic viral illness [181]. In contrast to malignant histiocytosis, VAHS is usually a benign, self-limiting illness in which histiocytic proliferation is reversible. Hemophagocytosis is not uncommon and occurs in the setting of a wide range of infections, not only viral but also bacterial, rickettsial, fungal, and parasitic [182]. However, in many patients, there is underlying immunosuppression, usually iatrogenic, so that the role of the incriminated pathogen as an etiologic agent or coincidental opportunistic infection remains unclear.

Parvovirus B19 infection has been detected in 15 cases of hemophagocytosis syndrome among children and adults [183]. The majority of patients were previously healthy, but four patients were immunosuppressed by drug therapies. In all but one case, there was a favorable outcome (one immunosuppressed patient died of fulminant aspergillosis). Further studies are required to determine whether parvovirus B19 is a major cause of VAHS as well as the rate of VAHS in otherwise uncomplicated parvovirus B19 infection.

2.7.7 Vasculitis

The role of parvovirus B19 in vasculitis remains unclear. Several case reports have described positive B19V serology in patients with vasculitis and/or polyarteritis nodosum [184–187]; however, in each individual report, it was uncertain as to whether the association was coincidental or causative. More recently, parvovirus B19 infection has been associated with acute systemic necrotizing vasculitis [188]. Recent infection with parvovirus B19 was indicated in three patients by the presence of both B19V IgM in the serum and B19V DNA in serum and tissues. Treatment with intravenous immunoglobulin led to clearing of the virus and resolution of the patients' symptoms.

A similar report linked parvovirus B19 to Kawasaki disease, a multisystem vasculitis of early childhood. In a study from Italy, parvoviral B19 DNA and/or IgM antibodies were found in 10 of 15 patients with Kawasaki disease compared to 0 of 36 control children [189, 190]. The authors do not report on treatment of their cases, but immunoglobulin therapy is known to be beneficial in Kawasaki disease. However, other studies have not shown a relationship between Kawasaki disease and parvoviral infection [189, 191, 192].

2.7.8 Myocarditis

There have been several case reports of myocarditis associated with B19V infection in both children and adults [193, 194]. Many of the case reports attributed the syndrome to B19V as the cause based simply on the detection of B19V DNA genome; but given the known persistence of B19V

DNA in tissues, this may be erroneous. The putative role of B19V in the pathogenesis of myocarditis nevertheless warrants further investigation, particularly because P antigen is found on fetal myocardial cells and B19V appears to cause myocarditis in the fetus [110, 111].

Less convincing is the evidence for parvovirus B19 as a cause of cardiomyopathy [195]. Many parvovirus-associated cases are based simply on the detection of B19V DNA in cardiac tissue. However, when control studies have been performed, B19V DNA is also found in control cardiac tissue [196–198].

2.8 Diagnosis of B19V Infections

The detection of B19V viremia is based on quantitative PCR. High titer parvovirus B19 DNA (>10^9 IU/ml) can be detected in serum at the time of TAC (Fig. 27.2). In immunocompetent individuals, high titer B19V DNA is only detectable for 2–4 days and then drops to between 10^4 and 10^6 IU/mL as the immune response develops.

Within 1 day of onset of the rash of EI, parvovirus B19 IgM and IgG are detectable by ELISA in serum samples, and diagnosis of EI is therefore based on IgM assays, ideally performed by the capture technique [199, 200]. (Indirect assays to detect B19V IgM often give false positives due to cross-reacting antibodies or rheumatoid factor.) IgM antibody remains detectable for 2–3 months following infection.

Parvovirus B19 IgG can also be detected by ELISA. With current IgG assays, IgG is also detectable within 1 day of onset of rash and is probably present for life thereafter. As more than 50 % of the population have IgG antibody to B19V infection, detection of B19V IgG is not helpful for the diagnosis of acute infection.

Immunocompromised or immunodeficient patients with chronic infection may not mount an immune response to B19V, and therefore, quantitative PCR is required for diagnosis. As with TAC, patients generally have high titer B19V DNA (>10^9 IU/mL) detected.

The sensitivity level of detection of B19V has greatly increased by the use of PCR, but at the risk of possible contamination and false-positive results confusing interpretation. Even in immunocompetent persons whose recovery from acute infection is uncomplicated, a sensitive assay can probably detect B19V DNA for the rest of their lives [53, 129]. Thus, simple detection of B19V DNA in serum or tissues does not indicate active infection.

2.9 Control and Prevention

The humoral immune response plays a dominant role in the normal immune response to parvovirus, and antibodies are protective in both passive and active immunizations. Human convalescent-phase antisera [121] and commercial immunoglobulin preparations [201, 202] contain neutralizing antibodies to parvovirus, as assessed in vitro using erythroid colony systems or tissue culture. In addition, commercial immunoglobulin from normal donors can cure or ameliorate persistent B19V infection in immunosuppressed human patients [18, 112].

Prospects for a B19V vaccine should be good, as baculovirus-produced B19V capsids induce neutralizing antibodies in experimental animals [203], even without adjuvant. The presence of VP1 protein in the capsid immunogen appears critical for the production of antibodies that neutralize virus activity in vitro, and capsids with supranormal VP1 content are even more efficient in inducing neutralizing activity in immunized animals [123]. Candidate vaccines have been produced, but the most recent study was halted due to unexplained cutaneous reactions in 3 of the 43 patients enrolled [204]. All patients had produced neutralizing antibodies to parvovirus B19. Whether the cutaneous reactions were due to the presence of insect cell proteins in the preparation of the phospholipase activity of the VP1 was not resolved. Further efforts to develop a safe and effective parvovirus B19 vaccine are currently on hold, but given the possible severe consequences of parvovirus B19 infection, production of such a vaccine continues to be an important goal.

2.10 Unresolved Problems

2.10.1 Full Spectrum of Disease

Although B19V is associated with a wide variety of diseases, almost certainly the list is not complete. In addition, the discovery of P antigen as the receptor for B19V suggests a possible role for the virus in diseases previously unsuspected as related to parvoviral infection. P antigen is found on thyroid tissue [102], and B19V has been found in patients with Hashimoto's disease and thyroid malignancy [205–207]. Whether it triggers disease or is a bystander remains unclear [208].

Neurological disease has also been associated with parvovirus infection. Pruritus is not uncommon in fifth disease, and in one study, 50 % of patients with serologically confirmed fifth disease experienced neurological symptoms, especially neurasthenia in fingers or toes [209]. One patient developed more significant disease with progressive weakness of one arm. Brachial plexus neuropathy has been described in other patients with B19V infection [210–212].

In those illnesses where B19V is known to be involved, the full spectrum of disease is still uncertain. Parvovirus B19 is recognized as the cause of acute polyarthropathy, but its role in chronic arthropathy and as a possible trigger for rheumatoid arthritis is still undetermined. Similarly, the role for B19V as a cause of autoimmune disease, kidney disease, vasculitis, and in utero B19V infection inducing constitutional

bone marrow failure such as Diamond–Blackfan anemia is still under investigation.

2.10.2 B19V Vaccine Policy

Apart from the problems in developing a vaccine without the cutaneous side effects, the target populations for such a vaccine also remain to be determined. Should only patients at high risk of severe or life-threatening disease, such as sickle-cell patients, be protected? Or, in view of the wide variety of disease manifestations affecting all strata of the population, should a universal vaccine policy be pursued? A universal vaccination policy would have the added advantage of possible eradication of B19V from a community but would have the disadvantages of high cost–benefit ratio.

3 Human Bocaviruses (HBoV)

3.1 Historical Background

Human bocavirus was discovered in 2005 as part of a virus discovery program to identify the causes of lower respiratory tract infections in children [213]. Human bocavirus sequence was first identified in two large pools of nasopharyngeal aspirates submitted for diagnosis of respiratory tract infections in young Swedish children. After sequence-independent specific primed amplification (SISPA) two libraries of DNA were obtained, and 62 of the >800 clones analyzed contained bocavirus sequences. A specific human bocavirus PCR was developed, and testing confirmed the presence of the novel bocavirus in 17 of 540 (3.3 %) of clinical samples. This respiratory human bocavirus is now classified as human bocavirus 1 (HBoV1). Subsequently related viruses, human bocaviruses 2–4, have been detected in fecal samples [214–216].

3.2 Methods Involved in Epidemiological Analysis

3.2.1 Sources of Mortality Data

There have been no reported cases of fatal infection due to any of the human bocaviruses to date, although there is a single case of fatal type 7 adenovirus reported in a patient who also had bocavirus detected on a throat swab [217]. However, no viral titer or serology results were provided, and the significance of the detection of the bocavirus is unknown. There has also been an additional case of life-threatening infection with bocavirus in a 4-year-old, with no other pathogen identified [218].

3.2.2 Sources of Morbidity Data

Most studies of human bocavirus infection are performed based on the detection of viral DNA in respiratory (HBoV1) or fecal samples (HBoV2–4). However, the bocavirus DNA is also commonly found in asymptomatic individuals, raising concerns that they may simply be "passenger" viruses, acquired early in life and not necessarily pathogenic [219–223].

3.2.3 Serological Surveys

As with Parv4, several groups investigating human bocavirus have expressed viral capsids in insect cells [224–229] and developed serological assays to detect both IgM and IgG. However, most assays probably measure cross-reacting antibodies to any of the four different human bocaviruses, and few studies have tried to distinguish between the antibody responses [230, 231].

3.2.4 Laboratory Methods

Although HBov1 can be grown in vitro in well-differentiated airway epithelial cells [232], replication is inefficient, and for most cases, viral DNA is detected by PCR. HBoV1 DNA is commonly found in between 2 and 33 % of hospitalized children with respiratory symptoms (see Jartti et al. [233] for a review of published work), making it the fourth most common infection identified. However, in many of the cases (13–19 %) [233], the virus is found with other pathogens, raising questions as to whether it is the main cause of symptoms. Patients with active or symptomatic bocavirus infections have higher concentrations of virus; therefore, in order to identify primary infection, it is important to perform quantitative PCR to determine the viral loads in respiratory secretions and in serum or plasma samples, in combination with detection of bocavirus-specific IgM or IgG seroconversion.

3.3 Biological Characteristics of Human Bocaviruses

HBoV1 has the typical structure of a member of the *Parvoviridae*, with nonenveloped icosahedral particles of ~25 nm on diameter [234]. The full-length genome, including the inverted terminal repeat sequences, has been cloned into a plasmid and, when transfected into cells, leads to the production of infectious virions with typical features [235]. Similar culture or reverse genetics have not been described for the other human bocaviruses.

The full-length genome of HBoV1 is 5,543 nucleotides, with dissimilar hairpin sequences at the 5′ and 3′ ends. The genome has three large open reading frames (as seen in other members of the bocavirus genus) encoding the nonstructural protein (NS1), the capsid proteins (VP1 and VP2), and a second nonstructural protein, NP1 [236].

The capsid protein, VP2, has been expressed in insect cells and self-assembles to form virus-like particles that are the basis of most serology assays. The three-dimensional structure of these virus-like particles has been solved to

<0.79 nm. Although it shares the β-barrel structural core, a depression at the twofold axis, a protrusion at the threefold axis, and a channel at the fivefold axis common to parvoviruses, the surface morphology is different and appears much "flatter" than other members of the group [237].

The coding sequences for the other human bocaviruses (HBoV2 [214], HBoV3 [238], and HBoV4 [216]) is known, but apart from HBoV3, the terminal sequences have not been elucidated. The different human bocavirus species show between 10 and 30 % divergence, with increased genetic variation and evidence for recombination between HBoV2 and HBoV4 [216].

3.4 Descriptive Epidemiology

Human bocaviruses have a worldwide distribution and have been identified in every country where a search has been undertaken. HBoV1 is predominantly found in respiratory secretions and has been observed in 2–20 % of samples from children with upper or lower respiratory tract disease [233]. Although HBoV1 DNA can be detected throughout the year, primary infection occurs predominantly in the winter and spring months [213, 221, 239], as is true of many other respiratory infections. Based on serological studies using HBoV1 as antigen, most, if not all, individuals are infected in early childhood before the age of six [224, 225, 240]. More recent studies have indicated that some of the antibody detected will be cross-reacting to the other human bocaviruses [226, 230], but even so, HBoV1 infections appear to be almost universal in childhood.

HBov2–4 are predominantly identified in fecal samples, both in patients (children and adults) with gastroenteritis and in healthy control subjects [216, 233]. HBoV2 appears to be the most commonly identified species, followed by HBoV3 and then HBoV4 [226, 233]. This is also reflected in the seroepidemiology, with seroprevalence showing species-specific frequencies as follows: HBoV1 > HboV2 > HBoV3 > HBoV4. Adult sera from individuals in China, Pakistan, and Finland showed similar seroprevalences: HboV2, 30–50 %; HBoV3, 8–38 % (8 % in Finland); and HBoV4, 1–4 %.

3.5 Mechanism and Routes of Transmission

HBoV1 appears to be transmitted predominantly by the respiratory route, although detection of HBoV1 DNA in urine [241, 242] and fecal samples [243, 244] suggests that it may also be spread by fecal–oral route.

HBoV2–4 are found mainly in fecal samples and appear to be spread by the fecal route. Studies of sewage confirm that HBoV2 at least is commonly found there [245].

Although low levels of human bocavirus DNA have been detected in serum from blood donors [246], it is not known if this nucleic acid represents infectious virus, and there is no evidence of transfusion-transmitted infection.

3.6 Pathogenesis and Immunity

HBoV1 can be grown in human airway epithelia [235], and cells in the respiratory tract are presumed to be the main site of replication during infection. Virus can also be detected in serum during primary infection [226, 247], and this finding almost certainly reflects high-level virus replication in the target tissues.

Following primary infection and local replication, there is a serological response with IgG seroconversion and production of a short-lived bocavirus-specific IgM [226, 242, 248, 249].

Little is known about the pathogenesis of the fecal human bocaviruses.

3.7 Patterns of Host Response

Many of the published associations of bocavirus and disease associations are difficult to interpret because it is now clear that there can be prolonged persistence of bocavirus DNA in respiratory tract (or fecal) samples. Thus, early studies often did not find a clear association with clinical presentation. However, now that the criteria for diagnosing HBoV1 are defined (high viral load in respiratory secretions, DNA in serum, and a serological response), many groups have shown that acute primary infection is associated with both upper and lower respiratory tract infection, and specifically with wheezing [247, 250, 251].

In a study of 109 Finnish children followed up at 3–6 monthly intervals, primary HBoV1 infection diagnosed on virological and serological grounds was associated with symptoms of respiratory tract infection in 61 % of children [249]. Upper respiratory tract infection was the most common (60 %), with lower respiratory tract infection only being seen in 5 % of children. Acute otitis media was reported in 46 % of children, and further studies are needed to see if this is a true clinical association.

Similar criteria for the diagnosis of infection due to the fecal bocaviruses have not been identified, and although HBoV2–4 can be found in patients with acute gastroenteritis, in controlled studies, they are found in healthy controls at similar rates [222, 252].

3.8 Diagnosis

It is now recognized that diagnosis of active human bocavirus infection should not be based on the detection of bocavirus DNA in respiratory or fecal samples alone, because of

the persistence of DNA at these sites. For HBoV1, a firm diagnosis should be based on the detection of viral DNA in the serum, along with a serological antibody profile of recent infection. This diagnostic profile generally includes evidence of IgG seroconversion, or detection of IgM, or low avidity IgG antibody. If serum is not available for serology and PCR, then high titer (>10^4 genome copies/mL) HBoV1 DNA in respiratory secretions should be used [233].

Similar criteria have not been developed for HBoV2–4 infections.

3.9 Unresolved Problems

There is still much to be learned about human bocavirus infections, not least their pathogenesis and role in human disease. With the recently developed criteria for HBoV1 diagnosis, the true role of HboV1 as a human respiratory pathogen can be determined. The significance of finding human bocaviruses in the elderly or immunosuppressed is still unknown.

4 Parv 4

4.1 Historical Background

Human parvovirus 4 (Parv4) was also discovered in 2005 as part of a virus discovery program looking for new viruses in plasma samples using sequence-independent single primer amplification. The positive sample was from a daily injection drug user (IDU) with signs of acute viral infection who was HIV-RNA negative.

Testing of pooled plasma products from Europe and North America readily detected this virus in 4–5 % of pools, with viral loads varying from <100 copies/mL to 4×10^6 copies/mL. The prevalence may be significantly higher in other parts of the world.

4.2 Methodology Involved in Epidemiologic Analysis

4.2.1 Sources of Mortality Data

Parv4 infection is not known to be a fatal disease, although the virus has been found in two patients with encephalitis of unknown etiology [253]. However, Parv4 infection is rarely looked for.

4.2.2 Sources of Morbidity Data

Very little information is available on the clinical features of acute infection with Parv4 because cohorts of recipients of blood products and IDUs, who are at high risk of acquiring infection have received only limited attention.

4.2.3 Serological Surveys

Several groups have expressed the Parv4 capsid protein in insect [254, 255] or bacterial cells [256] and used these to develop assays to detect antibodies for Parv4. However, these assays have generally only been used to test for antibody levels in high-risk populations and not to look at seroprevalence in the general population.

4.2.4 Laboratory Methods

Parv4 has not been grown in culture, and thus, diagnosis of Parv4 infection is by the detection of Parv4 DNA by molecular techniques, usually PCR. As with parvovirus B19, low levels of Parv4 DNA in serum or tissues likely represent previous infection and are part of the "bioportfolio" as described for erythroviruses [53].

Acute infection can also be established by detection of Parv4-specific IgM by ELISA [254, 257].

4.3 Biological Characteristics of Parv4

Although the virus has not been grown in culture, and the full-length sequence is not known, it appears to have the typical features of a member of the *Parvoviridae*. On EM the particles are nonenveloped 20–22 nm icosahedral particles [258].

So far 5,268 nucleotides of the sequence have been identified, and although this sequence represents the full-length genome, the inverted terminal repeat sequences are incomplete [259]. The genome has two large open reading frames, similar to B19V, but a very different transcription profile, with a spliced RNA encoding the nonstructural protein, and in the middle of the genome a second promoter from which the RNA for the two capsid proteins (VP1 and VP2) are transcribed [260]. The ability to transmit Parv4 infection with virally inactivated blood products [257] suggests that Parv4 will have a similar inactivation profile to parvovirus B19.

As with B19V, Parv4 does show sequence variation, and virus sequences can be divided into three main groups or genotypes. Genotypes 1 and 2 (previously known as Parv5) have been found in many countries [261–268]; genotype 3 appears to be predominantly found in sub-Saharan Africa [269, 270].

4.4 Descriptive Epidemiology

In Europe and North America, Parv4 appears to be a rare infection outside certain high-risk groups—those who share needles or receive blood products. However, only been limited seroprevalence studies have been conducted in the general population, and the true prevalence is not

known [254, 255]. Within high-risk groups, seroprevalence varies widely with up to 95 % of HCV-HIV coinfected intravenous drug users having detectable antibody in one study [271]. Parv4 DNA has also been detected in a variety of different tissues from patients who fall into these high-risk groups, confirming previous Parv4 infection [255, 272, 273]. Together these results suggest that the main route of acquisition of the virus in these countries is through parenteral transmission.

However, the epidemiology appears to be very different in other parts of the world. In one study, Parv4 DNA was frequently detected in the sera of young children, with approximately 12 % of 2-year-olds having detectable Parv4 DNA [270]. Following this study, further serological assays for Parv4 IgG have been performed, and seropositive rates of 20–37 % have been found in adults in sub-Saharan Africa [274]. Similar high levels of Parv4 antibody have been described in elderly patients from the Cameroon [275]. Parv4 DNA has also been detected in nasal secretions and fecal samples from young children in Ghana [276].

4.5 Mechanism and Routes of Transmission

In Europe and North America, the contrast between high seroprevalence and detection of Parv4 DNA in tissues of individuals at high risk of blood-borne infection compared to a virtual absence of seropositivity in the general population is striking. It suggests that the main route of transmission in these countries is parenteral. However, that seems less likely in other parts of the world, such as in Africa. Although the high seroprevalence and detection of Parv4 viremia in young children do not rule out parenteral transmission, the identification of Parv4 DNA in nasal secretions and fecal samples from young children in Ghana [276], as noted above, does add additional support to the possibility of respiratory or oral–fecal routes of transmission in some countries.

4.6 Pathogenesis and Immunity

Virtually nothing is known of the pathogenesis of Parv4, not even which cells the virus targets and replicates in. Low levels of viral DNA have been found in numerous tissues, including the bone marrow and liver, but as with parvovirus B19, this may not reflect the true site of replication.

Acute infection with Parv4 appears to correlate with high levels of viremia and the development of Parv4 IgM and Parv4 IgG [257]. This pattern resembles that seen with acute B19V infection. As with parvovirus B19, there seems to be a rapid drop in Parv4 DNA titers, but with low levels ($<10^4$ copies/mL) being detectable for many months.

4.7 Patterns of Host Response

In the study of acutely infected hemophiliacs, the only repeatedly observed clinical presentation was a rash in three patients and unexplained hepatitis in two of them [257]. However, no further information on the rash was provided, and no significant increase in liver enzymes was reported. The original index case had symptoms of an acute viral syndrome.

4.8 Diagnosis

Diagnosis is generally by the detection of Parv4 viral DNA by PCR. Patients with acute infection appear to have transient high levels of Parv4 DNA in serum. However, the duration of the high-level viremia before the development of an IgM and IgG response is not known.

4.9 Unresolved Problems

There are still many unsolved questions about Parv4, including its route of transmission and the main clinical presentation of infection. Although this virus appears to be transmitted by the parenteral route, there are very few data on the seropositivity rate among blood donors in most countries.

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Suggested Readings

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Rhabdovirus: Rabies

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1 Introduction

Rabies is an acute, progressive encephalitis of mammals and has the highest case fatality proportion of any conventional infectious disease. This disease is an ancient, reemerging global zoonosis, caused by highly neurotropic viruses in the family *Rhabdoviridae*, genus *Lyssavirus* [1].

Illness develops following a productive infection with bullet-shaped, enveloped virions that contain single-stranded, non-segmented, negative-sense RNA [2]. The usual route of virus transmission is via the bite of a rabid animal. Non-bite exposures, such as direct mucosal contact, inhalation of virus, inoculation with improperly inactivated vaccines, or transplantation of infected corneas, tissues, and organs, have occurred [3]. Historically, in the United States, the primary source of human exposure to rabies virus was the domestic dog, which still predominates as the major reservoir in developing countries. In developed countries, multiple wildlife species are affected. For example, current rabies reservoirs in the United States include raccoons, skunks, coyotes, foxes, and bats [4]. The incubation period usually ranges from 1 to 3 months after exposure, but can range from days to years [5]. Once virus is deposited in peripheral wounds from a bite, centripetal passage occurs toward the central nervous system [6]. The virions may replicate locally or enter unmyelinated nerve terminals and migrate by retrograde axonal transport to the neuronal cell body. After replication in the cell body of a primary neuron, infection proceeds via retrograde axonal transport and transsynaptic spread through several neurons to the central nervous system (CNS), before infecting acinar cells of the salivary glands and excretion by saliva into the oral cavity [7]. Onset of disease is initially nonspecific, consisting in humans of signs and symptoms compatible with a "flu-like illness," such as fever, headache, and general malaise. Following the prodrome, an acute neurologic phase may include intermittent insomnia, anxiety, confusion, paresis, percussion myoedema, excitation, agitation, hallucinations, cranial nerve deficits, chorea, dysphagia, hypersalivation, piloerection, priapism, paralysis, and sometimes maniacal behavior [8]. Clinical presentation may also include paresthesia at the site of the bite exposure, and classically hydrophobia or aerophobia, manifesting as phobic pharyngeal spasms following provocative stimuli [8]. The clinical course is progressive, with death ensuing usually within days. One form of the disease, termed paralytic, or "dumb rabies," may also present as part of the clinical spectrum, with the general sparing of consciousness, together with ascending paralysis, progressive unresponsiveness, coma, and death. Once clinical signs are present, there is no proven cure. Intensive medical support may prolong life, but typically death ensues due to cardiac or respiratory failure. Exceptions to this general scheme are exceedingly rare, with few well-documented cases of human survival from clinical rabies, usually (but not always) with a history of either pre- or postexposure prophylaxis (PEP). Survivors may have significant residual neurologic impairment, but this is not found in all patients. Serological surveys and documented occurrences under laboratory conditions in the animal reservoir species have supported the existence of acquired immunity, which presumably follows subclinical exposure, abortive infection, or survival of overt clinical rabies [9, 10].

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2 Historical Background

Rabies is an ancient disease of uncertain origin and one of the oldest recognized infectious diseases [11]. As such, it is difficult to provide more than highlights, given the fascination

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and dread it evoked in historians of the past. For a more thorough treatise, the interested reader is referred to Steele and Fernandez [12], Baer [13], and Wilkinson [14]. Most global reference languages singularly denote the terms damage, violence, fury, madness, or rage as literally synonymous with this affliction. Many great civilizations refer to a disease akin to rabies, weaving a tapestry of overstated fact, legend, and nightmare, exceedingly out of proportion to its actual pathos. In the not-too-distant past, extreme ostracism, torture, or execution were inflicted on those even suspected of having hydrophobia. Democritus in 500 BC recognized rabies in dogs and other domestic animals, as did many other Greek and Roman scholars. Thus, it can be assumed that this viral disease, or a similar contagion, was well recognized and occurred throughout Asia, Europe, and, perhaps, Africa, during centuries of recorded history.

Early historical accounts focused primarily on individual human or animal case reports. For example, wildlife rabies was recognized throughout the Middle East since biblical times [11]. However, few epizootics were well documented until the Middle Ages. Dramatically, there were multiple accounts of rabid wolf attacks in Franconia during 1271 [12]. Perhaps such incidents were one of the origins of the "big bad wolf" of the fairy tale. Throughout Europe, outbreaks were recorded in wolves, foxes, and dogs, especially during the eighteenth and nineteenth centuries. These episodes continued sporadically until World War II, when another major epizootic in foxes swept Europe, from east to west, at a rate of approximately 30–50 km/year, the epidemiological repercussions of which are still felt.

Quite likely, lyssaviruses evolved in the Old World. In most of the New World, the disease was largely unknown, at least among dogs, until the early eighteenth century; one of the first descriptions dates to 1703, from Mexico. Rabies was subsequently identified in the Caribbean by the mid- and late 1700s. By 1753, dogs in Virginia had been affected, and most of the Mid-Atlantic American and New England colonies shortly afterward. Pioneer expansion in the early 1800s was replete with tales of "madstones" and "phobey cats." In South America, one of the first reports of rabies was in Peruvian dogs during 1803. Besides typical incidents involving carnivores, Baer cites Spanish reports from the 1500s onward that associated bat bites and human mortality in Latin America [13]. Thus, rabies viruses were likely imported repeatedly into the New World, perhaps from the Old World circumpolar invasions predating recorded history, and more extensively during European colonization in the sixteenth through eighteenth centuries, contemporaneous with major domestic animal outbreaks in Europe. Later documentation of antigenic and genetic similarities between both Old and New World rabies viruses [15, 16] supports trans-Atlantic introduction by infected animals, in whom incubation periods could exceed the length of the voyage [17, 18]. Continued isolation of distinct viruses in Africa, serologically and genetically related to rabies virus [19], supported the hypothesis of an African genesis [20], with adaptive radiation of several lyssaviruses throughout the Old World.

Beyond mere geographic documentation, the history of rabies was also marked by a series of observations and speculations regarding its cause, prevention, treatment, and diagnosis. For example, about 100 AD, Celsus treated animal bite wounds by cauterization. In 200 AD, Galen recommended amputation of the bitten limb. Both had limited success (later supported by laboratory animal research) [12]. During 1804, Zinke demonstrated transmission of rabies virus to a normal dog by inoculation of infected saliva, considering it a toxin [12]. These observations helped lead to institution of muzzle laws and stray dog control, which resulted in rabies elimination from several countries such as Denmark, Norway, and Sweden by 1826, before the invention of rabies vaccination.

Animals have been central to progress in applied rabies research since the late nineteenth century. For example, in 1879, Galtier used the domestic rabbit as a suitable laboratory host for rabies [12]. His observations enabled the later classic 1881 experiments in which Pasteur (with important primary contributions by collaborators such as Roux, Chamberland, and Thuillier) reported the characterization of a "microbe of infinite smallness" attenuated for the dog but having a uniform (fixed) incubation period in the rabbit [21]. Pasteur distinguished this adapted laboratory form of disease from that of the agent in nature, or "street virus." He used this laboratory-adapted "fixed" virus (based upon derived characteristics, not to be confused with inactivation), grown in rabbit spinal cords, and dried for varying periods, to give graded doses of noninfectious to fully infectious virus for the immunization of animals. The first successful human rabies vaccination was administered in 1885 to a boy aged nine years, Joseph Meister, severely bitten by a presumably rabid dog, based on the animal's clinical derangement and pica [21]. This single historic event ushered in the era of rabies vaccinology, which was reasonably successful by accepted standards of the day. Occasional failures were attributed to either prolonged delays before vaccine initiation or to the particular severity of an exposure.

Since Pasteur's time, numerous improvements in safety and efficacy of the early nerve tissue origin (NTO) biologics have been attempted. Such gradual attempts were partially frustrated, until the 1940s, by the lack of standardized vaccine potency evaluation [22]. For example, in 1908, Fermi developed the first chemically treated rabies vaccine, unfortunately still with residual live virus. By 1919, Semple showed that phenol might more fully inactivate rabies virus without destroying its antigenicity. Semple's vaccine was used extensively for at least 65 years but was replaced gradually in Latin America and elsewhere by a suckling mouse NTO vaccine, by the efforts of Fuenzalida and Palacios in 1955. By circumventing the sensitization to myelin basic proteins found in

adult animal brains, the suckling mouse NTO vaccine had a lower rate of neuroparalytic reactions. A number of studies in the 1940s suggested that, besides vaccine, rabies immune serum was also effective in preventing disease. The importance of combined therapy consisting of serum plus vaccine, in combination with local wound treatment, had been demonstrated most convincingly in those few cases where human rabies occurred, despite intervention, when one of these facets of an accepted protocol was altered or delayed [23]. Other major vaccine initiatives during the 1950s and 1960s moved away from NTO vaccines, with the development of avian embryo vaccines, initially of dubious potency. Such alternative strategies eventually culminated with the first true cell culture vaccine in the 1970s, the human diploid cell vaccine (HDCV), with improved safety and efficacy [22, 24, 25]. Although the original Pasteurian use of an attenuated virus vaccine in rabbit CNS tissue was discontinued by the 1950s, inactivated NTO vaccines, from small mammals or livestock, were still used in parts of the developing world during the twenty-first century, despite the availability of safer, potent, albeit more costly alternatives. The further development of purified avian embryo vaccines and Vero cell vaccines today should compete economically and displace historical NTO vaccines altogether over the next few years.

Before the development of routine laboratory tests for rabies diagnosis, the clinical presentation of the biting animal provided a primary motivation for human PEP. At the beginning of the twentieth century, scientists such as Babes and Van Gehuchten described lesions in the CNS, presumptively related to rabies [12], but which were actually nonspecific, and applicable for a set of other etiologies as well. However, in 1903, Negri described intracytoplasmic acidophilic inclusion bodies (which he believed to be protozoa) in neurons of rabid humans and other mammals, facilitating the early diagnosis of rabies when brain tissue was collected, fixed, and stained appropriately. Histopathologic identification was a key procedure despite the relatively high rate of false-negative observations until Goldwasser and Kissling introduced the immunofluorescence diagnostic technique in 1958 [26]. This technique is still in routine use today, as the global gold standard for rapid, sensitive, and specific rabies diagnosis.

These historical accomplishments reveal that rabies research efforts focused primarily on the human as victim, rather than on primary prevention in the animal reservoir. While animals did serve as critical experimental subjects, focused human vaccination preceded the serious consideration of dog immunization, which was originally deemed impractical. It was not until 1919 that an attempt at mass vaccination of dogs occurred with the use of Fermi vaccine in Japan, with the focus shifting to the source of exposure, compared with the preceding three decades, during which many thousands of persons were treated with Pasteurian postexposure prophylaxis (PEP). However, problems associated with potency of early inactivated vaccines plagued these first trials. By 1948, a live virus vaccine using an attenuated Flury strain of egg-adapted virus was successfully applied to dogs. A variety of both inactivated and live virus vaccines was shown subsequently to be effective in preventing rabies in most of the major domestic animals. During the 1950s, animal management and mandatory dog vaccination programs resulted in the gradual elimination of canine rabies in the United States and other developed nations [27]. This, coupled with still-ongoing improvements in epidemiologic surveillance, diagnostics, and preexposure immunization and PEP, has markedly decreased the number of human rabies cases in developed countries. Clearly, an extensive public health infrastructure and proper allocation of resources are required to minimize domestic animal rabies and maintain the vigilance necessary to recognize potential human rabies exposure for the prompt initiation of prophylaxis.

3 Methodology Involved in Epidemiologic Analysis

3.1 Sources of Mortality Data

There is no single definitive depository for human rabies data at a global level. Data, if collected, may be available in national summaries, regional reports, or the peer-reviewed literature. Human rabies is a notifiable disease in the United States, as in most other developed countries. Statistics for humans and animals are compiled from local and state health departments by the Centers for Disease Control and Prevention (CDC) and published in the *Morbidity and Mortality Weekly Report*, as well as in an annual summary. In Europe, numbers and distribution of human rabies cases and cases in animals are published in the *Rabies Bulletin of Europe*. The World Health Organization (WHO) in Geneva and the associated Pan American Health Organization (PAHO) in Washington, DC, together with the World Organization for Animal Health (OIE), collect data from countries or political units on deaths attributable to rabies. These data are distributed from national governments as a source of information on global rabies occurrence and the general relative risk of infection, which, provided that proper epidemiologic surveillance is ongoing, should be nil in countries with no reported cases during any one particular year (Table 28.1). Notably, the amount of human PEP administered in some countries, such as the United States, may be lacking in these global summaries, because reporting may not be required by most localities or on a national level. Obviously, the temporal and spatial occurrence of rabies in animals will affect exposure patterns in people. For example, the emergence of a raccoon rabies epizootic in the mid-Atlantic and northeastern United States during the 1970s

Table 28.1 Global disease surveillance: selected countries/political units reporting no cases of rabies during 2012

Region	Places
Africa	Cape Verde, Libya, Mauritius, Réunion, São Tomé and Principe, Seychelles
Americas	
North	Bermuda, St. Pierre and Miquelon
Caribbean	Antigua and Barbuda, Aruba, The Bahamas, Barbados, Cayman Islands, Dominica, Guadeloupe, Jamaica, Martinique, Montserrat, Netherlands Antilles, St. Kitts (St. Christopher) and Nevis, St. Lucia, St. Martin, St. Vincent and Grenadines, Turks and Caicos Islands, Virgin Islands (the United Kingdom and United States)[a]
Asia and Middle East	Hong Kong, Japan, Kuwait, Lebanon, Malaysia (Sabah[a]), Maldives[a], Qatar, Singapore, Taiwan[b], United Arab Emirates
Europe	Austria, Belgium, Cyprus, Czech Republic[a], Denmark[a], Finland, Gibraltar, Greece, Iceland, Ireland, Isle of Man, Luxembourg, Netherlands[a], Norway, Portugal, Spain[a] (except Ceuta/Melilla), Sweden, Switzerland, United Kingdom[a]
Oceania[c]	Australia[a], Cook Islands, Fiji, French Polynesia, Guam, Hawaii, Kiribati, New Caledonia, Northern Mariana Islands, Palau, Papua New Guinea, Samoa, Vanatu

Sources: CDC [154], as well as PAHO/WHO reports
Bat rabies may exist in some areas that are reportedly free of rabies in other mammals
[a]Bat lyssaviruses are known to exist in areas that are reportedly free of rabies in carnivores
[b]Taiwan remained rabies-free during 2012, however, in July 2013 reported its first case of rabies in over 50 years to OIE with the identification of a rabid ferret-badger, *Melogale moschata* (http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=13775, cited 24 July 2013)
[c]Most of Pacific Oceania is reportedly "rabies-free"

significantly affected the epidemiology of human PEP, diverting critical health resources as animal rabies cases increased. Moreover, if predicted effects of climate change models occur, an expansion of vampire bat distribution in the Americas could subsequently result in an increase in the number of human exposures requiring PEP administration [28]. To properly assess global and national needs and implement appropriate use of PEP, efforts toward proposing a nationally notifiable reporting system for PEP should be encouraged.

When rabies surveillance is "adequate," human death rates may begin to approximate incidence rates of human infection. Generally, for statistical purposes the disease can be regarded as 100 % fatal, because the acute clinical course incidence rates are also virtually equivalent to prevalence rates. However, in most developing countries, human rabies is grossly underreported. When it is reported, human cases may outnumber documented cases in animal species. Such limitations are not limited to developing countries, as human rabies is underreported even in the United States, considering that human cases have been diagnosed retrospectively from autopsy material.

Where canine rabies occurs, human exposure and rabies incidence rates appear related to the local epizootiology of the disease. However, the relationships between human rabies mortality, virus exposure, and wildlife rabies cases are less well established. For example, since most animal reservoirs in the United States are free-ranging wildlife species, the number of animal rabies cases is variable and biased by surveillance efforts first developed for canine rabies detection, prevention, and control. Surveillance, typically based on the intensity and occurrence of animals with suspicious clinical presentations and encountering humans or domestic animals, would tend to be more reliable in detecting cases during an outbreak but less so during expected enzootic periods. This bias may be greatest in localities in which the responsibility for suspect animals, such as predominantly sick or nuisance wildlife rather than domestic dogs, does not rest with any one particular agency. Historically, as vestiges from the time of enzootic canine rabies, an animal control officer may respond to domestic animal problems in a community. However, without a mandated and funded local response to suspect wildlife, a "shoot-and-bury" practice may evolve to cope with rabies among wildlife species, with the result that fewer cases are recorded. Disease-specific incidence (or death) rates for animals are generally unavailable, since free-ranging wildlife populations are not well enumerated, and one cannot approximate the true populations at risk. Thus, in the United States, representation of the public health magnitude or burden of the disease, solely by absolute case numbers of animal rabies reported, is inherently biased and should be qualified in view of its obvious historical, scientific, and logistical limitations.

Predictably, predominant animal reservoirs change over time and in geographical distribution. For example, before World War II, dogs were the major rabid animal in the United States. After dog rabies management began, the advantages of surveillance in other species became more apparent, first during the 1960s with foxes and skunks, until the latter 1970s. Thereafter, since the incursion of raccoon rabies into the eastern United States, the annual totals of rabid wild animals have surpassed historical records, demonstrating the magnitude of

the wildlife rabies problem in such particular geographic regions [29]. Thereafter, the total areas affected, and the proportion diagnosed rabid among raccoons tested, appear stable. Based upon such observations, when animal outbreaks occur, administrations of human PEP will increase dramatically.

3.2 Sources of Morbidity Data

Human rabies case morbidity is largely reflected in mortality data. In contrast, only a few documented cases of humans surviving clinical rabies exist, some with serious neurological sequelae. Morbidity that has otherwise been averted could be assessed through numbers of human PEP administered. However, because reporting PEP in countries such as the United States may not be required, information on potential morbidity prevented by PEP is not available readily. Such occurrence may be approximated, as in developing countries, by the number of vaccine doses and human rabies immune globulin (HRIG) sold or used. Based on these imperfect data (because of the vaccine's utilization in both pre- or PEP protocols and administration of HRIG on a per-weight basis), current national estimates range between 10,645 and 35,845 (average = 23,415) human administrations of PEP per year in the United States [30]. In some localities, PEP data are reportable and partially quantifiable, whereas in many other places, vaccine and HRIG are available only through the state or local government, or private providers, from which the number of PEP administrations may be approximated. Representative reporting of human PEP would be advantageous for proper assessment of the epidemiology of rabies, for national and regional PEP needs, and to assess risk factors for exposure, as well as for further definition of the overall economic impact of rabies. These data tend to be more rabies-specific, as opposed to all of the other requisite morbidity factors associated with the trauma of the bite occurring after a rabid animal exposure, and the secondary effects that may result thereafter (such as death, or serious morbidity, associated with the severity of the attack itself, rather than from viral induced mortality or morbidity, per se).

3.3 Serological Surveys

In general, serology may be used to assess natural exposure to viral antigens and evaluate an induced response to immunization. Historically, rabies serological evaluation [31] had been performed by antigen-function assays, such as a mouse neutralization test, by antigen-binding assays, such as an indirect immunofluorescence test, or by antibody-function assays, such as the hemagglutination test. Currently, one of the most widely used serological tests is the rapid fluorescent focus inhibition test (RFFIT). Results are typically reported in relation to a known standard, as international units (IU) per milliliter of sera. The RFFIT requires trained personnel and appropriate laboratory equipment for cell culture and manipulating live rabies virus, in addition to being somewhat complex and time-consuming [32]. Alternatively, commercial enzyme-linked immunosorbent assays (ELISA) have been applied for simple, rapid screening of large numbers of sera. However, specificity may be less reliable, especially at low antibody levels, and utility is determined by the source and quality of crude antigen. The fundamental difference between these tests is that the ELISA is based on primary recognition of antigens, whereas the RFFIT and other neutralization assays (such as the fluorescent antibody virus neutralization test) are functional tests.

The absolute interpretation of such rabies serological survey data is not straightforward. Titers do not directly correlate with protection against disease, because other factors, more difficult to measure and interpret, play a role in prevention of disease [4]. Further, minimal differences in the reported values of rabies antibody results might occur between laboratories that provide antibody determination using variations in tests such as the RFFIT [5]. Obviously, there is no known absolute "protective antibody" level for all humans or other animals [31]. Minimum standards for humans are based empirically on presumed protective activity of rabies-specific antibodies, e.g., virus-neutralizing antibody (VNA), for a given exposure scenario and on repeatable values for paired sera, as could be readily detected by reference laboratories.

With regard to rabies in animal reservoir species, a subset of animals may be exposed to virus, may develop measurable viral antibodies, and are then immune to subsequent challenges. Such data do not support the existence of a "carrier state" in animals. Thus, serological surveys of wildlife in areas with enzootic rabies may reveal a low prevalence of antibodies [33]. These incidents may occur among animals that are infected and will ultimately die of rabies or more likely among individuals that have developed immunity to rabies, presumably following natural sublethal exposures. These naturally occurring antibodies are distinct from those due to purposeful immunization of free-ranging animals via limited trap–vaccinate–release programs, regional oral rabies vaccination programs, or occasional parenteral vaccination of wildlife with domestic animal vaccines by well-intentioned wildlife rehabilitators [34].

Clearly, multiple ecological factors will affect rabies epizootiology, as related to host, agent, and environment. Among carnivores, the small Asian mongoose provides one clear example of outbreaks influenced by host biology and distribution [35]. Mongooses were introduced for rodent control onto most of the larger Caribbean Islands, including Puerto Rico, Dominican Republic, Haiti, Cuba, and Grenada. Though mongooses are wild, their population density is

usually directly proportional to human activities [35]. As such, mongooses proliferated and are now deemed undesirable due in part to predation on native fauna and the risk from rabies in this introduced reservoir species. For example, 56 % of the 73 rabies cases diagnosed in Puerto Rico in animals during 2012 were attributed to mongoose [29]. Although poisoning has been practiced, such control was undesirable and ineffective over time. Many mongooses (as many as 55 % at the end of an epizootic cycle) have evidence of rabies antibodies, possibly as a result of sublethal virus exposure, and such animals may be immune for life [48]. Removal through poisoning or other lethal means appears detrimental, which may simply enhance reproductive turnover, dispersal, and increase the number of naive, susceptible animals. In view of such levels of naturally occurring disease and probable consequent herd immunity, the nearly closed populations of these nonindigenous mammals should be ideal candidates for oral vaccination. This would represent one means of disease management to limit human and domestic animal exposures, especially in a circumscribed island environment [36].

3.4 Laboratory Methods

Several etiologies which cause encephalitis may be confused with rabies. Epidemiologic studies should be based on laboratory confirmation of suspected disease or death from rabies. As summarized in the above historical description, laboratory techniques have evolved gradually over the last century. Although variations are practiced in many laboratories, the standard techniques of the WHO Expert Consultation on Rabies (2005), and the current edition of the WHO *Laboratory Techniques in Rabies*, are recommended and widely used [37]. Included in these texts are methods for the collection, preparation, and shipping of specimens; tissue and organ removal; laboratory animal inoculation; immunofluorescent antibody (FA) procedures for detection of virus antigens; serological determinations; cell culture propagation of virus; electron microscopy; vaccine production; potency and safety determinations; and specific virus identification by antigenic and genetic techniques. For regulations governing specific international or national shipment of potentially infectious specimens, such as rabies, the local or national public health laboratory should be consulted for current updates.

Historically, brain tissue from the rabid subject was examined for Negri bodies, which are specific intracytoplasmic inclusion bodies observed under the light microscope. These inclusions contain viral nucleoproteins, which accumulate in quantities large enough to be detected after staining by the Seller's, Giemsa, Mann, or other methods, and can be visualized microscopically. Within the hippocampus, Ammon's horn is one particular portion of the CNS usually examined for Negri bodies, as are the Purkinje cells of the cerebellum and pyramidal cells of the cerebral cortex. Limitations from this technique include a concern that Negri bodies may be absent in up to one quarter to one third of rabies cases, while artifact and inclusions produced by other agents could be confusing and differentiated only after considerable laboratory experience.

Classically, animal inoculation with suspect diagnostic material was once used widely to support rabies diagnosis, particularly because of the potential dramatic ramifications of false-negative results in human rabies exposure situations. Laboratory rodents were used routinely for viral isolation, as suckling and weanling mice are highly susceptible to infection by intracerebral inoculation. Typically, the time from inoculation to illness varied from 7 days to 4 weeks, but viral antigens in brain may be confirmed as early as 4 days following inoculation. Confirmation in the inoculated animals of rabies, as the source of illness and mortality, should always be performed, because laboratory species may die of comorbid conditions or as a result of infections by other agents in the original clinical specimen.

Besides the use of mice, murine neuroblastoma cell cultures are as sensitive as laboratory animals for isolation of field strains from saliva and brain of rabid animals and are the method of choice in laboratories where cell culture facilities are available [38]. The presence of antigens in infected cells is demonstrated by the FA test.

In brief, the direct FA test uses a fluorescein dye conjugated to rabies immune serum, which in turn is reacted with acetone-fixed impressions from CNS tissue of the presumed rabid subject, including the brainstem and cerebellum or hippocampus [26]. The antigen–antibody reaction is detected by microscopic observation of fluorescence under light of the appropriate wavelength. The direct FA test is very rapid and reliable when used by an experienced laboratorian. The direct FA method is the test of choice for fresh or frozen tissue, because of test sensitivity, specificity, and economy. Immunohistochemical testing, or a modified FA technique, can be applied on formalin-fixed tissues; however, the method is more cumbersome and cannot distinguish between different lyssavirus species. Also, RT-PCR methodologies can be used for diagnostic support but may be unreliable due to RNA degradation in these fixed tissues [39]. Brain tissues from suspect rabid animals continue to be submitted in formalin, and research continues to develop an ideal molecular methodology to confirm rabies and to determine variant characterization [39]. Significantly, in the Old World, other lyssaviruses related to rabies virus will cross-react in the direct FA test, depending on the quality of the particular diagnostic conjugate. Specific monoclonal antibodies (MAbs) can be used to distinguish rabies virus from these related lyssaviruses, but the public health consequences of identification are the same, regardless of exposure to any one

of these etiologic agents, in that appropriate medical care and PEP considerations are necessary.

Rather than reliance upon a history of relevant viral exposure and compatible clinical signs, proper laboratory-based diagnosis is needed for case confirmation. Besides the direct FA test from the 1950s, during the early 2000s, a rapid immunohistochemical test (RIT) to detect lyssavirus antigens was developed. Modifications of a former indirect test led to a direct test, the dRIT, which uses a cocktail of highly concentrated and purified biotinylated anti-nucleocapsid MAbs produced in vitro, in a direct colorimetric staining approach, and allows a diagnosis to be made in <1 h. Currently, the dRIT is an experimental procedure designed for consideration as a potential confirmatory test of the direct FA test. Similar to the direct FA test, the dRIT can be performed on brain touch impressions, and based upon the reagents used, viral antigens may appear as magenta inclusions against a blue neuronal background. The test recognizes all variants of rabies virus and all representative lyssaviruses [40]. One advantage to the dRIT, compared with the direct FA test, is that the dRIT can be conducted by light microscopy, compared with the FA test, which must employ the use of a fluorescence microscope [41]. The dRIT may be used to enhance field surveillance among suspect animals, particularly in support of national, regional, state, or local oral vaccination programs. However, until the concept is validated as sensitive and specific as the direst FA test, the dRIT should not to be used for public health decisions, in those situations in which human or animal exposure has occurred or is suspected, and local public health authority or other officials should be contacted for immediate and appropriate diagnostic testing [41].

While animals are diagnosed postmortem, rabies in humans can be diagnosed while the patient is still alive. Human antemortem diagnosis can be made by virus isolation from saliva, antigen detection in biopsy tissue, or demonstration of virus-specific antibody (without prior vaccination). Selected FA tests are performed on human brain or skin biopsies, the latter usually taken from highly innervated areas, such as the nape of the neck, where piloerector nerve plexi are prominent [42]. Also, in some human rabies cases, corneal cells obtained from touch impressions may be antigen-positive during life, care being taken not to abrade the delicate corneal tissue (a rationale to caution against this method vs. other stated procedures). Antemortem diagnosis may also be made by showing a fourfold or greater rise in serum antibody titer during the illness in the absence of vaccination or administration of rabies immune serum. Demonstration of virus antibody in the cerebrospinal fluid (CSF) is also a reliable indicator of infection, even after vaccination. Vaccination alone does not elicit CSF antibody. During viral infection, other pathophysiologic changes in the CSF, such as an increase in specific proteins, and pleocytosis, particularly a monocytosis which is compatible with a viral encephalitis, may also occur [43]. Repeated samples should be taken for antibody and antigen detection, because negative results in early initial samples do not rule out rabies. Further, because of the risk of false-negative results with a potential human exposure, intravitam testing of the rabies-suspect animal is inappropriate.

In addition to classical laboratory procedures for detection of viral antigens and antibodies, recent progress has been made in the application of molecular techniques, particularly nucleic acid detection and amplification of cDNA by RT-PCR [44–46], with subsequent generation of viral nucleotide sequences, leading to a greater understanding of lyssavirus epizootiology and phylogeny [19, 43, 47]. However, the extreme sensitivity of molecular methods, such as the RT-PCR technique, also greatly increases the probability of a false-positive diagnosis from laboratory contamination. Moreover, because of lyssavirus heterogeneity, false-negative results can occur if primer selection is inadequate to compensate for heterogeneity. To date, universal primers for all known lyssavirus variants have not been clearly defined or standardized. Considering these factors, related costs, and the considerable expertise required for proper analysis and interpretation, such molecular techniques are not recommended for routine primary rabies diagnosis alone when CNS tissue is available, as opposed to laboratory confirmation in concert with other techniques.

The routine sequence characterization of lyssaviruses from formalin-fixed tissues is possible but may be difficult, partially as a function of the fixation time and resultant short fragments of nucleic acid [16]. Optimization of these methods, beyond mere qualitative diagnostic methods for viral antigen analysis preserved in such tissue, may be feasible by the adaptation of techniques that would employ MAbs for retrospective virus identification in archival material [48]. Besides determination of optimal specific reagents, corroborative data would have to be generated on the sensitivity and specificity of such technique in comparison to fresh tissue analysis. Because of increased utilization for other viral diseases, such as HIV, influenza, polio, etc., additional development of appropriate modern molecular methods is anticipated to improve the application to basic rabies diagnosis in developing countries.

4 Biological Characteristics of the Agents That Affect Epidemiologic Patterns

Rabies virus is the type species of the genus *Lyssavirus*; additional and proposed members of the genus *Lyssavirus* include those listed in Table 28.2.

Rabies is essentially a “dead-end” disease in humans and several other species. While possible, human-to-human

Table 28.2 Viruses currently included in the genus Lyssavirus

Recognized and proposed species	Predominant natural hosts	Geographical range
Rabies virus (type species)	Chiroptera, Carnivora	Worldwide
Australian bat lyssavirus	Pteropodid and insectivorous bats	Australia
European bat lyssavirus, type 1	Insectivorous bats	Europe
European bat lyssavirus, type 2	Insectivorous bats	Northwestern Europe
Khujand virus	Insectivorous bat, *Myotis mystacinus*	Central Asia
Aravan virus	Insectivorous bat, *Myotis blythii*	Central Asia
Bokeloh bat lyssavirus	Insectivorous bat, *Myotis nattereri*	Germany
Irkut virus	Insectivorous bat, *Murina leucogaster*	Eastern Asia
Duvenhage virus	Insectivorous bats	Sub-Saharan Africa
Lagos bat virus	Pteropodid bats	Sub-Saharan Africa
Mokola virus	Unknown (shrews, rodents?)	Sub-Saharan Africa
Shimoni bat virus	Insectivorous bat, H. *commersoni*	Kenya
West Caucasian bat virus	Insectivorous bat, *Miniopterus* sp.	Southeastern Europe
Ikoma lyssavirus	Unknown (isolated from civet, *C. civetta*)	Tanzania

Recognized viral species are listed in italics, as adapted from Kuzmin and Tordo [155]

transmission via bite has not been reliably established [49]. However, likely cases of human-to human transmission continue to be reported [50]. A marked exception to the apparent rarity of direct human contagion from exposure to patient secretions, such as saliva, consists of at least eight human rabies cases resulting from the surgical implantation of infected corneas from donors that had succumbed to undiagnosed rabies [51, 52]. In addition, other cases have occurred in Europe and the United States from other organ and tissue transplants. For example, in 2004, four recipients of kidneys, a liver, and an arterial segment from a common organ donor died an average of 13 days after the onset of neurologic symptoms following the development of encephalitis, and antibodies against rabies virus were later found to be present in three of the four recipients and the donor [53].

Biological characteristics of lyssaviruses have a measurable, although incompletely understood, effect on epizootiologic patterns among their animal host species [54]. Initially, through classic serology, and later by MAb and genetic analysis, translational research was possible to differentiate serotypes, variants, and genotypes of rabies virus and related lyssaviruses [2, 15, 16]. All mammals are believed susceptible to lyssaviruses; however, prominent reservoirs among bats and carnivores perpetuate largely independent cycles of infection. Epizootiologically, such variant viruses are maintained by different host species. Complex virus–host interactions lead to the emergence of viral characteristics that are beneficial to self-perpetuation among these particular species that manifest as regional epizootiological "compartments" [55–57]. Thus, in countries with adequate laboratory-based surveillance, such as the United States, there are discrete geographic zones of raccoon rabies, skunk rabies, and so forth. Such viruses are fully transmissible to other mammals within a region but genetically and antigenically distinguishable as a single variant in a particular geographic area, regardless of affected host, be it the reservoir species (e.g., raccoon) or mere "spillover" into an essentially dead end, but susceptible host (e.g., domestic cat). In short, all animal reservoirs are also vectors, but not all potential vectors are reservoirs for rabies.

Not all mammals are equally involved in rabies maintenance from an epizootiological perspective, but for poorly understood reasons. Among the Carnivora, meso-carnivores, ranging in size from the mongoose to the jackal or coyote, are most significant. As one specific example of virus vagility, coyotes possess many host qualities ideal for the initiation of a rabies epizootic. Historically, during 1915–1917, an extensive outbreak of coyote rabies extended over large portions of southeastern Oregon, northeastern California, western Utah, and Nevada [58]. Thereafter, rabies among coyotes was reported only sporadically in the western United States, despite the coyotes' widespread distribution and abundance. Another major focus was detected in Texas during 1988 [59], and the virus spread throughout southern Texas. Antigenic and genetic analysis of isolates obtained from this outbreak suggested the involvement of a rabies virus variant associated with canine rabies along the United States–Mexico border but apparently capable of sustained coyote-to-coyote perpetuation. Intensive oral vaccination progress initiated during 1995 was able to eliminate this virus in the United States [60], which contributed to the United States being declared free of canine rabies virus transmission in 2007 [61].

Another unique characteristic of virus infection is a prolonged incubation period during which the infection is virtually undetectable. In humans, although the incubation period is generally several weeks to months, unusually long incubation periods of 6 or more years have been described [17]. This potential for long incubation periods influences directly the management of exposed domestic animals. An exposed, unvaccinated domestic animal may be euthanized or held in

quarantine for 6 months [4], which extends beyond the majority of documented incubation periods for dogs and cats.

Mechanisms for successful in vivo productive infections are not fully appreciated. Neurotropism appears to be a critical facet of a successful strategy to partially evade immune detection [62, 63] with the ultimate end to ensure future viral progeny. Under the constraints of mammalian anatomy, there are fairly limited routes available to a virus for transit from an initial portal of entry (i.e., a bite in a peripheral muscle) to a primary portal of exit (e.g., saliva). One of the most common mechanisms for other viruses to transit to such distant sites is by viremia or spread via lymphatics. The passage along both of these latter pathways may also initiate a vigorous immune response. An effective blood–brain barrier would limit the utility of viremia in enhancing the spread and perpetuation of a neurotropic virus. In contrast, all known lyssaviruses utilize neurotropism. Direct evasion, and suppression of host immune surveillance, appears to be a critical evolutionary strategy [64]. Long incubation periods may be one consequence of reliance on flow within neural circuitry [65]. Adaptation to the CNS may also be a mechanism to lower the risk of extinction in a host that may pursue a more solitary existence for short periods of its life history. Since the reservoir host must eventually seek a breeding partner, an opportunity for transmission is readily available, considering the common use of teeth and oral mucosal contact preparatory to typical mammalian copulation, especially among carnivores and bats. Following bite inoculation, covert access to the CNS via neuron-to-neuron passage would be one unique mechanism for minimizing immune detection while reaching distant sites important for replication and transmission [62, 66]. Lyssaviruses are rather fragile, maintained by direct transmission, and do not survive readily in the external environment, where inactivation is operative. Dependence on fluid-sucking arthropod vectors is unnecessary, as are additional structural proteins to ensure extra-mammalian environmental survival, because in vivo transmission is largely a direct host-to-host event. For example, virus may perpetuate fox-to-fox, bat-to-bat, and so on, irrespective of firm constraints imposed by either geography or season.

5 Descriptive Epidemiology

Rabies is a zoonosis with diverse natural mammalian reservoirs [54]. Predominant hosts are bats and mammalian carnivores. These mammals include canids, such as the domestic dog (as the principal global reservoir, particularly in equatorial regions of Asia, Africa, and Latin America [27]), foxes (in the circumpolar Arctic, Central and Eastern Europe, the Middle East, and scattered foci throughout the western United States [29, 67]), raccoon dogs (in eastern Europe [68]), jackals (in Africa and Asia), and coyotes (historically in the western United States [29]); skunks (primarily in the central United States and Canada [29]); procyonids, such as the raccoon (in the southeastern, mid-Atlantic, and northeastern United States [29]); and mongoose species (in Asia, Africa, and several Caribbean islands [35]). Other conspicuous carnivores, such as bears, wolves, and all of the felids, can serve as effective short-term viral transmitters intra- and interspecifically, but insufficient documentation is available to suggest that these groups can continue to propagate virus or serve as reservoir hosts for unique viral variants, as opposed to primary infection by the primary host. "Bat" rabies from infection by rabies viruses per se is a New World phenomenon only, described primarily among the insectivorous species of North America and the hematophagous vampires and frugivorous species from Latin America [69, 70]. Ultimate evolutionary and ecological perpetuation of lyssaviruses appear to be driven by bats [71]. Distinct from the type species rabies virus, related lyssaviruses have been diagnosed in Australian, African, and European bat species [72–75]. While the disease is naturally maintained by relatively few taxa, rabies may affect any mammal, such as ungulates, and result in a largely "dead-end" infection. Contrary to popular belief, small mammals, such as rodents (mice, rats, etc.) and lagomorphs (rabbits, hares, etc.), are not important in rabies, and cases are uniformly rare [76]. Further, rodents have not arisen as "missing link" reservoirs in any region in which domestic dog or wildlife rabies has been controlled.

5.1 Incidence

To properly understand the occurrence of human rabies, the context of animal rabies is fundamental. Rather than a global rabies pandemic, single or multispecies animal host groupings are apparent when appropriate disease surveillance is practiced systematically. Combined with historical temporal and geographical data, both antigenic characterization and nucleotide sequence analysis can be used to "compartmentalize" viral isolates with different animal reservoirs responsible for their perpetuation and to estimate relative risks of human disease from differential animal exposures (Table 28.3). For example, during 2012, only one human fatality occurred; however, 6,162 animal rabies cases were reported in the United States (including the District of Columbia and Puerto Rico) [29] (Fig. 28.1). In contrast to fulminant canine rabies pre-World War II, >90 % of current animal rabies cases in the United States are from wildlife (Fig. 28.2, top). Most of this increase resulted from continued spread of a predominant raccoon rabies virus variant, following unrestricted progression of an outbreak initiated by animal translocation during the late 1970s to the Virginias from a nidus in the southeastern United States [77, 78]. Cases rise as infected individuals within raccoon populations encounter naive conspecifics with dramatic results.

Table 28.3 Rabies immunization: preexposure and postexposure

Criteria for preexposure immunization			
Exposure category	Nature of risk	Typical populations	Preexposure regimen
Continuous	Virus present continuously, often in high concentrations. Specific exposures may go unrecognized. Bite, nonbite, or aerosol exposures	Rabies research lab workers.[a] Rabies biologics production workers	Primary course. Serologic testing every 6 months. Booster immunization if antibody titer falls below acceptable level[ab]
Frequent	Exposure usually episodic with source recognized but exposure also might be unrecognized. Bite, nonbite, or aerosol exposures	Rabies diagnostic lab workers,[a] cavers, veterinarians and staff, and animal control and wildlife workers in areas where rabies is enzootic. All persons who frequently handle bats	Primary course. Serologic testing every 2 years. Booster vaccination if antibody titer is below acceptable level
Infrequent (greater than population at large)	Exposure nearly always episodic with source recognized. Bite or nonbite exposures	Veterinarians and animal control staff working with terrestrial animals in areas where rabies is uncommon to rare. Veterinary students. Travelers visiting areas where rabies is enzootic and immediate access to appropriate medical care including biologics is limited	Primary course. No serologic testing or booster vaccination
Rare (population at large)	Exposure always episodic. Bite or nonbite exposure	US population at large, including individuals in rabies-epizootic areas	No vaccination necessary

Adapted from CDC [5]

Preexposure immunization. Preexposure immunization consists of three doses of HDCV or PCEC vaccine, 1.0 ml, IM (i.e., deltoid area), one each on days 0, 7, and 21 or 28. Administration of routine booster doses of vaccine depends on exposure risk category as noted

Postexposure immunization. All PEP should begin with immediate thorough cleansing of all wounds with soap and water. *Persons not previously immunized*: HRIG, 20 IU/kg body weight, as much as possible infiltrated at the bite site (if feasible), with the remainder administered IM; 4 doses of HDCV or PCEC, 1.0 ml IM (i.e., deltoid area), one each on days 0, 3, 7, and 14. *Persons previously immunized*: Two doses of HDCV or PCEC, 1.0 ml, IM (i.e., deltoid area), one each on days 0 and 3. HRIG should not be administered. Preexposure immunization with HDCV or PCEC; prior PEP with HDCV or PCEC; or persons previously immunized with any other type of rabies vaccine and a documented history of an acceptable rabies virus-neutralizing antibody response to the prior vaccination

[a]Assessment of relative risk and extra monitoring of immunization status of laboratory workers are the responsibilities of the laboratory supervisor (see United States Department of Health and Human Services' *Biosafety in Microbiological and Biomedical Laboratories* [137])

[b]Routine Preexposure booster immunization consists of one dose of HDCV or PCEC, 1.0 ml/dose, IM (deltoid area), or HDCV, 0.1 ml ID (deltoid). The acceptable antibody level is a 1:5 titer (complete inhibition in the RFFIT at a 1:5 dilution, approximately equivalent to 0.1 IU/ml). Boost if titer falls below this level, as long as the person remains at risk of viral exposure

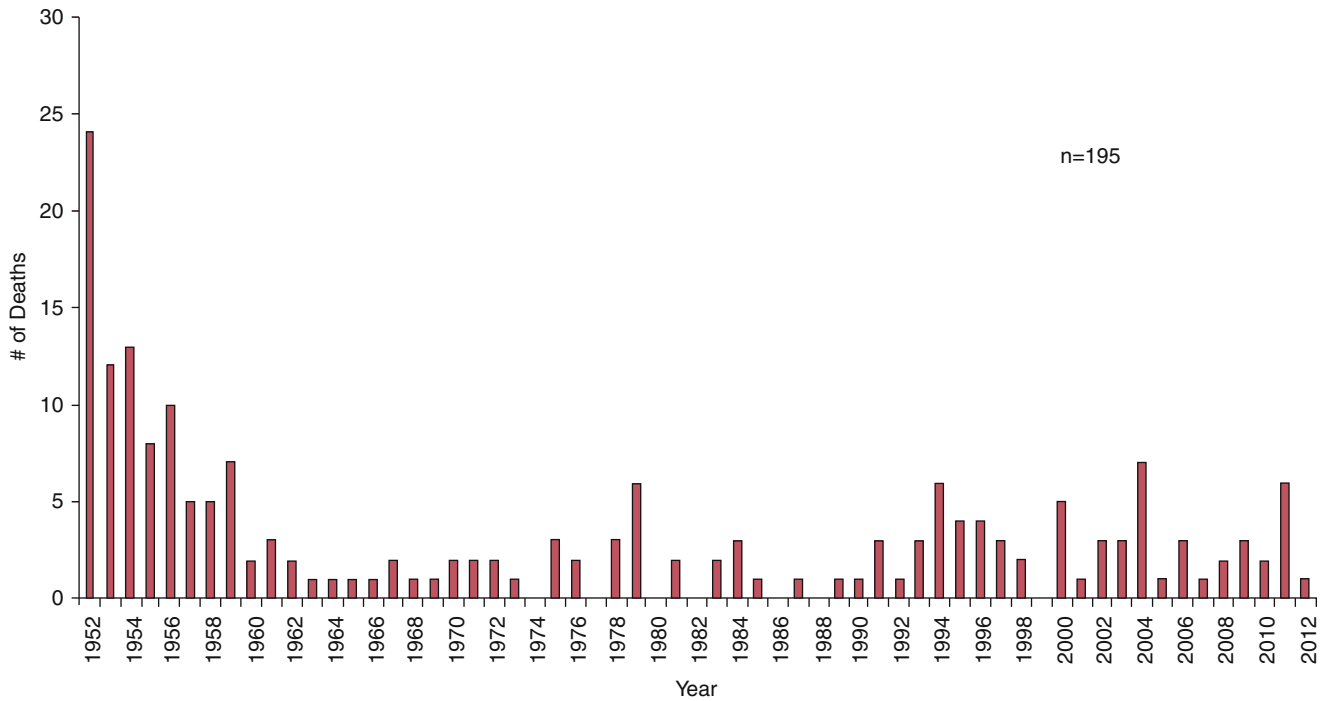

Fig. 28.1 Reported human rabies cases in the United States, 1952–2011 (Sources: Petersen and Rupprecht [81] and Dyer et al. [28])

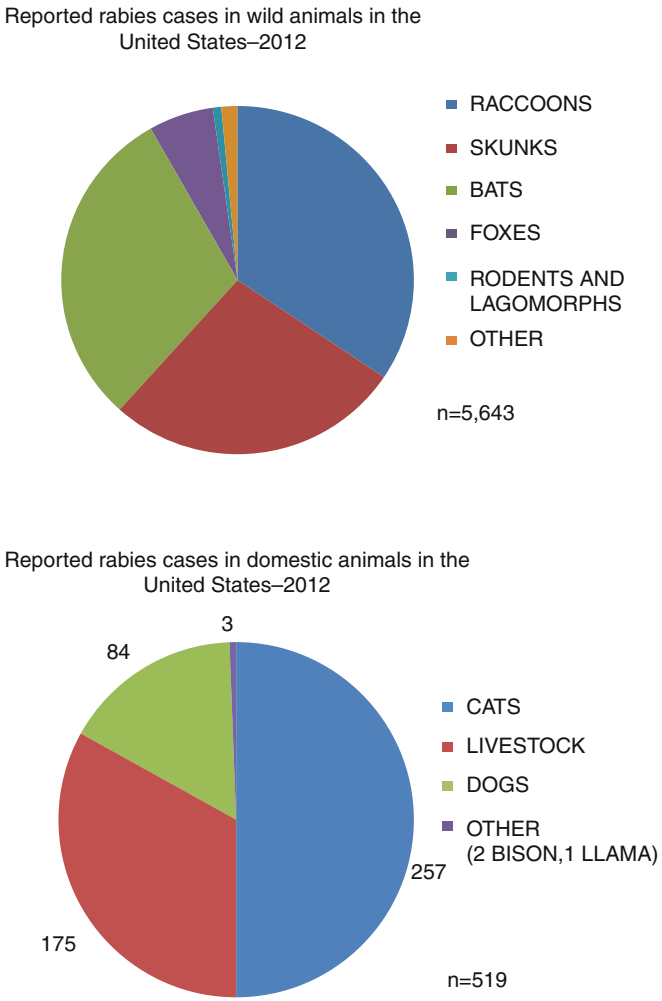

Fig. 28.2 (*Top*) Reported rabies cases in wild animals in the United States, 2012. (*Bottom*) Reported rabies cases in domestic animals in the United States, 2012 (Source: Dyer et al. [28])

During 2012, rabies was also reported in other important wildlife, primarily skunks (1,539 cases) and foxes (340 cases), in the Midwest and Alaska/northeastern United States, respectively, and rabies in bats (1,680 cases) was widely distributed [29]. In the eastern United States, rabies was reported in 1,953 raccoons during 2012 [29]. Historically, Hawaii is unique in the United States as never having reported a case of indigenously acquired rabies, attributable in part to its remote insular geographical isolation in Oceania and 120-day quarantine policy.

Domestic animals most at risk include those with a lower likelihood of parenteral vaccination but higher potential for virus exposure (especially if poorly supervised and free-ranging), such as cats. During 2012, 257 cases of rabies were reported in cats [29], compared with 303 cases during 2011 (Fig. 28.2, bottom) [79]. Cats are significant in part because they have begun to outnumber their canine counterparts, owing to the increased popularity of cats as pets, but also lower likelihood of routine vaccination. Difficulties in clinical recognition also increase the public health significance of rabies in cats, as early nonspecific signs may easily be compatible with unrelated differential diagnoses, and may be compounded by an abscess compatible with a bite wound in an outdoor cat [80]. By temporal comparison, 84 cases of rabies in dogs were reported in the United States during 2012, compared with 303 cases in cats reported during 2011 [79].

In contrast to areas enzootic for dog rabies with a consequent high case load (e.g., estimated at >20,000 annual human fatalities in India alone), human rabies is rare in developed countries. For example, between 1980 and 2011, only 71 human deaths were diagnosed in the United States, 20 of which were imported. Of the 45 cases for which viral variant data were available among those suspected of infection while in the United States during 1980–2011, 40 were attributable to bat rabies virus variants. By contrast during the same time period, of the 20 imported cases for which variant data were available, 18 (90 %) were attributable to dog virus variants [81].

5.2 Epidemic Behavior

Application of MAb and molecular technology to the study of the epizootiology of the *Lyssavirus* genus (previously felt to consist of a relatively homogeneous group of viruses) first provided substantive evidence for considerable variation within the group, based on the nucleoprotein (N) and glycoprotein (G) protein reactivities among both fixed and street rabies viruses and between rabies viruses and related lyssaviruses [15, 82]. The use of MAbs was particularly useful in determination of the extent of natural antigenic variation among lyssaviruses as well as epizootics—such as the raccoon epizootics in the eastern United States described below—either isolated from a variety of wildlife reservoirs within a fairly restricted geographical area or between continents. In particular, through the use of genetic sequence comparisons, distinctions became much more obvious between those viruses isolated from bats and carnivores [82].

5.3 Geographic Distribution

As illustrated in Table 28.2, rabies is distributed on all continents, with the exception of Antarctica, but varies greatly dependent upon the dynamics of certain viruses among particular mammalian hosts and the degree of operational surveillance, prevention, and control methods used in each geographical unit. Currently in the United States, the most prominent wildlife rabies hosts include raccoons, skunks, foxes, and bats [29].

Through MAb and molecular laboratory techniques applied to public health and agricultural surveillance, fox rabies outbreaks in Canada and Alaska appear related, as do the raccoon epizootics in the eastern United States [57, 78]. Skunk isolates group as distinct variants defining separate outbreaks in the north central/south central states and California. Such investigations, when extended over large geographic areas, lead to a better understanding of lyssavirus dynamics and improved animal rabies management programs, to distinguish recent viral introductions, potential vaccine failures, and so on.

Antigenic or molecular characterization can also be employed to investigate unusual or unexpected lyssavirus mortality, especially in domestic animals or humans that lack an obvious exposure history. The antigenic patterns or nucleotide sequences obtained can be compared with variants from known animal reservoirs. For example, analysis of recent human rabies cases from the United States implicates variants of virus associated with insectivorous bats (e.g., *Eptesicus*, *Tadarida*, *Myotis*, *Lasionycteris*, or *Perimyotis* spp.), indirectly or directly, in the etiology of infection. Unfortunately, bat rabies cases are not routinely identified to species. Thus, case surveillance data alone do not provide adequate clues about unique variants affecting different bat species or species groups. More rigorous reporting standards coupled with modern virological analysis will be necessary to provide further detail into epidemiologic facets of bat lyssaviruses.

In contrast to most countries in North America and western Europe, where wildlife rabies predominates, Southeast Asia, Africa, Latin America, and most developing countries, canids continue to be the principal causative vectors of transmission to humans [83]. Additionally, transmission by hematophagous bats, unique to the Americas, is an emerging public health problem, in addition to being a historically important disease of livestock, with widespread economic implications [69, 70].

5.4 Temporal Distribution

Human rabies occurrence is predicated in part by the seasonal patterns of the infected animal species. In the absence of an active or sensitive surveillance system for human PEP, epidemiologically it would be reasonable to investigate a potential association between increased animal movements, seasonality of human outdoor activities (adjusted for age), and exposures resulting in PEP. Due to the exceedingly low occurrence of human rabies mortality in the United States, there is no obvious meaningful historical trend in the temporal distribution of cases, with the exception of cases associated with bats. Most bat rabies cases appear in late summer. Associated human cases occur typically in autumn, after an incubation period of approximately 1–3 months [84]. Clearly, the overt limitations of a passive surveillance system for domestic animal rabies may obscure temporal trends in wildlife reservoir species and consequent risks for humans, as evidenced by the encroachment westward of the raccoon epizootic from the eastern seaboard, described above.

5.5 Age, Sex, Race, and Occupation: Socioeconomic, Nutritional, and Genetic Factors

There do not appear to be reliable trends associated with many risk patterns leading to viral exposures, with few exceptions. Racial, genetic, nutritional, or socioeconomic differences in risk of exposure have not been fully investigated, and there does not appear to be a trend in occurrence or susceptibility with regard to these factors, as well. If an active surveillance system for the reporting of PEP were established, these epidemiologic characteristics would be analyzed to further delineate sources of exposure and target preventive measures. For a variety of reasons, presumably related to exposure, there is a bias in gender (toward males) and in age (toward children) among human rabies cases. For example, in many developing countries children are the primary caretakers of animals and, accordingly, are more at risk for exposure to.

6 Mechanisms and Route of Transmission

Lyssaviruses are transmitted by saliva from the bite of a rabid animal, which may be infectious for days before obvious illness, but not prior to brain involvement [5, 17, 45, 65]. If the converse existed, thousands of human mortalities would be obvious following exposure to animals proven nonrabid by routine postmortem diagnostic examination of brain material. Moreover, if a true "carrier" state readily existed, in which most infected animals remained healthy while excreting large quantities of infectious material, human rabies would not be rare and the current practice of confinement and observation of the rabies-suspect biting dog, cat, or ferret would fail as an effective public health measure. Beyond the domestic dog, cat, and ferret, reliable observation periods have not been established routinely because the shedding period for virus is undetermined for most species [4].

Virus does not penetrate intact skin. Hence, touching a rabid animal does not constitute exposure. Other nonbite routes of natural viral transmission have been documented very rarely and largely remain unimportant epidemiologically [85]. For example, contamination of mucous membranes with infectious material is considered the major nonbite route of

exposure, and as previously discussed, transplant recipients of tissues or solid organs have succumbed, in which the donors had died of undiagnosed rabies [51–53]. In February 2013, a patient in the United States died of rabies 18 months after receiving a deceased-donor kidney transplant. The donor, in retrospect, had displayed signs and symptoms consistent with rabies upon death: vomiting, upper extremity paresthesias, fever, seizures, dysphagia, autonomic dysfunction, and subsequent brain death. At the time of his death, his symptoms were thought to have been caused by ciguatera, a foodborne toxin [86]. The rabies viruses infecting the donor and deceased recipient of the kidney were found to be consistent with the raccoon rabies virus variant and were more than 99.9 % identical across the entire *N* gene (1,349/1,350 nucleotides), thus confirming organ transplantation as the route of transmission [86]. Three additional persons had received organs from the deceased donor: the other kidney, heart, and liver. All three persons remained asymptomatic for the 18-month period between organ receipt and administration of PEP, recommended upon retrospective diagnosis of rabies in the donor, and to date have not shown signs or symptoms of rabies. A comprehensive public health investigation identified a further 38 contacts of the donor and 17 contacts of the recipient who were recommended to receive PEP; the investigation further revealed that the kidney donor was an avid hunter and trapper with frequent wildlife exposures and at least two raccoon bites, for which he did not seek medical care, in February 2010 and in January 2011 [87]. Four cases of apparent aerosol transmission have been implicated: two individuals working with extremely dense populations of bats in the confines of caves (vs. millions of cavers who may do the same) [88] and in two laboratory workers who were exposed to high concentrations of aerosolized rabies virus [89, 90], and in 2002 a bat conservationist in Scotland died of infection with European bat lyssavirus 2. It is posited that the individual acquired the virus via inhalation [91]. These considerations aside, there is a theoretical risk of human-to-human transmission resulting from contact with rabid patients [49, 50]. Although suspected on occasion in both wild and domestic animals, neither the oral route nor other unusual means, such as transplacental transmission, have been implicated definitively as important in human rabies cases to date, partially because of the difficulty in ruling out the traditional bite route as the primary means of exposure, especially in regions with enzootic dog rabies.

7 Pathogenesis and Immunity

The final outcome after viral exposure, leading to either death, or apparent immunity, is complex. Following local introduction at the site of a bite, the virus may remain localized and undergo limited replication at extraneuronal sites, such as in muscle tissue. Currently, it remains unclear if this period during which the virus is virtually undetectable represents a phase of extraneuronal infection [62] or coincides with direct infection of the nervous system [66]. During these early phases of virus infection, the neuronal synaptic transfer may occur in the form of ribonucleoprotein–transcriptase complexes [64], rather than the assembly and passage of complete virions per se, as regularly occurs in later phases of replication. After a relatively quiescent early phase, viral antigens can be found distributed in most parts of the CNS [92]. A temporal relationship between viral RNA synthesis and immediate-early gene mRNA expression suggests an activation and upregulation mechanism may be responsible for the burst of viral activity after a variable incubation period [93].

Rabies is often considered as a disease due to prototype neurotropic viruses. Multiple nonneural sites have also been associated with viral antigens following centrifugal transport from the CNS [62]. Studies suggest that the relative proportions of infectious virions will vary depending on the end organ examined [94]. Typical exit portal tissues important in transmission, such as salivary glands, support high viral titers and contain only a minimal accumulation of matrix and deviant viral production products, while multiplication in other nonneural sites, such as adrenal glands and mucous glands of the nasal mucosa, produce only moderate viral concentrations but large amounts of viral nucleocapsid and anomalous structures [94]. Further, antigens may be found in free sensory nerve endings of tactile hair in a skin biopsy, which is one of the best diagnostic methods of confirming an antemortem diagnosis of rabies in humans [95].

One relatively unique characteristic of lyssavirus infection is consequent behavioral changes that may favor transmission, most notably irritability and the predilection to bite. Other more subtle behavioral changes may occur as well. For example, in the case of raccoon rabies, induction of juvenile vocalizations in an infected adult host has been observed, which may enhance investigation by conspecifics, bringing into close proximity another susceptible host.

Mechanistically, behavioral manifestations of "dumb" or "furious" rabies in a given host may be dependent on the specific accumulation and differential effect of virus in selective brain areas, and these two extreme clinical presentations may likely reflect differences in viral infection within specific areas of the CNS. Further, an individual may alternatively manifest both of these generalized forms [96]. Host incapacitation during clinical disease may be obvious and dramatic. As such, lyssaviruses have been characterized as poorly adapted or imperfect parasites because infection typically results in host death. However, in an ecologic sense, the type of behavioral changes associated with classic rabies may ensure viral perpetuation regardless of ultimate host outcome, provided transmission of progeny virus has occurred before host demise.

While the phenomenon of differential susceptibility of mammalian hosts to various viral variants is clear, the mechanisms underlying such observations are not. For example, free-ranging raccoons may have serum VNA detected even in areas without raccoon rabies [33]. Moreover, while skunks inoculated with a Midwestern skunk isolate succumbed within 4 or 5 weeks, raccoons infected with several greater concentrations of virus did not succumb or shed detectable amounts of rabies virus; 2 of 12 raccoons developed VNA and were the only two that survived subsequent rabies virus challenge [97, 98]. Such observations may help to explain the differential occurrence of rabies in Midwestern skunks, without apparent perpetuation in raccoons. In the eastern United States, raccoons are competent hosts for a different variant, which does not appear to be independently supported by skunks. Additionally, the relative resistance of the only North American marsupial, the opossum (*Didelphis virginianus*), to rabies virus and its paucity of acetylcholine receptors provide experimental data suggesting that host susceptibility may be influenced in part by receptor occurrence and relative abundance, to which virus may bind and be internalized [99].

Rabies VNA, induced solely by the viral glycoprotein, is believed to play a major role in the pre- and PEP protection against rabies [22]. However, comparison of absolute VNA titers and mortality in laboratory rodents immunized with whole virus vaccine or viral glycoprotein did not delineate a clear role for VNA in vaccine-induced resistance to disease [100]. Specific discrepancies between VNA titer and rabies protection were also shown in wild carnivores where no firm correlation existed between any known titer level of VNA resulting from oral vaccination and protection per se [101]. Data from immunization experiments suggest that protective activity may ultimately correlate with a vaccine's ability to induce immunologic memory. Lack of firm correlation between absolute VNA titers and survivorship in immunized animals suggests that, in addition to VNA, other immune effector mechanisms may be involved in the protection against lethal rabies virus infection in either pre- or PEP situations [22, 31].

Studies have demonstrated that in addition to glycoprotein [2], the internal viral ribonucleoprotein (RNP) may also be important to the induction of protective immunity [102, 103]. In related studies following RNP immunizations, primates developed a strong anti-RNP antibody response, were protected against lethal rabies, and were primed for the induction of a VNA response following exposure to antigens [104]. These experiments and others suggest that the RNP plays an important role in inducing both primary protective immunity and cross-protective immunity against infection to heterologous strains. Since RNP is a strong inducer of nonneutralizing antibody, one of the functions of RNP-specific antibodies may be the promotion of viral RNP attachment via Fc receptors to phagocytotic cells, stimulated by virus to produce cytokines such as interferon, which inhibits viral replication.

8 Patterns of Host Response

8.1 Clinical Features

The bite of a rabid animal may often be too superficial to produce a productive infection. Even if exposure is effectively transdermal, the bite must be concomitant with the presence of virus from the infected host, which may be excreted only intermittently. A host is only considered potentially infected once the virus has breached the skin or mucous membrane barriers. This is not synonymous with disease but is partially dependent on viral load and variant characteristics within a host ranging from fully susceptible to immune. Host immune response following viral exposure may or may not result in the production of detectable amounts of VNA. While in theory only a single virion is necessary, a fully productive infection usually requires numbers of fully infectious particles several orders of magnitude greater to produce disease.

"Inapparent" infection of long duration may occur rarely, but has not been demonstrated to be of epidemiologic importance, regardless of widespread speculation to the contrary. Distinct from inapparent or so-called latent infection (which is a misnomer compared to the latency of other truly persistent herpesvirus or retroviral infections), the incubation period associated with overt disease can be highly variable in rabies and prolonged, during which the host is not infectious [85]. Productive infection, during which input virus replicates, may result in viral encephalitis that manifests with neurologic signs and symptoms. Perhaps the only consistent feature of the resulting clinical presentation is that it is rarely typical from one host to another. The prodromal stage is uniformly acute, lasting from 2 to 4 days, including moderate fever, malaise, anorexia, headache, and nausea. Presenting symptoms in humans are often nonspecific. However, paresthesia at the site of the bite and aerophagia or hydrophobia (spasms of pharyngeal or inspirational musculature induced by air currents or anticipation of swallowing) are highly suggestive of rabies. Physical and mental status deteriorates rapidly. Not discounting classical Pasteurian observations of spontaneous recovery in animals or the remarkable contemporary accounts of the few human survivors [81], the case fatality proportion in practice still approaches unity, once clinical signs are present. Death is usually attributable to respiratory or cardiac failure.

8.2 Diagnosis

The incidence of more common infectious agents that result in human encephalitis should be compared to the relative rarity of rabies in the establishment of a diagnosis. Pairing of a known animal exposure followed in several weeks' time with the appearance of classic signs and symptoms should alert the astute infectious disease professional. However,

rabies should also be considered in any suspected viral encephalitis of unknown origin, regardless of a definitive history of animal bite, especially once a compatible clinical syndrome is manifest. An exposure may have been unrecognized, forgotten, or discounted. In humans, differential diagnoses include herpesvirus and arboviral encephalitis and poliomyelitis, among others. Although lyssavirus infection and pathogenesis can be described experimentally, there remains no practical or reliable method for detecting exposure or infection in humans until the CNS has been extensively infected and there has been centrifugal distribution of virus via peripheral nerves. At this point, rabies may be diagnosed in humans through positive FA results on brain biopsy, full-thickness skin biopsy from the nape of the neck or on a corneal impression, virus isolation from saliva, detectable antibody in the CSF or serum (the latter from an unvaccinated individual), or demonstration of viral amplicons in samples of CNS, saliva, skin, or other tissues. Negative results do not rule out rabies. Optimal diagnostic material is fresh brain tissue for detection of viral inclusions in neurons. Use of appropriate laboratory-based techniques is the only method to confirm a suspected rabies case based upon clinical appearance alone and/or a history of likely exposure to infectious material.

9 Prevention and Control

9.1 Basic Epidemiologic Methods

A modern preemptive public health strategy against rabies encompasses prompt and proper PEP of humans bitten by infected animals, prevention of disease among companion animal species by preexposure vaccination, and the ecologically sound management and control of rabies among free-ranging reservoirs. While a focus upon the exposed person is an obvious and primary biomedical responsibility, the ultimate management of the host is the only way to selectively eliminate rabies in animals. Management of animal rabies extends beyond domestic animals. Since the conception of oral rabies vaccination (ORV) of wildlife during the 1960s at the CDC and the initial field experiment during 1978 in Switzerland with modified live rabies virus, the distribution of millions of vaccine doses in Europe and North America attests to the utility of this technique, with local disease elimination as one intended result [67, 105–109]; as evidenced by the elimination of fox rabies in many sites in Europe and southern Canada, and in the United States of a coyote rabies virus variant [61]; the suppression toward elimination of gray fox rabies, as exemplified in west-central Texas [60]; and the containment of raccoon rabies to the eastern states.

With regard to domestic animals, for millennia prior to the modern age and through the first half of the twentieth century in the United States, canine rabies was enzootic throughout the world [110]. In the early 1880s using virus obtained from a rabid dog and serially passed in rabbits by intracerebral inoculation, Pasteur vaccinated a series of dogs and subsequently challenged them with rabies virus resulting in acceptable results; additional challenge studies using monkey intracerebral inoculation allowed Pasteur to demonstrate that dogs became resistant to additional challenge with virulent wild rabies virus [111]. Post-Pasteur, refinement and evolution of vaccine designed to protect dogs against rabies resulted in extensive vaccination campaigns implemented during the 1940s and 1950s and subsequent decline of rabies reported in dogs in the United States [110]. Today in the United States, rabies vaccine is administered paranterally to dogs every 1 or 3 years depending on local or state requirements. However, despite the successes in the United States, canine rabies remains responsible for the most human rabies deaths globally. The domestic dog in developing countries is often unowned, "community" owned, or otherwise neglected by agricultural veterinary services [112]. Canine population mismanagement is one contributor: although vaccination campaigns in the developing world have been found to achieve the WHO recommended vaccination coverage of ≥70 %, high dog turnover rate in the population (i.e., introduction of new susceptible individuals) contributes to enzootic canine rabies [83]. Considering the success of the elimination of canine rabies virus transmission in the United States, the burden of rabies deaths attributable to dogs throughout the developing world, and availability of vaccine, surveillance, and laboratory diagnostics, the need for a global effort to achieve the elimination of canine rabies transmission is increasing. A tool developed in the context of promoting attention to the interdependency of human and wildlife health, i.e., One Health, aims to assist the global public health community in achieving this goal and eliminate the approximate 55,000 deaths per year attributable to rabies through The Blueprint for Rabies Prevention and Control [113].

With a concentration upon wildlife, a vaccinia-rabies glycoprotein (V-RG) recombinant virus vaccine [114–116] was developed that has proved to be an effective oral immunogen in raccoons [101, 117] and a variety of other important species [118–122] and provides long-term protection against rabies [120]. Advantages of this recombinant orthopoxvirus vaccine included greater thermostability than attenuated rabies viruses and the inability to cause rabies, because only the cDNA of the surface glycoprotein of the rabies virus was inserted within the recombinant virus [114]. The period of the 1980s was marked by intensive, collaborative, safety evaluations of the V-RG virus under laboratory conditions in North America and Europe [120, 123] that culminated in the initial limited field experiments by the end of that decade [124, 125].

Despite the successes of V-RG in conferring immunity against rabies among several wildlife species including raccoons, V-RG is not efficacious for control of rabies in several other important reservoirs, such as *Mephitis mephitis*, the striped skunk [121]. Further, less-attenuated, first-generation rabies virus candidates, such as the rabies virus strains SAD/ERA, may be associated with vaccine-induced rabies in these species [126]. For safe, effective, and more comprehensive wildlife rabies control, a human adenovirus type 5 (HAdV-5) vectored rabies virus glycoprotein recombinant vaccine (AdRG1.3; ONRAB®) has been tested for safety and efficacy in the laboratory and used in the field in Canada and is currently being considered for broader use throughout North America. Adenoviruses belonging to the family *Adenoviridae*, subfamily *Mastadenoviridae*, are nonenveloped DNA viruses found ubiquitously among many mammalian species, including humans [127]. The ONRAB® vaccine employs a replication-competent HAdV-5 vector into which a DNA copy of the ERA virus glycoprotein gene has been inserted [128, 129] and, similarly to V-RG, is packaged into sachets for oral administration to wildlife. Results from field trials in Canada have indicated that ONRAB is stable both in vitro and in vivo, with a limited ability to replicate in an established model for human adenovirus infection and is suitable for environmental use as an oral rabies vaccine for wildlife [130].

Some opponents to wildlife ORV espouse depopulation, rather than vaccination, as a control method, citing the effectiveness of dog rabies control via compulsory muzzling, movement restrictions, and stray animal removal in Europe and North America during the early 1900s, before the utilization of efficacious vaccines en masse. Two components of this technique—animal movement restrictions and muzzling—are inapplicable to wildlife, leaving only depopulation. As a sole mechanism of control, depopulation may decrease the local intensity of rabies or postpone the invasion of a new geographic area, but it is unlikely to completely eliminate rabies or permanently protect a region from an impending epizootic.

Several professional groups may view a number of wildlife species, including some rabies vector species, as nuisance or pest species, and hence oppose ORV, citing the undesirable potential for increased numbers following elimination of rabies, inappropriately viewing rabies as a form of wildlife population control. However, proportionate mortality due to rabies among the most prominent wildlife vectors in the United States, such as raccoons, skunks, and so on, has not been investigated. In Europe, collective data suggest that rabies may not be a significant regulator of population size; there has been no substantial increase in red fox numbers following the elimination of rabies over large geographic areas [131].

Addressing bat rabies control, the importance of bat exclusion from human habitations and better public education to facilitate recognition of potential exposures cannot be overemphasized. Overall, bat conservation is very compatible with basic public health tenets.

9.2 Basic Immunization Concepts and Practice

For practical purposes, rabies may be viewed as universally fatal once clinical symptoms manifest. In the same context, extremely effective human PEP consists of vaccine and RIG if administered promptly and properly, after thorough wound cleansing, if a bite is evident. Besides application in humans, the extension of PEP to companion animals and livestock has also been investigated, such as in dogs [132, 133] and sheep [134]. Moreover, further epidemiologic investigations to include the use of PEP for animals should be conducted, from a One Health perspective within the veterinary field, considering the critical role of domestic animals in human society for food, fiber, security, companionship, transportation, etc., among others.

As described earlier, minimum standards as opposed to an absolute "protective antibody" for humans are based empirically on presumed protective activity of rabies-specific antibodies and can be detected by reference laboratories. A RFFIT titer of ≥1:5 (approximately 0.10 international units [IU]/mL) or higher is evidence of adequate immunization in persons at either constant or frequent risk of exposure, at 6-month or 2-year intervals, respectively, as a measure of baseline immunity, as recommended by the United States Advisory Committee on Immunization Practices [85]. A single booster vaccine dose is administered if the level is lower, based on a determination of apparent risk (Table 28.3). Preexposure immunization simplifies human PEP because of priming of the immune response and is thought to provide some degree of protection against unrecognized exposures, which by definition should be negligible if proper protocols are followed. Nevertheless, when pre-immunized persons are knowingly exposed to rabies virus, two booster doses of vaccine, administered on days 0 and 3, are required to induce a sufficient anamnestic response to prevent development of disease. Also, rabies immune globulin (RIG) is contraindicated under such circumstances, because it may interfere with development of an adequate anamnestic response [135]. Pre-immunized individuals should remain vigilant in recognizing potential viral exposures and seek appropriate PEP. If actual viral exposures occur but are unrecognized, the pre-immunized individual may still die of rabies, albeit very rarely, as did one Peace Corps volunteer, who was bitten by her own puppy, that died of a disease compatible with rabies [136] (Table 28.4).

Table 28.4 Rabies postexposure prophylaxis (PEP) guide, United States

Animal type	Evaluation and disposition of animal	Postexposure prophylaxis recommendations
Dogs and cats	Healthy and available for 10 days observation	Should not begin PEP unless animal develops signs of rabies[a]
	Rabid or suspected rabid	Initiate PEP immediately[b]
	Unknown (escaped)	Consult public health officials
Raccoons, skunks, bats, foxes, other carnivores, woodchucks	Regard as rabid unless geographic area is known to be free of rabies or until animal is proven negative by laboratory tests	Initiate PEP[c]. Consider factors such as provocation, suggestive clinical signs, severity of wounds, type of exposure, and timeliness of test results (24–48 h) for decisions regarding immediate initiation or to delay pending test results
Livestock, rodents, and lagomorphs (rabbits and hares)	Consider individually	Consult public health officials; bites of squirrels, hamsters, guinea pigs, gerbils, chipmunks, rats, mice, other rodents, and lagomorphs almost never require PEP

Adapted from the ACIP

[a]If clinical signs compatible with rabies develop during the 10-day confinement and observation period, the animal should be euthanized and tested. Depending on circumstances, initiation of PEP may be delayed pending a positive report, if results may be obtained in 24–48 h

[b]If the bite was unprovoked or resulted in severe wounds, treatment of the bitten person should begin immediately with human rabies immune globulin (HRIG) and human diploid cell vaccine (HDCV) or purified chick embryo cell vaccine (PCEC). PEP may be discontinued if the test is negative

[c]If available, the animal should be euthanized and tested as soon as possible. Holding for an observation period is not recommended since the potential viral shedding period prior to clinical signs has only been determined for dogs, cats, and ferrets

Newer approaches to immunization from conventionally prepared products may also include subunits such as DNA vaccination. For example, mice immunized intramuscularly with a plasmid vector expressing the rabies virus glycoprotein under a SV40 early promoter developed specific cytotoxic lymphocytes, thymic lymphocytes (subset TH1), and rabies VNA and were protected against rabies virus challenge [138]. Although many questions remain regarding the consequences of persistent "infection" with a plasmid, the effects of long-term stimulation of the immune system, the genetic stability and safety of foreign promoters and nucleic acids, and the potential benefits are clear, which include simplicity and economy. Obviating the need for booster immunizations would be particularly useful for certain applications, such as livestock in extensive range situations, for example, where protection against bovine paralytic rabies via vampire bats in Latin America with current vaccines is prohibitive economically and logistically and could have similar utility for humans in remote locations.

Viral sources as substrates for vaccine development could also be expanded. Compared to the conserved fixed virus strains that form the basis for most rabies virus vaccine production historically, considerable diversity is obvious among lyssavirus antigens by MAb analysis. Nevertheless, even recombinant rabies virus vaccines that only express one viral glycoprotein are able to fully protect animals from severe experimental and field exposure against all of the major rabies variants. Moreover, after considerable investigation, there is no firm evidence to suggest that current human rabies deaths are attributable to antigenic variation of street rabies viruses, but rather they appear due to serious omissions in basic PEP protocols [23].

Besides epidemiologic utility (for which they were originally generated), antirabies MAbs are also useful in the refinement of host immunization and PEP concepts. Rabies VNA are induced solely by the viral glycoprotein and play a major role in the immune protection. Hybridomas that secrete rabies virus antigen-specific MAb have been generated and can effectively neutralize fixed and street lyssavirus isolates. These MAbs were selected on the basis of isotype, antigen and epitope specificity, virus strain specificity, affinity, and neutralizing activity. Administration of such MAbs protected animals when challenged with a lethal dose of rabies virus in experimental PEP models [139]. These data strongly suggest that MAbs, alone or in combination with vaccine, may be an effective method of protection against clinically relevant lyssaviruses [140]. Such an approach has several theoretical advantages over presently used hyperimmune sera: first, in contrast to current products, comparatively small volumes of MAbs would have to be inoculated for equivalent active protein content, because specific neutralizing activity per mass of protein is higher, so MAbs may be optimal for lessening the trauma and pain of local wound infiltration with a source of passive antibodies, and second, safety issues arising from the possibility of adventitious agents associated with human or animal blood products would be alleviated by bulk production under modern GMP conditions in cell culture. Despite the experimental progress shown, the rationale and acceptability of heterogenous murine MAbs for future human rabies PEP have been questioned on the grounds that a human anti-mouse response to the MAbs would present a significant drawback by the consequent effects on kinetics and antigenic targeting. If murine or other heterologous MAbs are used, human anti-species responses might be expected, but these are not necessarily

deleterious, because MAbs would only be used once in rabies PEP. As is the case for HRIG, MAbs would not be readministered should the person be reexposed in the future. In the previously vaccinated subject, rabies PEP in these situations consists of vaccine only on days 0 and 3. Moreover, if such MAbs are recognized as foreign antigens, with an expected shortened serum half-life, this potentiated clearance may be advantageous by minimizing the opportunity for interference with an active immune response on behalf of the vaccinated host, while effectively neutralizing virus, prior to induction of host VNA. This is one of the primary reasons cited for homologous or human MAbs, as opposed to heterologous. As such, several homologous MAbs are under clinical evaluation [141].

Given the above considerations, what is an appropriate gauge for immunoprotection for extension to humans? Mere in vitro neutralizing capacity alone may not be fully indicative of protective efficacy in vivo. Some MAbs that do not neutralize viruses in cell culture are still effective when administered via PEP to animals [141]. Besides extracellular neutralization, other useful capabilities of MAbs may also be operative, such as the inhibition of intracellular viral spread and interference in transcription of rabies viral RNA. Such questions, as well as the ability to consider the conduct of phase III clinical applications, should require introspection as MAbs progress toward regulatory approval for use in human PEP.

10 Unresolved Issues

10.1 Rabies Management in the Free-Ranging Dog: Oral Vaccination (ORV) as a Solution?

Rabies management of animals is linked directly to human rabies prevention. One of the most cost-effective methods to prevent rabies in humans is the mass vaccination of dogs by the parenteral route. Whether oral delivery of vaccination is needed for application to dogs remains uncertain. During the past century, considerable progress has been made in the laboratory development and field testing of ORV for free-ranging wildlife disease prevention and control in Europe and North America. Major species have included the red and gray fox, raccoon, raccoon dog, and coyote. Oral vaccines tested in the field to date have included attenuated rabies (e.g., SAD/ERA/SAG) and recombinant glycoprotein (e.g., orthopox-, adeno-) viruses. Especially with regard to any application for domestic canine vaccination, intended biologics should at minimum be pure, potent, efficacious (against a virulent, epidemiologically relevant rabies virus challenge), and safe to target and nontarget species, especially humans, at risk for exposure to distributed, edible baits, or through direct, intimate contact with a vaccinated host.

Self-replicating attenuated or recombinant vaccines offer the greatest versatility for oral rabies vaccination of animals such as dogs. While a few candidate oral rabies vaccine preparations have demonstrated sound protective immunity in captive dogs, no self-replicating viral system, conventional or otherwise, may be considered completely apathogenic, especially in the context of the immunocompromised host [142]. As an alternative, inactivated oral preparations should maximize safety considerations, yet the significant quantities required for minimal efficacy may be cost-prohibitive unless novel adjuvants, delivery techniques, or production methods are found [143].

Applied research on the oral or enteric administration of affordable inactivated rabies biologics may diminish the public health concerns related to the current products under consideration for application to free-ranging dogs, particularly as they relate to human rabies deaths in developing countries. However, regardless of the approach, limited laboratory trials of oral canine vaccination may not directly equate to rabies control under field conditions, where a combination of ORV and traditional parenteral vaccination may be necessary. This may be especially applicable when considering that the majority of successful canine rabies elimination programs have only involved traditional parental application, and not canine ORV.

10.2 Alternatives to Current Biologics for Human/Animal Disease Prevention?

While current biologics to prevent rabies in humans, domestic animals, and wildlife are extremely effective, progressive development toward new paradigms is necessary. The vigilance necessary to minimize human rabies mortality necessitates an extensive public health infrastructure and requires the annual prophylaxis of tens of millions in the developing world and tens of thousands of potential exposure cases throughout a developed country, such as the United States. Following a bite from an infected mammal, PEP of rabies in humans includes proper wound care and the simultaneous administration of multiple doses of an efficacious rabies vaccine, together with a preformed antibody source, typically HRIG. However, this regimen presents certain obstacles. Modern inactivated cell culture vaccines and HRIG are vastly improved over historical NTO vaccines, but such products are relatively expensive (especially in the developing world where they are most needed), are often in scarce supply, and may carry a perceived theoretical risk of adventitious agent acquisition to the public. Major concerns in rabies prevention have concentrated on the need for potent, inexpensive PEP for humans, especially replacement for more costly HRIG, while retaining activity against a wide variety of diverse lyssaviruses.

In contrast to the relatively impotent equine antirabies serum used in the past that resulted in high adverse reactions, such as serum sickness in up to 40 % of human recipients, modern purified equine rabies immune globulin (ERIG) products are safer, more potent, and more affordable than older cruder products and may be at a fraction of the cost of HRIG [144–146]. The ERIG products have been used effectively in conjunction with vaccine in human rabies PEP, particularly in developing countries under the continued threat of enzootic dog rabies. In surveys, less than 1 % of humans have reported adverse events, coupled with adequate efficacy [147]. By comparison, no ERIG has been available in the United States market for several years, although it was still licensed into the late 1980s. Due to its potency and lack of apparent significant local or systemic effects, purified ERIG products were the only immediate alternative, should the supply of available HRIG be threatened by shortages, contamination, or other limitations. Such use might be considered a temporary antecedent until the availability of more novel replacements, such as MAbs.

To be most effective, conventional PEP should be applied prior to viral invasion of the nervous system. However, PEP may still be effective even after virions access the nervous system. For example, in one experimental animal infection study, analysis by RT-PCR has revealed the presence of rabies virus-specific RNA in the olfactory bulb and cerebral cortex of animals within 6 h of direct intranasal inoculation. Yet, when animals were administered a neutralizing MAb up to 24 h after inoculation, 80 % survived a challenge in which all controls succumbed [139, 140]. Neither virus nor virus-specific RNA was detected when these survivors were euthanized a month later. These data clearly demonstrate that virus "neutralization" and clearance from the CNS are complex processes, less than fully understood [148]. Thus, if produced in a cost-effective manner, antirabies MAbs may be useful in the future PEP of humans, as well as in economically or otherwise important, unimmunized domestic animals. Such biologics that abrogate infection even after virus enters the CNS present exciting new possibilities for intervention with significant advantages over historical polyclonal rabies immune globulin.

10.3 Treatment of Clinical Human Rabies?

While prevention of disease among reservoir hosts and victims alike is still the optimal approach, when this fails, further alternatives regarding treatment of clinical rabies may be warranted. Historically, the individual experimental application of RIG and Ig fragments, cytosine or adenine arabinoside, interferon, acyclovir, antithymocyte globulin, steroids, vidarabine, tribavirin, inosine pranobex, or ribavirin has not demonstrated any substantive utility to date [8]. However, case histories of recovery after clinical signs in animals suggest that host defenses may be exploited to alter the end stage of this otherwise fatal malady, if the pathophysiologic mechanisms of disease can be understood.

As one now classical example, during 2004, a girl aged 15 years in Wisconsin rescued and released a bat, which had bitten her on her left hand. The wound was cleaned with peroxide, but PEP was not administered. Approximately 1 month later, she developed a clinical syndrome consistent with infection with rabies virus, including generalized fatigue and paresthesia of her left hand, which later developed into diplopia, ataxia, nausea, and vomiting. On the fourth day of illness, blurred vision, left leg weakness, and ataxia were present which developed into fever, slurred speech, nystagmus, and tremors of her left arm [149]. She was admitted into a pediatric referral facility in Milwaukee. On the second day of care, CDC confirmed the presence of rabies virus-specific antibody in the patient's CSF and serum. However, attempts to isolate virus, detect viral antigens, and amplify viral nucleic acids from two skin biopsies and nine saliva samples were unsuccessful [149]. An experimental treatment protocol, later termed the "Milwaukee Protocol" that combined anti-excitatory and antiviral drugs, including ketamine, ribavirin, and amantadine, was administered in conjunction with supportive intensive care [149]. Neither rabies vaccine nor HRIG was administered because of the patient's demonstrated immune response and the potential for potential harm from a potentiated or altered immune response [150]. After more than 70 days of hospitalization, the patient recovered, with only minor neurologic sequelae at the time. While promising, this experimental protocol has been attempted in several additional human rabies cases without success. Clearly, prevention of exposure, or prompt appropriate prophylaxis after exposure, but before development of clinical signs, remains a primary focus in public health. While there is no established therapy that is effective for patients who develop rabies, efforts should continue on basic viral pathogenesis research, development of relevant surrogate animal models and protocols that mimic supportive intensive care, and experimental applications in human cases where ethical/legal approvals and modern facilities exist [151].

10.4 Animal Translocation and the Threat of Disease Introduction/Reintroduction?

People who reside in true rabies-free areas enjoy a luxury of not having to be concerned with PEP following an animal bite. However, given the availability and rapidity of modern transportation, alarming trends are evident in animal translocation on local, regional, and intercontinental levels. Such deliberate translocation, but often with unintended results, carries a significant risk of the emergence of infectious agents into new niches previously unavailable

because of zoogeographic barriers. There are limited legitimate reasons for purposeful animal translocation, such as for species reintroduction, research, education, or zoological captive breeding. Unrestricted importation of nonnative species, or translocation of native mammalian species during the incubation stage of the disease, poses the threat of rabies introduction into rabies-free regions. Within rabies enzootic countries, there is also a danger that other variants may be introduced into new areas, such as occurred in the late 1970s with translocation of infected raccoons from the southeastern United States, resulting in the initiation of the mid-Atlantic raccoon rabies epizootic, which perpetuates along the Atlantic seaboard states. The same potential looms over the transportation of infected carnivores to unaffected states for hunting, trapping or other recreational use. Translocation of infected wildlife (i.e., bats and canids), from enzootic rabies areas in one country to another, would hamper public health efforts at wildlife rabies control, if they escape. Little is known about the clinical signs, incubation periods, or potentials for transmission of related agents, such as other Old World lyssaviruses to New World hosts via importation of reservoir species; such establishment would be problematic, owing to the questionable vaccine efficacy against certain nonrabies lyssaviruses. Enlightened and targeted education, enforced legislation, improved regional surveillance, and a rapid, appropriate public health response will minimize the overall dangers associated with animal translocation and will hopefully prevent an infectious nidus from becoming an epizootic, as seen increasingly in island nations, such as Indonesia.

10.5 Reservoir Population Management: Immuno-Contraception of Animals?

As most humans are infected with rabies virus after a bite, new focused animal management strategies appear obvious to minimize the burden of disease in people. Rabies is but one of a multitude of factors that arguably may influence the density of free-ranging carnivores over time [131]. One criticism of wildlife rabies vaccination is a concern that by decreasing or eliminating rabies mortality, a program may increase local carnivore populations over time, which in many instances may be undesirable. Historically, lethal means to control carnivore populations have been employed extensively, primarily to decrease economic loss from predation on livestock. Additionally, limiting or reducing carnivore populations may be beneficial for the protection of other, more "valued" animals, including important game, endangered, and threatened species, and to decrease human nuisance carnivore interactions. Regardless of the potential benefits, sustained reduction of carnivore populations exclusively by lethal means over large regions is unacceptable due to economic factors, questionable efficaciousness, and ethical considerations. Besides the carnivore issue, there has been a significant renewed impetus to enact population management for other species as well, most notably humans. One of the major modern strategies under consideration has been fertility control by immuno-contraception [152, 153]. Contraceptive approaches have pursued several avenues, including (1) eliciting an immune response to ova, sperm, or zona pellucida antigens; (2) disrupting spermatogenesis; or (3) interfering with reproductive hormones. The ideal technique would entail a targeted approach, involving either a species-specific antigen or vector, although none has yet been identified. Application to dogs and wild carnivores may be an important adjunct to current management techniques. Although the need appears obvious and preliminary efforts are promising, considerations that must be addressed include (1) the levels of herd immunity needed to preclude the persistence of either a subpopulation that evades exposure to these agents (e.g., bait avoidance) and prevent "nonresponders" from gaining a significant reproductive advantage; (2) the nontarget species issue, because of bait competition; and (3) the escape of a transmissible agent and propagated spread beyond an intended "pest" target population (i.e., introduced red foxes in Australia) to other distant geographic regions (i.e., red foxes in North America) by deliberate or unintentional animal or agent translocation. If such concerns could be minimized, a recombinant vaccine could, in theory, not only immunize against an infectious agent of choice, but also simultaneously decrease host abundance as well. For these reasons, more limited, targeted parenteral application to animals may be more relevant than generalized bait distribution in the environment.

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Disclaimer

The findings and conclusions in this chapter are those of the authors and do not necessarily represent the view of the Centers for Disease Control and Prevention.

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Rhinoviruses

29

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1 Introduction

Picornaviruses, which include the human rhinoviruses (HRVs) and enteroviruses (EVs), are the most frequent cause of acute human illness worldwide [1]. HRVs are the most prevalent cause of acute respiratory tract illnesses (ARIs) which usually commence in the upper respiratory tract (URT). ARIs are the leading cause of morbidity in children under 5 years and occur in all seasons [2, 3]. ARIs linked to HRV infections are associated with excessive and perhaps inappropriate antibiotic prescribing [4] and with significant direct and indirect healthcare expenditure [5, 6]. ARI incidence is highest in the first 2 years of life, with up to 13 episodes per year including up to six positive for an HRV, and it is not uncommon to average one infection per child-month [3, 7–9]. In preschool-aged children, nearly 50 % of general practitioner visits are for ARI [10], many of which are self-limiting. ARIs can often be managed in the community with supportive care from parents, but complications can arise that require a medical visit for management of asthma, otitis media, or sinusitis [11]. HRVs replicate in nasal cells, sinus cells, bronchial epithelial cells (BECs) [12, 13], and smooth muscle cells [14] but not in monocytes [15] or dendritic cells (DCs) [16]. The inflammatory immune response they trigger very soon after infection has its greatest impact in the young, the elderly, those with asthma or chronic obstructive pulmonary disease (COPD), and in the immunocompromised. First infections usually elicit a stronger response. Antiviral interventions have been under development for decades; to date most have met with varying degrees of failure or unacceptability. Vaccines have been considered unachievable because of the large number of diverse and distinct viral types.

There are 100 classically defined and recognized HRV serotypes grouped into two species, HRV-A and HRV-B, and a recently defined third species, HRV–C, containing more than 60 genotypes identified and characterized entirely by molecular means. Their cousins, the four enterovirus species (EV-A, EV-B, EV-C, and EV-D), are also found in the airways at times. Most systematic and mechanistic studies of HRV etiology and pathogenesis have been informed by studies in adults, mostly prior to the discovery of HRV-Cs. Adults exhibit reduced symptoms from HRV infections because of prior exposure and the resultant protective immune memory which that imparts (see Sect. 7.3). Furthermore, many modern studies (1) draw conclusions about lower respiratory tract (LRT) disease using URT specimens and (2) infrequently sample, doing so across small cross sections of time. These limitations have hampered attempts to associate virus detection and disease. Current thinking is that HRV-Cs may be key players in asthma exacerbations although our inability to culture them routinely has hindered our progress in understanding their role. The impact of the HRVs has been underestimated for decades, and the concept of the HRVs as a very large assemblage of genetically, immunogenically, antigenically, and temporally distinct and stable viral entities remains rare; they are more commonly considered a single variable virus, a view that science does not support.

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2 Historical Background

The disease most commonly associated with the airways and resulting from HRV infection is the common cold, a self-limiting coryzal illness [17–19]. The term dates back to ancient Greece, but evidence that the syndrome and asthma, another disease most frequently due to HRV infection, has been with us since ancient times can be viewed in writings on the Ebers papyrus, a medical document written in the sixteenth century BC [20, 21]. In 1930 the common cold was considered either to be due to exposure to the elements or to infection by bacteria [22]. It was later understood to be largely due to something in bacteria-free filtrates, and so the search for viral causes began [23, 24].

The Common Cold Unit (CCU) was established in Salisbury, UK, to seek solutions to the mysteries of the common cold, mostly through adult volunteer infection studies and careful systematic science [23]. The CCU functioned for 44 years (1946–1990), and it was here in 1953 that the first in vitro culture of an HRV was achieved using lung tissue from a particular embryo (Fig. 29.1) [25, 26]. Propagation failed once this tissue was expended [22, 33].

Once HRV isolation was possible, viral serotyping developed and culture techniques were further refined. This leads to an international effort to characterize and name the HRVs [27–30].

In 2006 renewed interest in HRV research was triggered by the description of a distinct clade of HRV types [31] found using molecular typing. The resultant flurry of HRV research raised questions about many earlier paradigms of rhinovirology and of the role of established respiratory viruses in ARIs. The novel clade was proposed as a new species, HRV-C, which was taxonomically confirmed in 2009 [32–34]. Prior to the discovery of the HRV-Cs, the genus *Rhinovirus* had been abolished and the HRV-A and HRV-B species assigned to the genus *Enterovirus* within the family *Picornaviridae* [35]. The HRV-Cs have been assigned a new naming scheme based on genetic sequence in the absence of antigenic or serological data. While the sequencing of all serotyped HRV genomes was completed in 2009, few of the HRV-Cs or apparently novel HRV-As or HRV-Bs have been similarly characterized, so the full spectrum of HRV genomes, the rhinovirome, remains incomplete. In this chapter we have described individual serotyped HRVs as the "classical" types, a type being the description for a single, genetically stable, stand-alone HRV.

3 Methods for Epidemiologic Analysis

3.1 The Pre-molecular Era

The original clinical definition of an HRV infection was written using data from cell and tissue culture and adult human infection studies. After 1953 in vitro isolation methods employed a virus interference test to more easily determine successful isolation; cultures suspected of infection with an uncharacterized HRV prevented infection by another, readily titratable virus [36]. Later, Price (1956; the JH strain) and then Pelon and co-workers (1957; 2,060 strain) developed culture systems that permitted HRV replication to be more easily identified [37, 38]. The early HRVs were initially classified as echoviruses (ECHO 28; later HRV-1) [39]. At the same time, propagation of the HGP (HRV-2) strain resulted from using increased acidity, lowered cultivation temperatures, and constant motion (rotation) [40, 41]. Despite the challenges [42], virus isolation was a more sensitive indicator of infection than an antibody rise in paired sera [43].

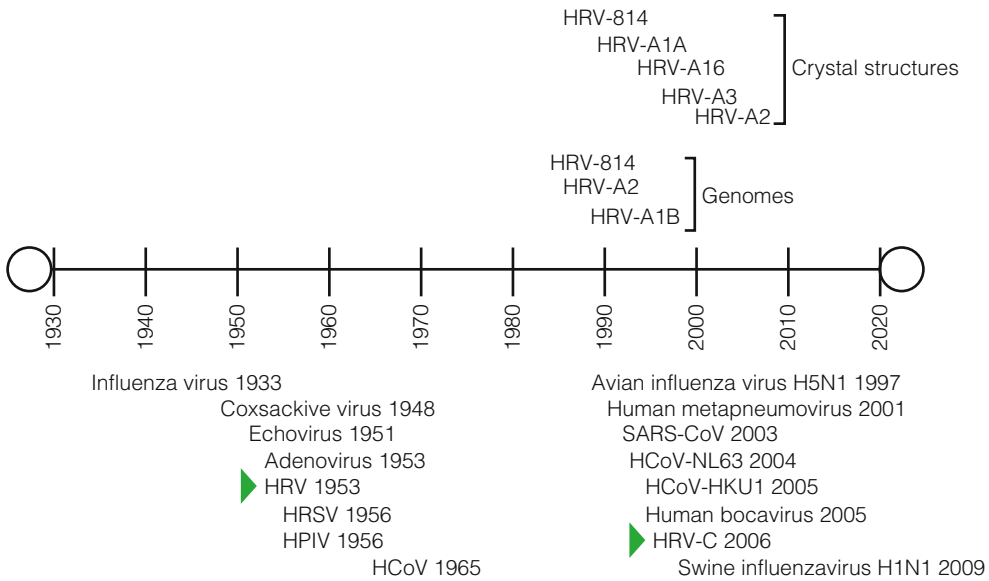

Fig. 29.1 A timeline of virus discovery from the human respiratory tract. The date of each virus's published description is shown, as are the dates the HRV crystal structures were defined and the first HRV genomes sequenced

It was found that several cell lines and methods were required to encompass virus concentrations ranging from 10^1 to 10^5 $TCID_{50}$/mL [44–47] and growth differences among the different virus types. Additionally, cell age after plating (<72 h), inoculum volume (relevant to the culture vessel), medium pH (6.8–7.3), and cell density were important factors for the reproducible appearance of HRV-induced plaques and for higher virus yields [48–51]. The HRVs can grow at temperatures above 35 °C (some prefer that under certain conditions) [52], but rolling at 33 °C, preceded by a 2–4-h stationary incubation period [41], has historically provided the highest yield and fastest in vitro HRV growth [36, 50, 53, 54].

Serodiagnosis grew increasingly impractical as the number of serotypes increased [49, 55]. However, antibody-based methods were essential for type-specific neutralization of infection [56] from which early epidemiology data were derived and around which the HRV nomenclature system evolved in 1967 [28]. The first classical strains were officially named in 1967 [57], the last in 1987 [30].

Today we know that cell culture-based methods are unreliable for accurately representing respiratory virus epidemiology; although enhanced by immunofluorescence, they are still used [58]. The HRV-Cs have not been successfully cultured in any cell lines or primary cell culture, although many attempts have been described [32, 59–62]. In 2011 HRV-C15 and W23 (another HRV-C) were shown to grow using organ culture [63]. Sinus tissue hosted increasing levels of viral RNA, as did adenoid, tonsil, and nasal polyp tissue, but much less effectively, as measured by in situ hybridization [63]. The sinus organ culture system also allowed testing of the first reverse engineered HRV-C (pC15) [63]. Isolation identified HRVs in ~23 % of adults with ARIs, associated with 0.5 illnesses per year [64].

3.2 The Molecular Era

Because culture is inefficient and subjective and requires expertise, even for the culturable HRV types, it is becoming an art lost to clinical laboratories the world over. It is unsurprising that PCR-based methods now prevail, providing a much improved understanding of the nature and scope of HRV infections. The virological and immunobiological cost of this improvement is a paucity of low passage "wild" HRV isolates to work with; thus, many research findings from recent years have employed easy to grow highly passaged and adapted HRV isolates. The impact of virus adaptation on the reliability of data from use of such viruses is unknown.

PCR-based assays have dramatically increased the frequency of HRV detection [65–70]. The improved sensitivity and reduced turnaround time have shown that HRVs, as a group, are usually the predominant viruses in ARI cases [71–73]. With reliable detection levels that extend from as few as 10^2 $TCID_{50}$/sample to well above clinically relevant loads, PCR can detect virus levels which are commonly shed during all stages of experimental infection studies [74, 75].

The common understanding of the systemic [76–78] or symptomatic [79, 80] context of HRV detections was established during the era of culture detection, and PCR has challenged these paradigms by detecting virus more often than culture. HRVs are sometimes found in "healthy controls"; however, it is likely that with more thoughtful definitions of "healthy," these detections would reduce. It is not uncommon to experience a feeling that one is "coming down" with something that never develops further. This is likely due to a transient infection or reinfection by an HRV or other respiratory virus that is eliminated quickly by the host response. It is possible to correlate viral nucleic acid load at the sampling site with disease severity; however, this is made difficult by the highly variable sampling efficiency of respiratory tract specimens which only permit the generation of reliable quantitative PCR (qPCR) data if serial specimens are available [81].

The 5′ untranslated region (UTR; Figs. 29.2 and 29.3) is the most common target for diagnostic oligonucleotides since the first HRV RT-PCR in 1988 [82], and the region has retained relevance for virus detection by its adaptation to reverse transcriptase real-time methods (RT-rtPCR) [53, 65, 66, 69, 74–76, 79, 83–103]. The 5′UTR is comprised of a number of conserved sequence "islands" (Fig. 29.2) that permit the robust detection of the majority of HRVs and those "respiratory EVs" which can be regularly detected in the respiratory tract [104, 105]. The detection of respiratory EVs in no way detracts from the importance of supporting clinical decision making using these assays. However, repositioning

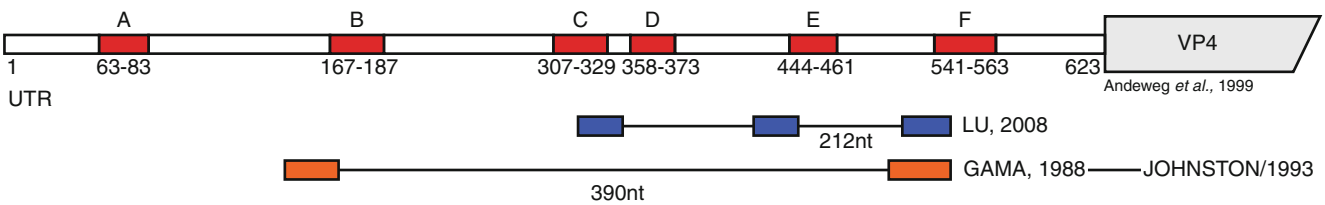

Fig. 29.2 A schematic of conserved sequence regions in a generalized HRV 5′ UTR, based on a map described by Andeweg et al. [68]. The PCR primers of broadly reactive conventional RT-PCR [82, 113] and RT-rtPCR [75, 114] assays are shown

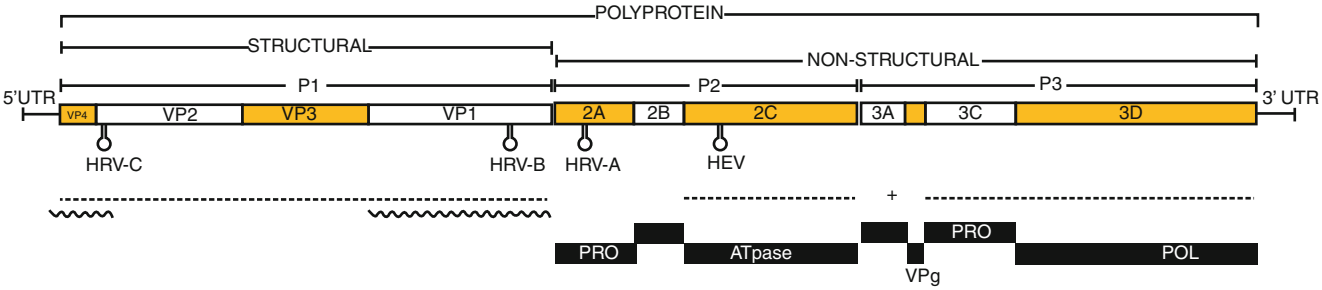

Fig. 29.3 A schematic of the ~7,200 nucleotide ssRNA genome and key regions of a typical HRV member of the genus *Enterovirus*. The polyprotein and precursory (*P1–P3*) and 11 matured peptides are named in genome boxes and functionally identified underneath. The RNA is polyadenylated at the 3′ end and covalently bound to the virion protein, genome (*VPg* encoded by *3B*) at the 5′ terminus. Regions essential for genus- and species-level identification are underlined (*dashed line*) as are those which are more commonly used in the clinical research setting (*wavy line*). The distinctively located HRV and EV cis-acting replication elements are shown as stem loop structures and protease (*PRO*) and polymerase (*POL*) functional regions are labeled (Adapted with permission from McErlean et al. [33])

these primers or changing the method of employing them [106–109] may undermine assay performance, as evidenced by predicted hybridization mismatches, uncommonly low detection frequencies [110], and by comparison of multiple primer sets using the same specimens [111]. The addition of an oligoprobe rtPCR method increases amplicon detection sensitivity and specificity, identifying 100-fold fewer $TCID_{50}$/mL or 10 fold fewer genome copies than agarose gel detection of amplicon [75, 79, 112].

Other molecular tools, capable of detecting multiple targets, have evolved in recent years [58, 70, 115–121], and some have gone on to be approved for clinical laboratory use [122]. Microarrays can detect thousands of viral targets, but are expensive for routine use (USD30–300 per sample) and not sensitive enough to avoid a pre-hybridization PCR amplification when using clinical specimens. At their most robust, microarrays, like PCR, rely on the existence of conserved regions of sequence to detect unknown viruses allowing them to detect previously unknown HRV types [123]. High-throughput or "deep" sequencing platforms have become less expensive and more readily available, and they have succeeded in finding new diversity within the HRV species [124]. The experiments remain costly so have not yet found a place for regular screening tasks and remain coupled to a need for pre-PCR steps. Rapid protein- or virion-based assays are not (yet) adequately sensitive [125, 126].

Because of the high number of HRVs and the high frequency of infections, genotyping methods have become an essential accompaniment for understanding HRV epidemiology. Nucleotide sequencing of the VP1, 5′UTR+VP4+VP2 (called hereafter VP4/VP2), or 5′UTR region has replaced traditional serological methods, because of its speed and need for fewer specialized reagents compared to serotyping. VP1 yields the most comprehensive subgenomic genotyping information and is essential for the minimal definition of a new HRV type [127]. The VP4/VP2 region (Fig. 29.3) is considered easier to use because it encompasses sufficient genetic diversity to confirm the identity of a clinical HRV type while also providing broad enough sensitivity to amplify the ~160 HRVs from a challenging biological substrate, clinical specimens [128]. Screening of airway specimens for HRVs is not routine [111] due to factors including cost and the perceived low clinical relevance of detection. Genotyping is mostly relegated to research facilities. Because of this, HRV molecular epidemiology studies tend to be smaller and focused on a specific disease or research question.

4 Biological Characteristics

Most in-depth molecular studies of HRV replication have focused on a single HRV type. Generally, it is presumed that results can be extrapolated to the other HRV types and to the in vivo situation. HRVs replicate in the cytoplasm (Fig. 29.4) [129] with membrane-associated replication structures containing double-stranded RNA (dsRNA) replicative intermediates (RI) which are formed in cells 4 h after infection [52, 130]. Single-stranded infectious RNA forms after RIs start to accumulate [130]. Genomic RNA (plus strand) is the template for complementary minus strand synthesis which in turn is the template for new genomic plus strands that become incorporated into virions [131]. Virions are synthesized from 4 to 7 h after infection and reach maximum release levels at 10–18 h [131].

HRV replication in epithelial cells may shut off host cell transcriptional activity via direct cleavage of transcription factors and nuclear pore complex components. Protease 2A ($2A^{PRO}$) of HRV-B2 may directly cleave eukaryotic initiation factor 4G (eIF4G) when bound to eIF4E [132, 133]. The eIFs have key roles in initiation and rate control of host cell translation [132]. Host cellular protein production is virtually replaced by HRV-B14 proteins after only 6 h of infection [134]. HRV-B14-infected cells also display reduced nuclear importing and degraded nuclear pore complex (NPC)

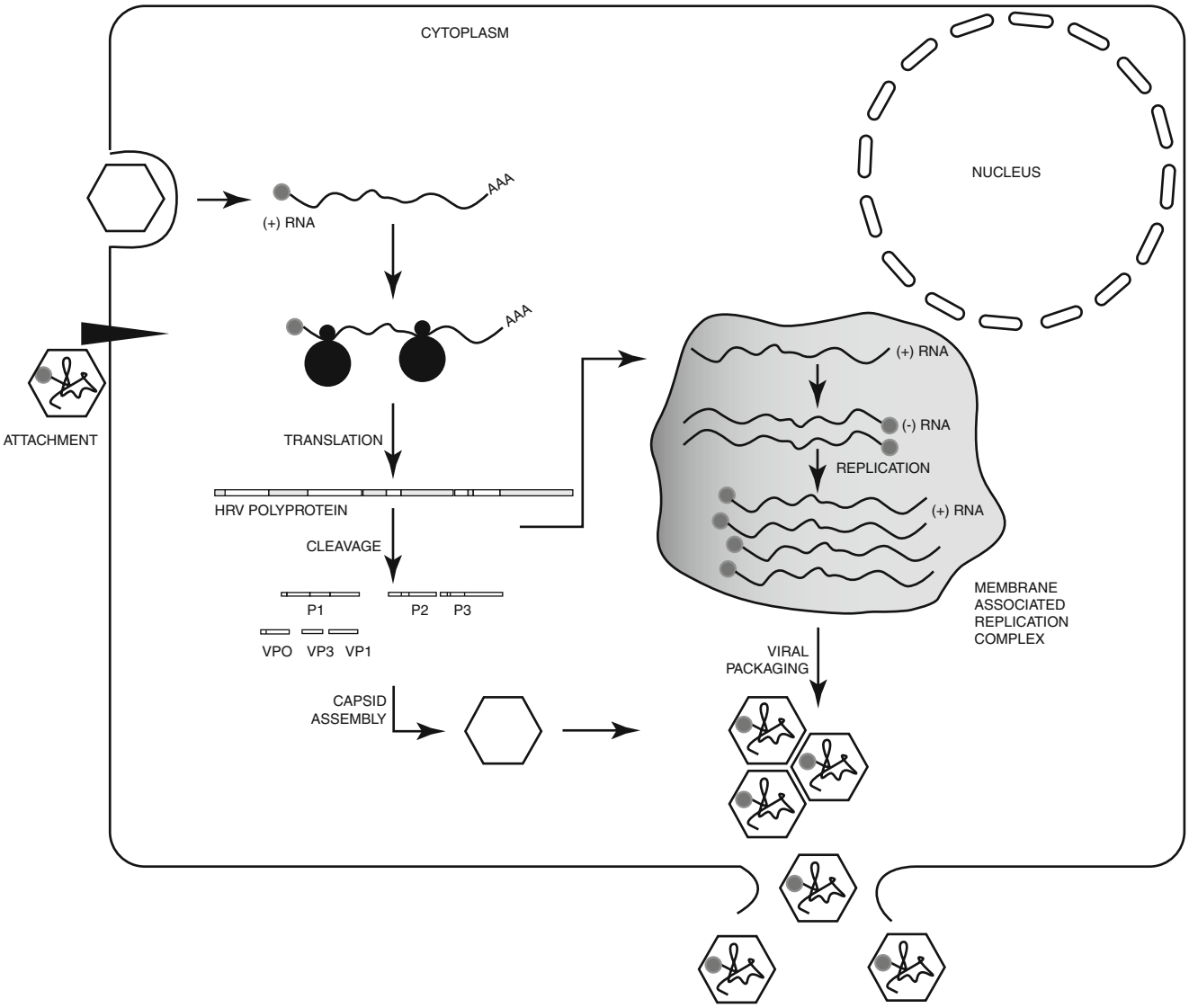

Fig. 29.4 Schematic of a general HRV attachment and entry process. Genome replication in association with membranes produces the viral polyprotein which is co- and posttranslationally processed by $2A^{PRO}$ and $3C^{PRO}$ into the proteins (*P1–P3*) and structural peptides (*VP1–VP4*; *VP2* and *VP4* derive from the VP0 precursor protein) that assemble into protomers, pentamers, and finally capsids. Nonstructural proteins are also released in these cleavages as well as through autoproteolytic cleavage. Mature HRV virions packaged with an ssRNA genome escape by cell lysis (Adapted with permission from Arden et al. [138])

components [135]. This may represent another HRV strategy for limiting the host response by preventing or reducing key signaling pathway molecules (e.g., IRF-3, STAT1, NF-κB) and shutting down host cell protein synthesis. Protease 3C ($3C^{PRO}$) from HRV-A16 targets the nucleus and can disrupt active and passive nucleocytoplasmic transport [129, 136]. Recombinant $2A^{PRO}$ protein from HRV-A16,HRV-A89, HRV-B4, HRV-B14, HRV-C2, and HRV-C6 exhibited differing specificities and kinetics against eIF4G as well as NPC components demonstrating functional diversity between HRV types [137]. This finding underscores the functional diversity within the HRV species and the risk of extrapolating too greatly from the study of single HRV types. It is apparent from a wealth of immunobiological data that HRVs still efficiently trigger a proinflammatory immune response that has considerable clinical impact among at-risk groups, and that their putative interruption of host cell machinery does little to hinder this.

4.1 The Rhinovirus Genome

The virion encapsulates an approximately 7 kb positive sense RNA genome (Fig. 29.3), which tends to be more adenine and uracil (A+U) rich than the EV genome [139]. In particular, A+U more frequently occupies the third or "wobble"

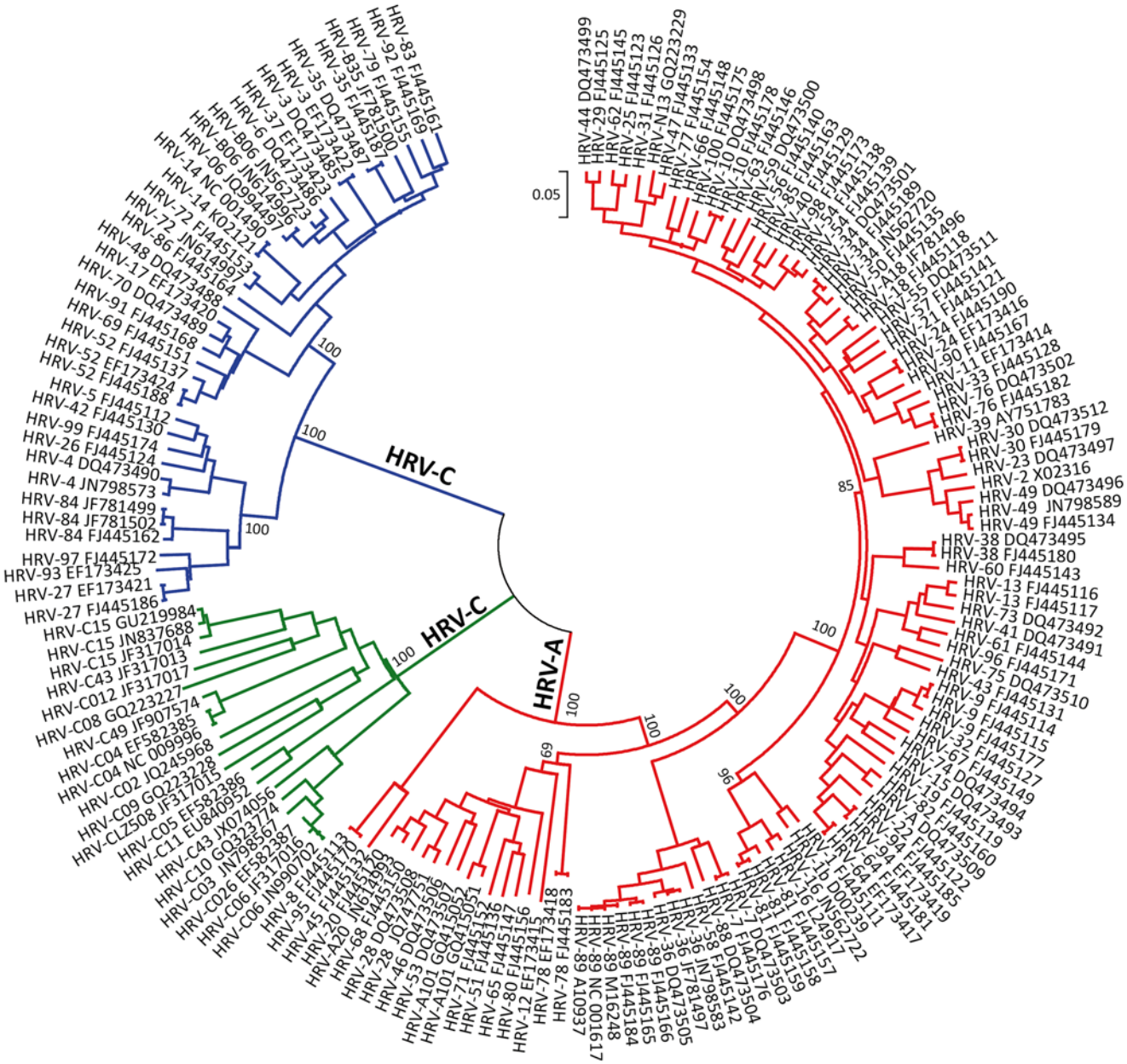

Fig. 29.5 The current spectrum of 168 complete HRV complete polyprotein amino acid sequences available on the GenBank database. The alignment was conducted using MAFFT within Geneious Pro v5.6 [148]. The phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Poisson model, 500 bootstraps with consensus support shown at the nodes where space permitted [149]) (Reprinted with permission from Miller and Mackay [150])

codon position. The single RNA "gene" acts as messenger RNA to encode the single multi-domain, proteolytically processed "polyprotein." The coding region is bracketed by UTRs which perform regulatory functions necessary for genome duplication [140]. These are very similar genomic, transcriptional, and translational features to those of their close cousins, the EVs. Most of the information currently required for virus identification by the International Committee on Taxonomy of Viruses (ICTV) can be found through analysis of the genetic features of HRVs (Fig. 29.3).

There are 158 complete HRV polyproteins on the GenBank database (Fig. 29.5). The first complete HRV genome sequence (HRV-B14) was described in 1984 [141] followed by HRV-A2 in 1985 [142] and HRV-A1b in 1988[143](Fig. 29.3). In 2007 Kistler et al. added 28 genomes [144] and Tapparel et al. 12, including one common to both studies [145]. Sequencing of the VP4/VP2 region was completed for all classical strains in 2002 [146], and the complete set of 1D regions were available in 2004 [147]. Currently there are at least 50 named HRV-C VP1 regions

available and 20 complete HRV-C genomes. Many more genomes are appearing as part of the Rhinovirus Consortium's efforts to complete and study the rhinovirome using high-throughput sequencing technologies to genetically characterize HRVs from their combined clinical specimen stores (http://www.international-rhinovirus-consortium.org/). Many 5′UTR and VP4/VP2 sequences reside on the GenBank database, most of which are labeled using in-house laboratory schemes rather than an approved nomenclature. Analysis of the full-length genomes supports the use of 5′UTR, VP1, and VP4/VP2 subgenomic regions for useful representation of HRV species and types [144, 147].

Recombination, the process of genetic exchange which results in a chimeric genome [151], can only be detected in mature viruses after the fact, and it must therefore be inferred indirectly through genomic analysis and comparison. Predictions of infrequent recombination among the HRVs [83] have been made based on examination of the available set of HRV coding and noncoding regions [152]. Intensive analyses reported that recombination is not a driving force for the evolution of HRV types [144, 153, 154]. Some discrepancies are likely because of the different number of sequences used, the different origins of the viruses used for sequencing, and the analysis methods employed. HRV-C evolution seems to have been more affected by prior recombination, than is apparent for members of HRV-A or HRV-B. This is similar to the EV species but with far fewer predicted recombination events than for EV evolution [114, 151, 155, 156]. Most of the recombination proposed to have affected the HRVs occurred between HRV-C and HRV-A and is often found within the 5′UTR or at the 5′UTR/VP4 junction [83, 157, 158] but rarely in coding sequence (2A [158] or 3C [83]). The high sequence diversity among the individual HRV polyprotein coding sequences may keep recombination events to a minimum in order to retain viral fitness [158]. The ability of HRVs to recombine in practice awaits empirical evidence; the extent of recombination among all HRV or EV types and the frequency with which viable recombinants arise are entirely unquantified.

4.2 The Rhinovirus Capsid

The 28–30 nm HRV virion has been visualized for only a handful of HRV-A and HRV-B types (including HRV-A1a, HRV-A2, HRV-B3, HRV-B14, and HRV-A16), but no HRV-C structures have been empirically determined to date. The first, HRV-B14, was described in 1985 [159] followed by HRV-A1a in 1989 [160], HRV-A16 in 1993 [161], HRV-A3 in 1996 [162], and HRV-A2 in 2000 [163]. HRV-C structure has only been predicted using computer modeling, but their basic structure seems to be that expected of an HRV (Fig. 29.6) [33]. The HRV capsid shell is composed of 60 protomers, each comprising one copy of the viral proteins VP4, VP3, VP2, and VP1. VP1, VP2, and VP3 (each ~30 kDa) are to some extent exposed on the capsid surface, whereas VP4 (~7 kDa) is internalized and associated with viral RNA. Five protomers come together at a point around a fivefold axis, and this cluster is called the pentamer. The fivefold axis is circumscribed by a cleft referred to as the "canyon." VP1, VP2, and VP3 are each formed by a convoluted set of protein sheets and loops [159]. The loops protrude beyond the external capsid surface and contain discontinuous antigenic sites. Of the HRV types studied, four neutralizing antibody immunogenic (NIm) regions have been identified on HRV-B14 and HRV-A16: NIm-1A (located in VP1), NIm-1B (VP1), NIm-II (VP2 and VP1), and NIm-III (VP3 and VP1) [159]. Antigenic sites identified on HRV-A2 are called A, B, and C [164]. The scope and location of antigenic and immunogenic moieties among the HRV-Cs is unknown. Using known receptor binding sequence as a guide for computer modeling (Fig. 29.6), it has been predicted that when discovered, the receptor for the HRV-Cs will differ from the major and minor receptors defined for the HRV-As and HRV-Bs [33].

4.3 Classification of the HRVs

The three HRV species within the genus *Enterovirus* are a genetically, immunogenically, and antigenically diverse assemblage of >160 viral types (Table 29.1). This accounts for the combination of HRV-A1a and -A1b, exclusion of HRV-87, which is actually EV-D68 despite confusion over acid liability [169–171] and combination of HRV-Hanks which is actually HRV-A21 [147]. Serological studies indicate that some HRV-A and HRV-B types may not be distinct enough to deserve a unique identity [147]. Species within the genus share >70 % amino acid (aa) identity in the polyprotein and in 2C+3CD and >60 % aa identity in P1 (Fig. 29.3) as well as their host cell receptors, a limited natural host range, a genome base composition (G+C) that varies by no more than 2.5 %, and a similar compatibility of proteolytic processing, replication, encapsidation, and genetic recombination [172]. A variant of the same HRV type shares 87–88 % aa identity or more in VP1 [129]. Much of the nongenetic criteria remain undefined for the HRV-Cs. In 2008 the genera *Enterovirus* and *Rhinovirus* were officially combined, retaining the former genus name *Enterovirus* with the *Human enterovirus* C as the prototype species. A genus in the order *Picornavirales*, family *Picornaviridae*, is at least 58 % different in its amino acid identity from any other genus. In 2009 a proposal establishing the species *Human rhinovirus C* was ratified by the ICTV. Formal HRV-C numbering commenced in 2010, and type numbers were initially assigned

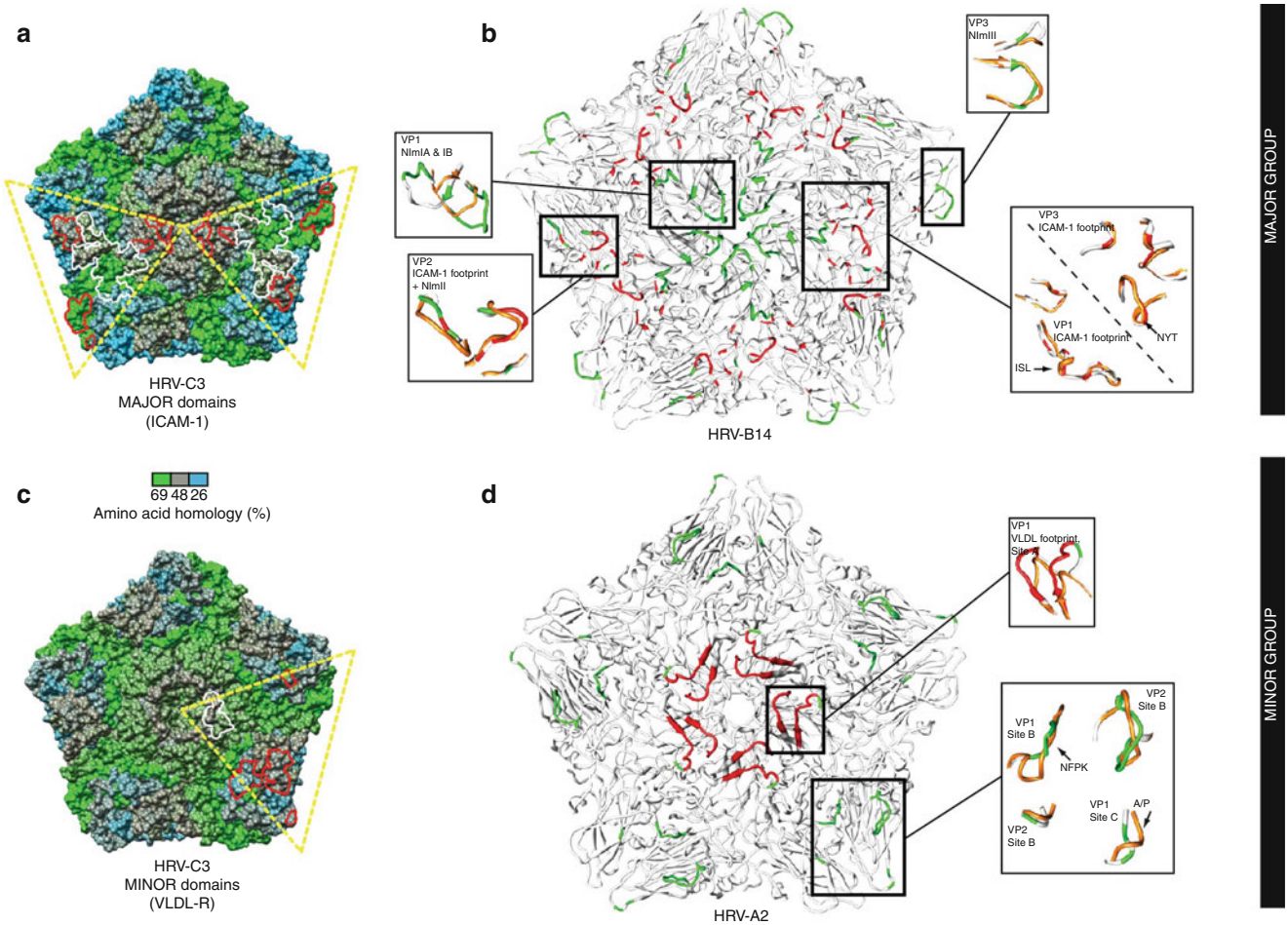

Fig. 29.6 Predicted HRV-C3 pentamers compared to major (HRV-B14) and minor (HRV-A2) group HRV pentamers which have been obtained from X-Ray crystallography. (**a**) HRV-C3 versus HRV-B14 SimPlot data projected onto a space filling depiction of the predicted HRV-C3 pentamer. Shading represents the amino acid identity (26–69 %). The *yellow-dashed triangle* represents a single icosahedral asymmetric unit ($T=p3$ conformation) composed of VP1 and VP2 from the same protomer and VP3 from an adjacent protomer. The major group domains of interest are divided between two asymmetric units for ease of viewing. Receptor (*white*) and antigenic (*red*) sites are shown in outline. (**b**) Bird's eye view of a major group HRV pentamer in ribbon form (HRV-B14, *gray*) with labeled antigenic neutralization sites (NImIA-III, *green*) and combined HRV-A16 and HRV-B14 intercellular adhesion molecule (ICAM)-1 receptor footprints (*red*) [165, 166]. Magnified areas of interest (*boxed*) highlight computer-based comparisons to the equivalent HRV-C3 (*orange*) predicted structures of interest. (**c**) HRV-C3 versus HRV-A2 SimPlot data projected onto the HRV-C3 pentamer. The domains of interest are mostly shown within a single asymmetric unit. (**d**) A minor group pentamer (HRV-A2, *gray*) including antigenic sites (sites **a**–**c**, *green*) and very-low-density-lipoprotein receptor (VLDLR) footprint (*red*) [167]. Attachment of the VLDL-R involves adjacent VP1 molecules. Magnified VP1 area represents one half of a VLDL-R footprint [168]. Amino acid substitutions (*arrowed*) contributed to the differences between minor group sites **b** and **c** (Adapted with permission from McErlean et al. [33])

based on the date of submission of relevant sequences to GenBank (HRV-C1, formerly NAT001; HRV-C2, f. NAT045; HRV-C3, f. QPM; HRV-C4, f. C024, etc.; Table 29.1) [127]. A clinical detection of an HRV-C can be considered a novel type principally based on its VP1 sequence or provisionally ("C_pat," Table 29.1) based on VP4/VP2 [146] and could be confirmed as a variant of a previously characterized HRV-C by identity thresholds to either region. The 5′UTR can be and still is used [173, 174] for HRV genotyping, but it is a more problematic region than VP1 or VP4/VP2 because of the recombination activity that affects this region, especially among the HRV-Cs [175]. This is presented as phylogenetic intermingling of some HRV-A and HRV-C types [114]. Nonetheless, careful application of sequence identity thresholds when comparing clinical sequences to the GenBank database (≥96 % identity required before assigning a clinical detection to a particular type) succeeds in characterizing HRV species and types [9]. There are currently 50 types within HRV-C (which includes the types once grouped together under HRV-"A2," HRV-X, and HRV-NY clades), 78 HRV-A types, and 25 HRV-Bs. The most up-to-date information on current taxonomic trends can be found at the ICTV Picornaviridae study group website (http://www.picornastudygroup.com/).

Table 29.1 ICTV-approved nomenclature for the members of the HRV species

Human rhinovirus						
A			B	C		
1[M,B]	**34**[B]	**64**[B]	**3**[H,A]	C3 (f. QPM)	C26	C_pat14 (f. SA365412)
2[M,B]	**36**[B]	**65**[B]	**4**[A]	C10 (f. QCE)	C27	C_pat15 (f. HRV-CO-1368)
7[H,B]	**38**[B]	**66**[B]	**5**[A]	C1 (f. NAT001)	C28	C_pat16 (f. RV1250)
8[H,A]	**39**[B]	**67**[B]	**6**[H,A]	C2 (f. NAT045)	C29	C_pat17 (f. RV1039)
9[H,B]	**40**[B]	**68**[B]	**14**[H,A]	C4 (f. C024)	C30	C_pat18 (f. RV546)
10[H,B]	**41**[B]	**71**[B]	**17**[H,A]	C5 (f. C025)	C31	C_pat19 (f. China/GDYY100/2008)
11[H,B]	**43**[A]	**73**[B]	**26**[H,A]	C6 (f. C026)	C32	C_pat20 (f. 202511)
12[H,B]	44[B]	**74**[B]	**27**[H,B]	C7 (f. NY074)	C33	C_pat21 (f. 202092)
13[H,A]	**45**[A]	**75**[B]	**35**[A]	C8 (f. N4)	C34	C_pat22 (f. 20264)
15[H,A]	**46**[B]	**76**[B]	**37**[A]	C9 (f. N10)	C35	C_pat24 (KR1868)
16[H,B]	47[B]	**77**[B]	**42**[A]	C11 (f. CL-170085)	C36 (f. NAT069)	C_pat27 (f. PV68)
18[H,A]	49[B]	**78**[B]	**48**[A]	C12	C37 (f. NAT059)	C_pat28 (f. Cd08-1009-U)
19[H,B]	**50**[B]	**80**[B]	**52**[A]	C13	C38 (f. tu34)	
20[H,B]	**51**[B]	**81**[B]	**69**[A]	C14	C39 (f. g2-11)	
21[H,B]	**53**[B]	**82**[B]	**70**[A]	C15	C40 (f. g2-25)	
22[H,B]	**54**[A]	**85**[B]	**72**[A]	C16	C41 (f. g2-23)	
23[H,B]	**55**[B]	**88**[B]	**79**[A]	C17	C42 (f. g2-28)	
24[H,B]	**56**[B]	**89**[B]	**83**[A]	C18	C43 (f. 06-230)	
25[H,B]	**57**[B]	**90**[B]	**84**[A]	C19	C44 (f. PNC40168)	
28[H,B]	**58**[B]	**94**[B]	**86**[A]	C20	C45 (f. PNC40449)	
29[M,B]	**59**[B]	**95**[A]	**91**[A]	C21	C46 (f. PNC40449)	
30[M,B]	**60**[B]	**96**[B]	**92**[A]	C22	C47 (f. K1091_301104	
31[M,B]	**61**[B]	**98**[B]	**93**[A]	C23	C48 (f. PNG7293-3193)	
32[A]	62[B]	**100**[B]	**97**[A]	C24	C49 (f. IN-36)	
33[B]	**63**[B]	N13	**99**[A]	C25	C50 (f. SG1,SO5986)	

M and *H* indicate early cell tropism-based classification (monkey, human) abandoned in favor of a sequential numbering system [177]. HRV types were later divided into the major and minor groups defined by receptor tropism [184, 185]. Receptor-designated minor group HRV types are underlined, and major group types are shown in bold. Antiviral groups (A and B) are labeled [165, 194]. HRV-A8 and HRV-A95 are also likely the same serotype [147]. A full list of genetically close serotype pairings was presented by Ledford et al. [147] HRV-C nomenclature was defined in 2010 and currently includes a number of *p*rovisionally *a*ssigned *t*ypes (pat) which are confirmed once preliminary VP4/VP2 data can be confirmed with VP1 sequence and the provisional number removed (e.g., C_pat1 to C_pat13 have already been reassigned)

Historically a key feature distinguishing the HRVs from the EVs was the instability of the HRV capsid in the presence of acid and their lower preferred laboratory propagation temperature (33–34 °C versus 37 °C for EVs). Over time HRVs have been subclassified in different ways. The first was based on tissue tropism and host range. HRVs that preferred growth using monkey cells were called "M" strains and those (the majority) that grew only in human cell cultures, "H" strains [56, 176–180]. These two groups correlate with receptor usage [131] (Table 29.1) and possibly with the titer of the inoculum employed [181]. In 1962 it was proposed to abandon this terminology in favor of a sequential numbering system [177].

Picornaviruses recognize a variety of cellular receptors [169, 182, 183]. HRV types are also subdivided into major and minor groups defined by use of one of the two main receptor molecules [184, 185]. The capsid of the majority of classical HRVs ($n=89$) [184] interacts with the amino-terminal domain of the 90 kDa intercellular adhesion molecule (ICAM-1; CD54) [186–189]. Receptor binding destabilizes the HRV capsid, probably by dislodging the "pocket factor," and initiates uncoating [164, 182, 190]. ICAM-1 interacts with its receptor, leukocyte function antigen-1 (LFA-1), and plays a role in recruitment and migration of immune effector cells [191]. The minor group [184] of classical viruses employ members of the low-density lipoprotein receptor (LDLR) family to attach to cells [167]. Binding of VLDL-R occurs outside of the canyon employing a different destabilizing and uncoating mechanism. Heparan sulfate may act as a receptor under specific conditions [183, 192, 193].

In 1990 Andries et al. defined, and Laine et al. refined, two "antiviral groups" (A and B) based on their susceptibility to a panel of antiviral molecules [165, 194]. These groupings reflected the nature of the amino acid (and hence nucleotide) sequence of the region interacting with the antiviral molecules. These antiviral groups can also be visualized using phylogeny [194]. When sequences from other subgenomic regions, including P1, 2C, and 3CD, were examined by phylogeny, the species were found, in most cases, to inversely correlate with antiviral grouping labels (Table 29.1).

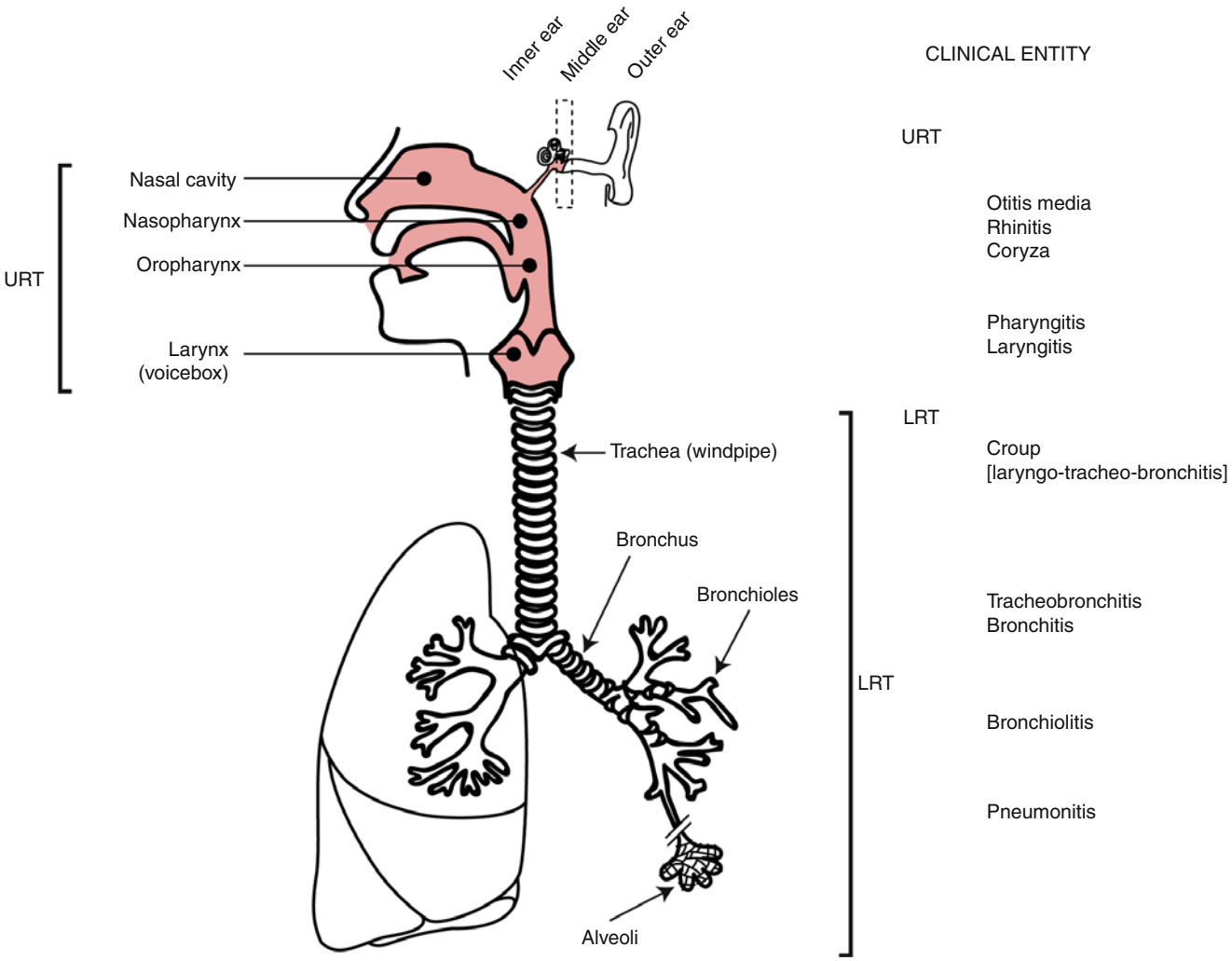

Fig. 29.7 A schematic representation of the human respiratory tract. The upper (*shaded pink*) and lower respiratory tract (*URT*/*LRT*) and the components of the ear are indicated as are the approximate locations of URT and LRT diseases associated with respiratory virus infection (Adapted from Mackay et al. [200] with permission from Caster Academic Press)

Today, sequencing and phylogeny play a central role in species classification within the genus, and together, they are surrogates for the important biological classification criteria [146, 147, 165, 195–197]. For the HRV-Cs, first described as the "HRV-A2" clade (not to be confused with the single virus, HRV-A2, this naming scheme appeared after the HRV-C clade's name was proposed) of viruses in 2006 [31], sequencing of 5′UTR and VP4/VP2 has provided the bulk of HRV information from clinical studies. While culture in primary sinus tissue has been reported [63], no receptor is yet defined.

5 Descriptive Epidemiology

HRVs are the most numerous and frequently detected of all the "respiratory viruses," so-called because of their predominant detection in and tropism for the human URT or LRT (Fig. 29.7). The circulation of HRVs varies with population age, underlying disease, immunocompromise, over time, and across distance. Circulation is influenced by the nature, strength, distinctiveness, and memory of the immune response HRVs trigger and by the nature and prevalence of other concurrently circulating respiratory, and perhaps non-respiratory, viruses. With the recent discovery of the unculturable HRV-Cs came the realization that previous HRV epidemiology was only reliable if conducted by one or more suitably broad-spectrum HRV PCR assays [111]; hence, prior to 1988, detection of the full spectrum of ≥160 HRVs did not occur. After 1988, the ability to detect all types very much depended on the nature of the PCR primers and detection methods used. The great number of distinct HRV types has burdened the search for answers to epidemiology-related questions. However, as for other important respiratory viruses including human respiratory syncytial virus (HRSV)

and the influenza viruses (IFVs), the virus types within a species show evidence of being both distinct and discrete viruses that are independently recognized by their host and consequently independently infect their hosts. Each HRV type is also genetically stable [144].

The HRV species circulate variably from year to year with evidence of epidemics of distinct types. A prospective longitudinal cohort study over 6 months examined HRV frequency and diversity in 272 specimens from 18 healthy children (0–7 years of age) [198]. A median of three HRVs and a maximum of six were detected per child. A similar outcome resulted from an Australian cohort study [9].

Genotyping reveals more of the HRV diversity at a single site than culture ever could with molecular studies finding between 34 and 70 distinct HRVs at a single location [9, 128, 199]. The number of additional HRV cases that occur in children outside of specifically defined symptomatic periods remain to be defined, with current studies indicating that a much higher number of HRV infections may occur. More comprehensive investigation of HRV type and illness will be undertaken during analysis of data from the Australian-based Observational Research in Childhood Infectious Diseases (ORChID) study (http://clinicaltrials.gov/show/NCT01304914).

Interestingly, the HRV-Bs are often underrepresented, even when accounting for the smaller number of known HRV-B types [128]. A number of studies have not found any robust patterns between the circulating HRV types or species and clinical outcome, but the majority of studies seeking this information are short and sample infrequently, limiting their ability to find the patterns they seek [128].

5.1 Specimen Collection

Studies into the relative sensitivities of nasopharyngeal aspirates (NPA) and swab sampling methods produce differing results, but generally, if seeking the best diagnostic yield for as many respiratory viruses as possible (i.e., seeking a laboratory diagnosis to support clinical decision making), NPAs are the sample of optimal choice. One study reported similar clinical sensitivities between swabs and NPAs for human coronaviruses (HCoVs), IFVs, and HRSV, but reduced sensitivities using swabs for HRVs, human adenoviruses (HAdVs), human metapneumovirus (HMPV), or parainfluenza viruses (HPIVs) [201]. A second study reported no difference in sensitivities for HRVs, HAdVs, and HPIVs but a reduced sensitivity for HRSV and IFVs when using swabs [202]. Nasopharyngeal washes also yield more viral culture success than either nasal or pharyngeal swabs. Nonetheless, many studies use nasal swabs as the sample of choice because they allow self-collection and involve much less discomfort than NPAs, and PCR has meant that infectious virus is not required, only viral nucleic acid which relaxes some limitations imposed by the need for rapid, careful, temperature-controlled, and expensive transport requirements [64, 203, 204]. Bronchoalveolar lavage samples are best for seeking LRT etiologies, especially in adults where nasal wash viral loads can be low compared to those in children, but this is an invasive method with some risk attached [205].

5.2 Host Population Distribution

HRVs infect all people, all around the globe. Spread of HRVs is most obvious and frequent from child to child and from child to parent [206]. In populations of mixed age, the majority of HRV detections occur in children [128]. Among 272 specimens from 18 healthy children, over a third (37 %) were HRV positive. Children less than 5 years of age (44 % of whom were HRV positive) were shown to have more HRV infections and a wider diversity of HRV types than children more than 5 years old (28 % HRV positive) [198]. Healthy adults in the military [54, 207], at university [208], at home [209–212], and in the workplace [209] have also featured prominently in historical, culture-based, and volunteer infection studies and heavily influenced our view of HRV infection outcomes [64, 206]. Although studies of children in hospital-based populations usually report more significant clinical outcomes (relating to the LRT) [213] than community-based studies, these data are still broadly applicable. Hospital populations originate from the community and reflect the more serious and perhaps first exposures to the virus. Hospital-based populations define the potential of a virus to cause severe clinical outcomes. Disease at this end of the spectrum has the strongest influence on future prioritization of therapeutic research and developments [214].

Modern air travel contributes to the rapid spread of respiratory viruses as seen in their often frequent detection among travelers [215] including those with febrile illnesses [216]. Apart from children, HRVs are found with the great clinical impact in the elderly (described as 60–90 years of age) with 50 % of ARIs positive for an HRV, sometimes with a greater burden of disease than IFVs [217]. Those with asthma or COPD are also affected by the ARI triggering exacerbations of wheezing illness (see Sect. 8.2). It is thought that this is not a different type of infection but rather a different response to infection by the host. Wheezing can also result from infection in atopic people who do not have underlying asthma or COPD. HRVs cause significant impact in the immunocompromised, and this group is the only population to date that has been found to host truly persistent HRV infections (see Sect. 5.7). Because the HRVs are the largest group of viruses to infect humans, it is not surprising that they confuse differential diagnoses during pandemics and have key roles in

co-detections and asymptomatic disease. The study of HRVs is the study of all respiratory viruses; while each can be considered in isolation, this will likely be detrimental to a greater understanding of respiratory virus pathogenesis.

5.3 Seasonality

HRVs circulate throughout the year but usually with a bimodal peak in temperate locations in both hemispheres. The highest peaks, mostly defined using adult populations, are in the autumn (fall) and spring [64, 66, 211] (and, peculiarly, on a Monday [218]). The major winter dip in HRV prevalence closely coincides with the peaks of other respiratory viruses, particularly IFVs [219] and HRSV [66]. One hypothesis states that a miasma exists in the school classroom, of particular relevance to those who suffer asthma exacerbations, and this miasma maintains immune stimulation, which subsequently wanes among school children during holidays, to be challenged anew upon return to school [220]. It is clear that an interplay or interference takes place between viruses at the population level, particularly evident among RNA viruses.

There is a correlation between spiking spring and autumnal HRV case numbers and an asthma exacerbation "season" 10–24 days after return to school from holidays, in a range of climates [220–223]. This was particularly obvious among asthma hospitalizations of children (5–15 years of age) in Ontario, Canada, which peaked at weeks 37–39 across a decade [223]. Upon investigation, HRVs were the most prevalent of the viruses found in a 1-year analysis of emergency room presentations in Ontario [223]. HRVs also predominate during "hay fever season" [172]. Although a defined seasonality is not always found in the tropics [224], this may sometimes be due to testing that does not include HRVs [222, 225] or only some HRVs [226].

5.4 Recurrence

All the HRV types continue to circulate today, including those named in the earliest of the nomenclature assignments. At a single site during 12–24 months, 70 or more types can co-circulate [8, 9] [174], dropping [198, 227] if the study time frame at the site is shortened. A recurring HRV type, defined using molecular tools, accounted for 1.6 % of any virus detected in a birth cohort followed for 12 months [8] and, in another cohort, occurred twice in two children, within a 6-month period [198].

Within a given year and across different years, it is apparent that HRV species exchange predominance [9, 36, 60, 227–229]. No evidence exists to satisfactorily explain this; however, herd immunity may be a factor.

5.5 Coinfection

The use of cell and tissue culture underestimated the frequency of multiple infections in patients, most likely because the dominant virus out-replicated any others, or due to viral load differences, specimen quality issues, differing cell tropisms, or the triggering of an antiviral state by the first virus. When the majority of respiratory viruses are sought using PCR techniques, multiple virus-positive specimens can comprise a third of those tested [230], dropping to around a fifth of ARI episodes when fewer viruses are sought [217]. There is sometimes an emphasis on the high number of HRV cases that are identified in the presence of another virus, and including HRV testing does raise the frequency of pathogen detection above one per sample [231]. Coinfections, or, more correctly for PCR-based studies, co-detections (since PCR cannot determine infectivity), have been found to either increase [71, 232–236] or have no impact on the clinical outcome in their host [237–241], and so the issue of clinical relevance of co-detections is still uncertain. In extreme cases, half of all HRV detections can be found concurrently with another virus. On the surface, this is a significant fraction, and yet 80 % or more of HRSV, HMPV, EV, and IFV detections and 71 % of HCoV-NL63 detections can be found in the company of another virus [242]. Other studies find different, but still higher proportions of co-detections involving non-HRVs [217]. Whether co-detections represent a particular synergism between the involved viruses, a differential capability to manipulate the host immune response, a sign of innocuousness for the most frequently involved virus [243], or a chance due to overlapping seasons remains unclear. It is clear, however, that co-detections are not an anomaly or an error due to "overly sensitive" PCR tests; they are evidence of further biological complexity that, until recently, remained hidden from us. Recent studies have shown that the initial impression of HRVs being overrepresented in these cases was incorrect. Closer analysis of viral co-detections has revealed patterns [231, 244]. These became clear when co-detections were examined bidirectionally, not just how many HRVs were positive for virus X but also how many of virus X cases were positive for an HRV. Whether in a hospital or a community setting, HRVs more often occur as the sole virus detected in ARIs [9, 244]. Considering their ubiquity, it is interesting that relatively low numbers of concurrent detections occur [245, 246], supporting the concept that HRVs have a direct role in the clinical outcome of their infection [247]. The HRV partnership with host immunity may be a mutualistic one, inadvertently imparting an advantage to the host by protecting against more cytopathic respiratory viral pathogens, while the host provides a vessel for HRV replication and transmission. Studies of single respiratory viruses without being in the context of the respiratory virome are of limited value in drawing conclusions about clinical impact.

5.6 Virus Interference and the HRVs

Much of the longitudinal epidemiology data previously relied upon to form assessments of HRV significance was acquired using culture-based techniques. With improved and more comprehensive testing, patterns can be seen among the interactions of HRVs and other respiratory viruses.

Virus interference is a type of virus-virus interaction (VVI) that has been known for decades. VVI has recently been categorized into types [248]. At the population level, it has been noted that during trials of live attenuated IFV (LAIV) vaccines, an interferon (IFN) response was triggered that protected vaccinees against off-target viruses for 7 days postvaccination [249]. This 1970 study went so far as to suggest such effects could be maintained for a prolonged period using a regime of consecutive schedule vaccinations, each separated by 7 days or more, during times of a prolonged epidemic [249]. A similar effect was produced using live EV vaccines (LEV) to replace pathogenic EV types and interrupt outbreaks [250]. Orally administered LEVs succeeded in their principal task but also reduced the incidence of ARIs during epidemics by 50 % overall [250]. This shows that immune activation in the gastrointestinal system generates an anatomically distinct protective effect and there may be a similar effect on the gut's inflammatory status after respiratory virus infection. In contrast to the LAIV results, the off-target protective effect was reversed in a study using a trivalent inactivated IFV vaccine [251]. The mechanism underneath these opposing outcomes is unclear.

During the heyday (1960s) of tissue culture for virus studies, a common biological assay for infection with HRV involved attempted infection of the culture with an enterovirus (EV) or HPIV-1 [252, 253]. Failure of the superinfecting virus to grow heralded the likely presence of a non-cytopathogenic HRV. Virus interference has been used to measure IFN in specimens through its inhibition of HRV growth [254]. More recently HRV-HAdV dual PCR-positive cases were found less often than expected and harbored lower viral loads of HRV than did specimens from cases of sole HRV infections [255]. Significantly, the majority of these instances of VVI involve RNA viruses [244]. It has been shown that dual infections of peripheral blood mononuclear cells (PBMCs) with viruses other than HRSV (including HRVs) induced immune responses similar to those of single infections, but coinfections including an HRSV resulted in reduced IFN-γ responses [71]. VVIs are affected by the ability of each to moderate the host response against them.

Virus interference has also been identified in virus positives as a series of patterns among respiratory specimens tested for up to 17 respiratory viruses (Fig. 29.8) [9, 244]. Statistical analyses supported that many of the co-detections occurred in patterns, in particular that fewer co-detections involved an HRV than would have been expected by chance alone ($p \leq 0.05$). For some period, RNA virus infection, especially the HRV group, may render the host less likely to be infected by other viruses and, by extrapolating to the community level, help constrict the epidemic periods of other viruses by reducing the number of fully susceptible hosts.

Virus interference as a feature of respiratory virus epidemiology can also be seen in results of other studies [256]. During an 8-week period that spanned peak 2009 H1N1 pandemic influenza season in Wisconsin, it was influenza A virus (IFAV) that seemed to dominate HRV in children with asthma who were sampled weekly [236]. Whether this reflects all IFV-HRV interactions or just those involving a novel IFV such as 2009 H1N is unclear. It was found that PBMCs from these children exhibited normal immune responses [236].

5.7 HRV Shedding and Persistence

Reports of subjects with continuous and extended (greater than 2–3 weeks) periods of HRV positivity [3, 257] increased as PCR methods replaced cell culture for HRV detection. This had only rarely been recorded using culture [54]. HRV RNA has been detected days prior to symptoms commencing and for as long as 5 or more weeks after they cease [3, 258–261]. Studies that only define the period between ARIs in children as that time when specimens are RT-PCR negative [3] will not detect overlapping serial infections (Fig. 29.9). Epidemiology that incorporates HRV typing generally does not find chronic shedding [204]. HRV shedding normally ceases within 11–21 days, after signs and symptoms have stopped [3, 9, 44, 75, 85, 260]. Thus, the perception of persistence is probably due to serial or overlapping infections by multiple untyped strains [8, 54, 210, 262]. Few studies [263] have suitably addressed persistence in HRV infections involving healthy subjects since pre- and post-sampling clinical data are rarely described [80, 264].

To date, true persistence—an ongoing detection of a single confirmed HRV type—has been limited to individuals with underlying immunosuppression or immune dysfunction [260]. HRV-Cs were detected more than three times longer in immunocompromised young patients than in immunocompetent children, with a mean of 16 versus 53 days [265]. Multiple detection of the same HRV type (100 % identical HRV-1a sequence in each patient over time) extended to 4 months in hematopoietic stem cell transplant recipients.

5.8 Asymptomatic Infections

The proof of causality is as difficult to achieve as the proof of innocuousness when it comes to respiratory viruses and

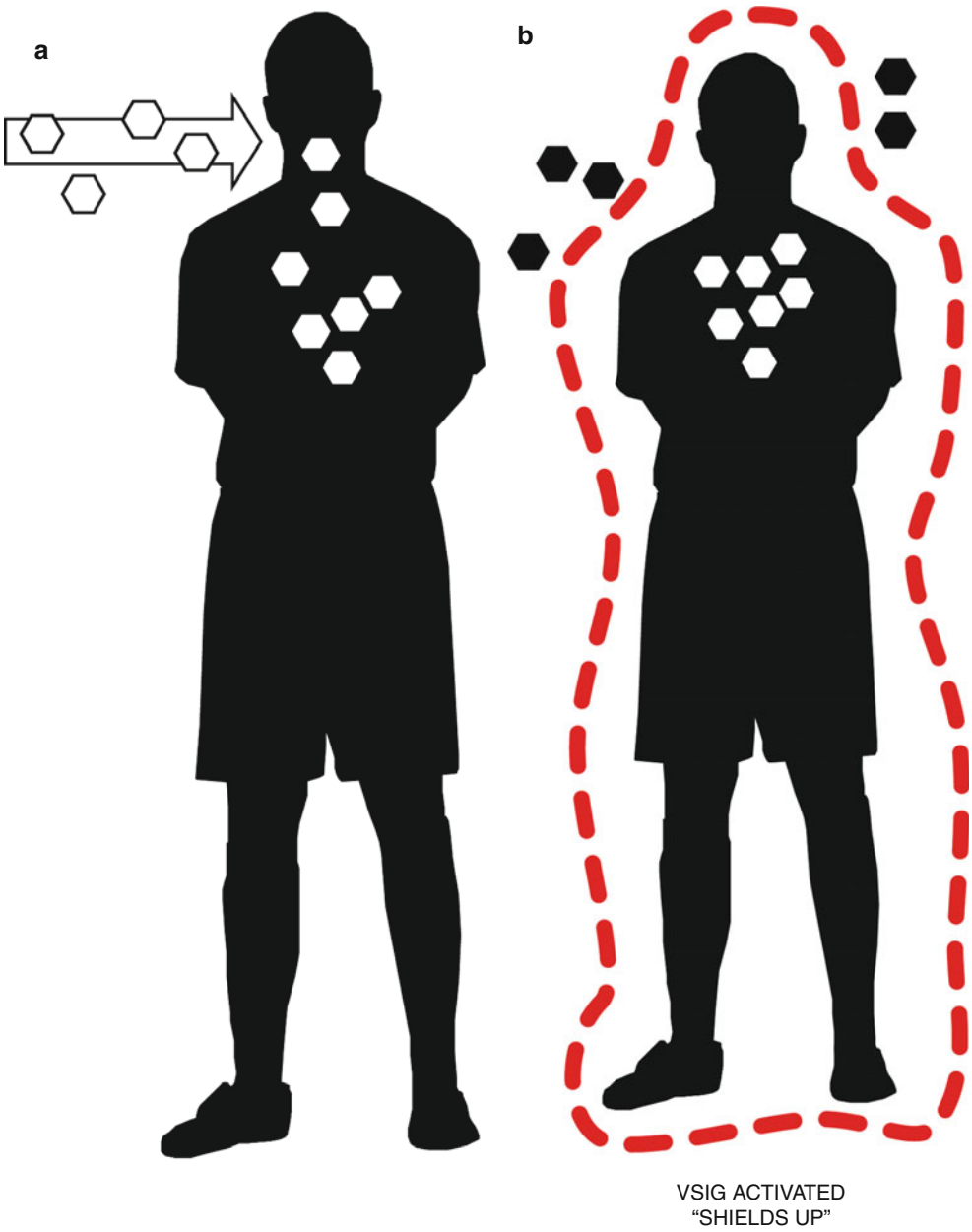

Fig. 29.8 A simplified representation of the impact of a first respiratory virus infection on subsequent respiratory virus superinfections. Very shortly after the host is infected, (**a**) the local early innate immune response creates an antiviral state in neighboring cells (see Sect. 7.1), perhaps also in distant epithelia, mediated by circulating immune cells. The resultant inflammatory response (**b**) creates a shield of sorts, reducing the likelihood of infection by a superinfecting virus mediated by viral stress-inducible gene (*VSIG*) products

ARIs. The definition of "well" subjects prior to or at the time of sampling or inoculation is sometimes not clear, especially for young children who cannot reliably report symptoms [3, 96, 204]. Often parents notice a symptomatic illness before an infection is detected in the laboratory [3], supporting the importance of diaries in longitudinal home-based community studies. Nonetheless, even with the support of telephone interviews and home visits, milder cold symptoms may be missed. It is not uncommon for an asymptomatic control to subsequently become symptomatic or have been symptomatic before sampling [8, 266]. Some studies employ sensitive symptom scoring systems [267], but the criteria for being symptomatic are usually designed to describe and clearly discriminate overt or more "severe" illnesses, those with obvious and measurable signs. Strict definitions help improve patient management and the commencement or better direction of treatment or cohorting. However, in research studies the arbitrary degree of severity required for reporting a symptomatic event often overlooks very simple changes in host biology due to a virus's replication. These changes to the norm are mild but nonetheless represent disease (a disorder of structure or function that produces specific symptoms or that affects a specific location and is not simply a direct result of physical injury) in the literal sense. Such minor or short-lived, often unrecorded [3], indications of infection include sinus pain, headache, sore throat, earache, watery eyes, fatigue, muscle aches and pains, and mood changes. Within families, HRVs are frequently transmitted from

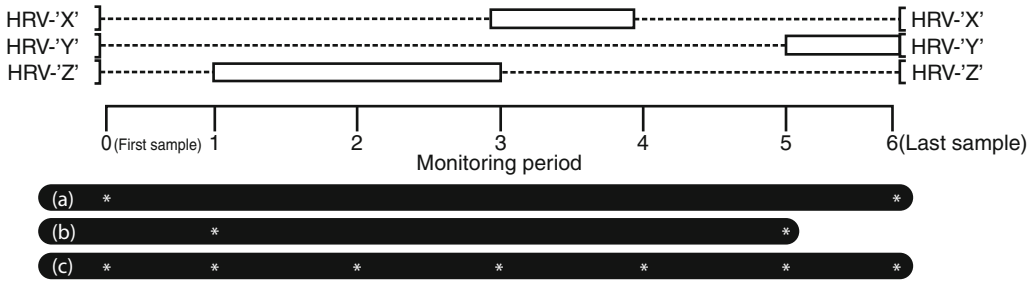

Fig. 29.9 The impact of HRV typing and of sampling based only on symptoms. The example provided here diagrammatically represents a single, hypothetical monitoring period, starting at time = 0, for a single individual. The period of potentially detectable HRV is indicated by an open box. If sampling occurred at each time point (*0–6*) and HRV positives were genotyped, it would be apparent that three different strains infected the individual, although discerning HRV-X from HRV-Z at time point *3* would require a molecular cloning approach. Illness, in different forms, may have continued over the entire period depending on the symptoms required/recorded and the period of time represented by the monitoring period. In this case a clinical diagnosis may record only a single symptomatic episode. Genotyping may not be performed, and sampling may be intermittent, and so association between viral type or species and disease is impossible. In the study examples indicated by (*a*) start and finish sampling or (*b*) symptomatic sampling, (*asterisks* mark sampling times in filled bars), the laboratory data would have made only one or two identifications, respectively. In the third example, (*c*) frequent sampling of this type has previously led to conclusions of HRV persistence or chronic shedding; when combined with genotyping, it becomes apparent that different HRV types are present

children who are usually symptomatic [204]. Infants frequently exposed to other children have more asymptomatic viral infections [8]. Among infected adult family members, asymptomatic infections are more likely [204]. Among older parents, whether their children live at home or not, asymptomatic infections are more frequent following HRV challenge than among adults without children or in younger parents [268]. In a study of viral species in age-stratified cases and controls, significantly lower viral loads were found in those without the required symptoms [269]. QPCR may prove useful to determine viral load cutoffs to address this issue in the future, although the respiratory tract is a difficult tissue for qPCR [200].

The high sensitivity of PCR-based methods has raised concerns over the clinical relevance of a virus-positive result [269]. It is clear that a proportion, around five to 28 % of study-defined asymptomatic control populations [90, 91, 269], are virus positive using sensitive PCR-based methods. This may vary up to nearly 50 % of cases when stratified by age, virus, and season or when including high-risk populations [8, 269]. Every respiratory virus, even IFVs and HRSV, can be found in cases without symptoms at the time of specimen collection even after specific inoculation of adults [137, 269, 270]. This is a complex and incomplete story in need of more research, and so it is frustrating that positivity in asymptomatic people is often used to rank viral importance. Better data are required from asymptomatic controls for any conclusion to be drawn about causality [266], but this requirement often disregards the memory of a normal functioning protective host immunity. It is the host response that defines the degree of clinical severity for the inflammatory disease that is the hallmark of an ARI [271]. It is well known that previous exposure to a virus affords protection from the full clinical spectrum of disease upon repeat exposure to that virus. It should come as no surprise then that HRVs, which usually cause brief infection anyway, could well produce only minor signs and symptoms upon reinfection. The unique and extremely personal infection history of each member of a control group cannot be determined unless they are part of a longitudinal cohort. So, what do cohort studies, supported by comprehensive PCR-based testing, tell us about asymptomatic virus infections?

Some cohort studies do not look in asymptomatic children, seeking samples only at times of symptomatic illness [66, 246, 272]. A birth cohort of children enrolled and sampled when ill and every 6 months for 24 months identified HRVs 14–28 % of infants and toddlers who had no nasal symptoms (defined solely by the presence of rhinorrhea) [273]. The Childhood Origins of ASThma (COAST) birth cohort followed 285 infants at high risk for allergies and asthma for 12 months and identified HRV infections as preceding (mean age of first detection, 4 months) those of HRSV (mean age at least 6 months), and HRVs were found in 35 % of asymptomatic versus 61 % of moderately to severely ill patients; the most frequently symptomatic children also had the greatest proportion of asymptomatic infections [8]. In a study of 58 children with asthma sampled weekly for 5 weeks during each of two peak HRV seasons, nearly two-thirds who were virus positive but not sensitized to at least one allergen showed no asthma symptoms, and nearly half showed no ARI symptoms; in the children who were sensitized, less than one-third showed no asthma symptoms, and only a fifth had no ARI symptoms [227]. A convenience population of 15 healthy children (1–9 years old) without asthma were followed during at least three seasons, and picornaviruses were detected in 5 % of 740 specimens (21 % of infections) not associated with symptoms,

although 9 of the 25 infections came from households with an infected sibling [3]. In summary, there is clear evidence for the presence of HRVs in asymptomatic controls. A precise proportion cannot yet be defined. Some study controls show signs of a "lead-in" period where RNA positivity precedes an ARI defined on follow-up, while others may have been defined as symptomatic if more symptoms had been accounted for.

6 Mechanisms and Routes of Transmission

6.1 Source of Infectious Virus

HRVs have been found at extra-respiratory sites. Viremia was determined in the blood of children with LRT infection or pericarditis [274, 275], and HRV-C was more commonly associated with viremia than was HRV-A, supporting possible increased pathogenicity [274]. Blood was also positive for HRV RNA and infectious virus from infants at necropsy [276, 277], and HRV RNA was detected in the plasma of children with asthma, bronchiolitis, or common cold [76]. An HRV was once isolated from feces [203], and more recently higher than expected loads of HRVs were detected in fecal specimens from children with suspected meningitis and fever of unknown origin [77], with gastroenteritis [278], and in a child with pericarditis [275]. Nonetheless, the nasopharynx is still considered the main site of focal virus production [279], regardless of inoculation route [280], and most studies of transmission routes have centered on the URT. In contrast to IFV and HRSV, HRV infection involves less destruction of tissue. Ciliated epithelial cells are sloughed off in proportion to the severity of an HRV ARI, but this damage is minimal and does not occur during the viral incubation period or with subclinical infections [137, 281]. The incubation period between infection and onset of virus shedding into nasal secretions is 1–4 days with shed viral titers peaking in adults between days 2 and 10 [44, 282]. The time until successful HRV transmission among adults in a childless family setting is usually 5–8 days and requires the donor to be shedding at least 10^3 $TCID_{50}$ at some stage, to have recoverable virus on the hands and in the nares, enough shared time, and a moderate to severe ARI [283].

The lungs have been shown to host replicating HRV [260], and the reader of such reports may be left with the perception that detection of HRV replication in the LRT explains all LRT symptoms. However, relatively few studies seek or identify true HRV replication in the LRT. While the overwhelming majority of LRT cases detect HRV from the URT, a correlation between URT positivity and LRT disease does exist [284].

6.2 Self-Inoculation and Virus Survival

It is well known from experimental inoculation studies that HRV infection can result from inoculation of the conjunctival sac after virus is moved through the nasolacrimal duct [280]. In these studies virus was commonly delivered by aerosol or intranasal instillation of 0.25 mL to 5 mL of suspension [43–46, 280, 285–287]. In the laboratory, HRVs can retain infectivity for hours to days on suitable, nonporous solid surfaces, especially if the inoculum remains damp [47, 287], which supports direct self-inoculation especially in the family setting and indirect inoculation via fomites [288]. In a trial to define the movement of virus from a contaminated donor to a recipient via multiple surfaces or by hand-to-hand contact, 13 % (donor to objects to recipient) and 6 % (donor to recipient fingers) of the virus recoverable from the donor's fingertips were recoverable from the recipients' [289]. Even under observation, eye rubbing (0.37 h^{-1}–2.5 h^{-1}) and nose-picking (0.33 h^{-1}–5.3 h^{-1}) occur frequently [47, 290], suggesting self-inoculation could outpace personal hygiene, particularly in the young.

6.3 Airborne and Intimate Contact Transmission

It was once thought strange that ARIs were so common, but isolation rates for the expected viruses were so low [36, 291]. With a better understanding of the importance of preexisting antibody (something common among the predominantly adult volunteers used by many studies), the discovery of a third, unculturable species of HRV (still causing ARIs but impossible to isolate or detect using antibody-based systems for which no reagents existed), and a vastly improved diagnostic sensitivity, this is much less confounding. In the past, household cross infection, determined by ARI, was low, about five exposures to infected members required for infection [17] despite viral loads in nasal washings peaking at 1.6×10^5 $TCID_{50}$/mL [44]. Experimental transmission was also reportedly inefficient [45]. In contrast, "naturally" close-quartered military populations, interacting over 1–4 weeks, experienced rapid spread of HRVs to >50 % of the group [54]. The use of PCR recently clarified this discrepancy, confirming that frequent transmission in families is more common than culture-based studies had identified, often resulting in asymptomatic infection among older siblings and parents [204]. PCR has helped define the scope of viral RNA, if not actual infectious virus, survival, and spread.

Transmission studies require infectious HRV, and so the HRV-Cs do not contribute to the historical data. Under crowded or intimate conditions and with more severe colds, transmission reaches 38–100 % [283, 292]. In some studies, both large- and small-particle aerosols proved inefficient,

supported by a low isolation rate from saliva (39 % compared to 65 % of hand washes and 50 % of nasal swabs)[44, 47, 293] and from only 8.3 % of participants exposed to large-particle aerosols [293]. In other human donor-recipient model studies however, aerosol proved to be the main transmission route among antibody-free adults [46, 282]. The discrepancy may have been due to insufficiently long or intense exposure in the earlier aerosol experiments [45, 267]. Apart from particle size, spread of virus by aerosol is affected by existing nasal obstruction which can divert secretions from the nares to contaminate saliva, the presumptive source of virus in coughs and sneezes [44]. When exposed to 10 liters of a small-particle aerosol, 10^1 $TCID_{50}$ of HRV-15 was associated with fever and prominent tracheobronchitis in antibody-free (<1:2) adult volunteers but not when delivered via nasal drops or a coarse aerosol [46]. It has also been found that simple breathing releases HRV RNA (the same type was also identified from nasal mucous) from at least a third of adults and children with symptomatic ARIs and infectious HRV could be isolated from a fifth [294, 295].

It is apparent that HRVs accumulate at sites with heavy human traffic, potentially forming a secondary source of infection. HRV RNA can be detected from 32 % of ~47-hour-old filters placed to sample air in office buildings [296]. In aircraft, high efficiency particulate air (HEPA) filters have been found to harbor HRV RNA more than 10 days after they were removed for servicing [297].

7 Immunity

HRV infections trigger a vigorous proinflammatory immune response that is thought to drive the symptoms experienced as illness [271, 298, 299], but they do not seem to actively prevent or interfere with the host's immune response the way most other viruses have evolved to do. There may be a role for repeated challenge by HRVs and other respiratory viruses leading to inflammation and tissue remodeling. The host response to HRV infection can be broadly broken into the innate (very fast, encoded in the germ line, nonadaptive) and adaptive (slower to develop, reliant on T cells, B cells, and the generation of antibody) responses. While the innate system is "always watching," it is significantly amplified by virus infection. The adaptive response is initiated by the host's first infection with a particular virus and then functions to limit subsequent infections through the production of neutralizing antibodies and amplification of existing cell-mediated immunity.

7.1 Innate Immunity and Interferon

After virus-receptor binding and internalization, the earliest host cell immune response to an HRV infection is elicited by the innate immune system (Fig. 29.10). Epithelial cells represent the front line against HRV invasion although alveolar macrophages and DCs are better equipped to respond [300] and do so despite not hosting HRV replication directly [16]. Virus detection is mediated by pattern recognition receptors (PRRs) that have evolved to recognize conserved molecular structures shared among diverse pathogens. Internal- or surface-mounted PRRs include sentinels that specifically recognize picornavirus RNA and protein and, in doing so, trigger an immune circuit that results in the production of IFNs and subsequently hundreds of IFN-stimulated gene products. The innate response to viral infection hinges on inducing two type I IFNs (initially IFN-ß then IFN-α), secreted cytokines that produce antiviral, antiproliferative, and immunomodulatory outcomes [301]. The type III IFNs

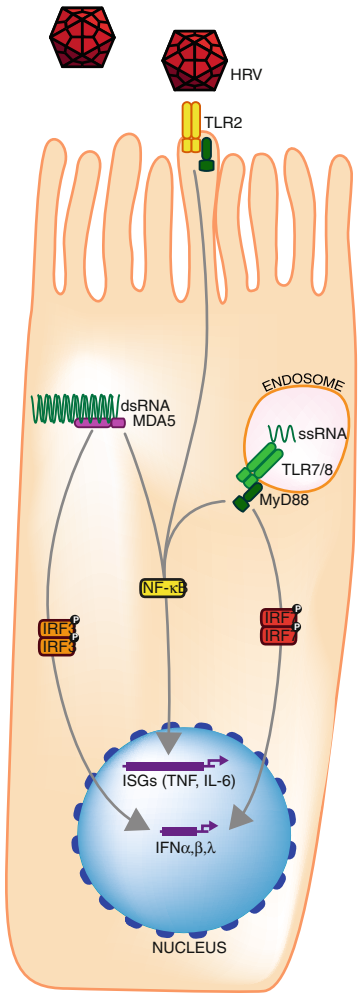

Fig. 29.10 A simplified representation of molecules involved in or that respond to the recognition and response to HRV infection of airway epithelial cells [302, 312–317]. *IFN* interferon, *IRF IFN* regulatory factor, *ISG IFN*-stimulated gene, *TLR* Toll-like receptor, *MDA5* Melanoma Differentiation-Associated protein 6; *NF-κB* Nuclear factor kappa-light-chain-enhancer of activated B cells, *MyD88* myeloid differentiation primary response 8

(IFN-λ1 or IL-29, IFN-λ2 or IL-28A, and IFN-λ3 or IL-28B) are also produced in response to viral infection in a range of cells, although their receptor is not as widespread [302]. The type II IFN, IFN-γ, is produced by activated T cells and natural killer cells rather than in direct response to virus [303]. Detection of viral components triggers protein signaling cascades that regulate IFN synthesis through the activation of viral stress-inducible genes (VSIGs) [301, 304]. These are sometimes expressed constitutively but upregulated after IFN induction following HRV infection [305]. Released IFN-ß binds to the IFN-α/IFN-ß receptor in an autocrine (the same cell) and paracrine (neighboring cells) manner, starting a positive feedback loop for type I IFN production, the "second wave." VSIGs include the antiviral proteins protein kinase R (PKR), 2′5′OAS/RNaseL, and the Mx proteins [306]. IFN-α upregulates expression of MxA, 2′4′-OAS, and PKR [307]. The Mx pathway is also induced after virus infection but is not constitutively expressed [307]. Depending on the sentinel system stimulated, there are different pathways to VSIG activation. Those VSIGs with antiviral properties (e.g., MxA, PKR, 2′5′OAS/RNaseL) inhibit different stages of virus replication and strengthen an antiviral state in the host. While this state is well known, the nature of its induction by different respiratory viruses and the impact of induction upon the replication of other respiratory viruses are topics for considerable ongoing research.

One pathway to IFN induction relies on the IFN-upregulated cytosolic sentinels retinoic acid inducible gene RIG-I-like receptors (RLRs) RIG-I (specific for IFAV and others) and melanoma differentiation-associated gene 5 (MDA5, specific for picornaviruses and others) [306, 308]. These RNA helicases recognize either RNA with a 5′-triphosphate or distinct dsRNAs, which results in activation of NF-κB leading to "classical" type I IFN induction [301, 306]. Studies into the innate response to HRV infection have been limited to the use of a very few easily cultured types. It is presumed that the result can be extrapolated to most if not all types. This is yet to be tested. RIG-I is degraded by HRV-A16 [309], IFN regulatory factor (IRF)-3 homodimerization is interfered with HRV-B14 which limits IFN-β induction [310, 311], and MDA5 is degraded by HRV-A1a but not HRV-A16 [312].

Another pathway for recognizing HRV infection involves the Toll-like receptors (TLRs), transmembrane PRRs that terminate in an intracellular signaling region. The endosomally localized TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids and are also involved in innate antiviral responses. TLR7 and TLR8 identify G/U-rich ssRNA from endocytosed viruses, while TLR9 recognizes unmethylated CpG DNA present in DNA viruses [301, 318]. TLR2 and TLR4 are found on the cell surface and recognize HRV or HRSV proteins, respectively [318, 319], and TLR3 recognizes dsRNA. TLRs operate mainly, but not exclusively, in plasmacytoid DC [301]. The particular TLR that notifies of an HRV incursion may depend on the method of virus approach [319]. TLR7 activation can reduce 2′5′OAS and MxA mRNA expression and IP10 protein in adolescents with asthma compared to healthy controls [320]. TLR3 activation did not result in a similar disparity [320].

It has been suggested that HRVs may have evolved with humans to such an extent that their symbiotic relationship serves to help train the human immune system [321]. Intriguingly, within the HRV species, there are differences in the type and level of host response induced [322] which may reflect receptor usage, route of entry and cell type infected, HRV species, or the degree of laboratory-adapted virus used during in vitro studies.

7.2 Cellular Immunity and Inflammation

After initial HRV infection, the innate response results in production of proinflammatory cytokines, vasoactive peptides, and chemokines that attract leukocytes, granulocytes, DCs, and monocytes (Table 29.2) [321, 323, 324]. The T-lymphocyte response to viral intrusion can be broadly categorized as T_H-1-like and T_H-2-like. Other T-cell subsets exist, but most work in relation to HRV has been conducted on the earliest defined subsets. The T_H-1 cellular response is important in managing cellular immunity and producing interleukin (IL)-2 and IFN-γ. The T_H-2 cellular response manages humoral immunity and stimulates B cells via IL4 (initiating production of IgE), IL5 (influencing eosinophils), and IL13 (crucial component of allergen-induced asthma). These two T-cell responses act in concert with epithelial-derived chemokines (e.g., eotaxin) to promote the recruitment and activation of eosinophils and mast cells, contributing to chronic airway inflammation and the hyperresponsiveness of airways to a variety of nonspecific stimuli [325]. T_H-2 lymphocytes, opposing T_H-1 lymphocytes, contribute to an allergic inflammatory cascade, akin to what occurs to rid humans of parasites [326]. The T_{H-1} response can also be repressed by binding of microRNA, which leads to an altered balance favoring a T_H-2 state in mice and probably in humans [327]. Regulatory T cells (T_{reg}) suppress allergic inflammatory pathways and are therefore fundamental in protecting the airway from allergen sensitization [326].

Considerable immunobiological research has focused on asthma exacerbation, with which HRVs are intimately involved. Although upregulated by HRV infection, the T_H-1 response is comparatively deficient in people with asthma [328, 329]. This is problematic as an increased T_H-1-like cytokine response, deduced from higher sputum mRNA IFN-γ/IL5 values, speeds clearance of HRV and symptom amelioration [85]. One possible cause of the T_H-1 deficiency in people with asthma is inadequate maturation of type I and III IFN responses due to reduced exposure to infections early in life [330]. The

Table 29.2 Some important molecules involved in the response to HRV infection

Molecule	Role
IFN-α/IFN-β (type I IFN)	Produced by leukocytes and BECs; numerous subtypes; immunomodulator
IFN-γ (type II IFN)	Produced by many cell types after viral infection, especially BECs, PBMC, and DCs; a key T_H1 cytokine in intracellular defense through stimulation of antiviral molecules; macrophage and NK cell activation and B-cell proliferation
IFN-λ (type III IFN)	Participates in creation of an antiviral state; produced by and influences the maturation of DCs
IL-1β	Proinflammatory properties; enhances adhesion molecule expression including ICAM-1; induces IL-2 receptor
GM-CSF	A granulocyte and monocyte growth factor
IL-2	Stimulates growth and differentiation of T and B lymphocytes and cytotoxic activity of NK cells and monocytes
IL-4	T_H2 differentiation, promotes IgE synthesis
IL-6	Activation, differentiation, and proliferation effects on T and B lymphocytes; induces C-reactive protein stimulating pyrexia
IL-8/CXCL-8	Neutrophil chemoattractant resulting in neutrophilic, monocytic, and lymphocytic recruitment and degranulation activity
IL-10	Anti-inflammatory factor produced by monocytes that acts by inhibiting proinflammatory cytokines IL-1, IL-6, and TNF-α
IRF7	A master hub, regulating antiviral immunity
IP10/CXCL10	Chemoattractant for activated T_H1 and NK cells
TNF-α	Proinflammatory activity similar to IL-1 β; activates neutrophils; induces vascular permeability
MPC-1	A monocyte attractant
Bradykinin	Potent inflammatory mediator, increases vascular permeability
TSLP[3]	An IL-7-like cytokine that activates myeloid DCs to induce naive T cells into T_H2 cells producing IL-4, IL-13, and TNF-α; induced by HRV in the presence of IL-4

BEC bronchial epithelial cells, *DC* dendritic cell, *IRF* interferon regulatory factor, *IFN-γ* inducible cytokine protein, *NK* natural killer, *PBMC* peripheral blood mononuclear cells, *IL* interleukin, *TNF* tissue necrosis factor, *TSLP* thymic stromal lymphopoietin

"hygiene hypothesis" [331, 332] posits a pathway for an asthma etiology described [325] in terms of the young, unchallenged immune system, dependent on infections to stimulate the development of its T_H-1-like functions. One theory suggests that HRVs play a central role in developing that efficacious antiviral immunity, particularly in infancy, via their frequent, usually mild self-limiting infections [333].

Genome-wide expression analysis of BECs from healthy and asthmatic adult subjects after HRV-A1a infection revealed some significant differences that were found between cell types and response to infection [61]. These included immune response genes (IL1B, IL6, IL8, IL1F9, IL24) and airway remodeling genes (LOXL2, MMP10, FN1) and an overall proinflammatory response and metabolic slowdown consistent with proteolytic cleavage of transcription factors by some HRVs [133, 334–336] in the infected cells. This study further noted some similarities to gene expression changes observed in brushings from people with mild asthma after allergen exposure and in BAL cells from subjects with corticosteroid-resistant asthma [61]. Overall, HRV replication and the host transcriptional response to it were similar in normal or asthmatic BEC cells [61]. This indicated, at least in adults, that something beyond the epithelial cell is an important contributor to more severe clinical outcomes in asthma.

The application of inactivated HRV-B14 was found to promote release of IL10 from monocytes (an immunosuppressive cytokine) and to inhibit the stimulation of IL12 (drives T_H-1 development) [337]. However, neither IL10 nor IL12 was significantly induced in asthmatic adult volunteers in response to HRV-A16 compared to healthy subjects [338]. While IFN-α was detected after transfection of DCs with HRV-B14 ssRNA, low TNF-α and IL12 levels were also noted [16]. It was posited that the reduced IL12 could indicate negatively affected local immunity possibly predisposing to secondary infections [337]. Infection of stromal lung cells by HRV-B14 triggered exaggerated levels of the pleiotropic IL11 (an IL6-type cytokine), akin to those triggered by HRSV, which were also detected in nasal secretions from children with wheezing [339].

Other cytokine changes have been identified in atopic adult volunteers challenged with HRV-A16. G-CSF and IL8 (chemo-attractant for neutrophils) levels rose in the URT (as examined by protein detection in nasal lavage) and LRT (mRNA detection in sputum) with concomitant rises in blood and nasal neutrophil numbers [85, 203, 340]. The nasal epithelial cells of atopic individuals, especially in season, express more ICAM-1 than those of nonatopic adults [341] as do normal subjects infected by the major group HRV-B14 [341]. By contrast, IFN-γ and IL8, which appear later postinfection, downregulate ICAM-1 expression in infected cells [191] and encourage infiltration of neutrophils [342], respectively. Changes in ICAM-1 levels may modify

T-lymphocyte-mediated cytotoxic or T_H interactions with HRV-infected cells, upregulating receptor expression and encouraging eosinophil and T-cell infiltration into the lower airways of asthmatic individuals [187, 343].

7.3 Antibodies

Before an HRV can enter a cell, it must pass through a defensive barrier of secreted anti-HRV antibody, mostly IgA. The ease with which this passage occurs is proportional to the progression of clinical disease. Healthy adult volunteers were found to develop IgA by at least 3 days to 2 weeks after inoculation—about the same time as serum antibody—and retain peak levels for at least 8 weeks [178, 344–346], falling faster than serum levels [347, 348]. There is also some evidence for a degree of nasal immune memory [347]. Volunteers with pre-study serum antibody could still be infected in some studies [46, 210, 292], but not in others [349]. Infection is more clear in volunteers without preexisting nasal antibody to experimental challenge virus; they become infected, exhibit more severe ARI, and shed more virus for longer [292, 347]. IgA does not seem to modify illness severity or virus shedding, but high levels prevent reinfection by the initiating virus type. Low levels or absence of IgA does not prevent reinfection by the same HRV type, which may manifest as symptomatic or asymptomatic disease [349].

Older children, adolescents, and adults have greater amounts of HRV-neutralizing antibody than young children [42], accompanying a trend toward decreasing numbers of symptomatic ARIs with increasing age [218, 350]. This feature raises an issue: did the use of older subjects in many common cold studies underplay the pathogenic potential of the HRVs because protective or partially cross-protective antibodies moderated the impact of infection? Consequently, quantifying levels of type-specific serum antibody became routine practice prior to some studies. Adult volunteer studies determined that no infections resulted if preexisting neutralizing antibody titers ≥1:16 existed; as levels grew from 0, so did levels of resistance to infection [43, 210]. Adults were protected by serum titers of 1:3–1:8 [209, 210]. The trend was interrupted by adults in the 20–39 year age group, presumably because they had begun families and their young children acquired and amplified currently circulating types from the community and transmitted them into a household that was either immune naïve or lacking sufficient antibody or cell-mediated memory for protection [351].

Traditional vaccine strategies were quickly ruled out as a prophylactic intervention for HRV illness because of the extensive antigenic variability that is a hallmark of the genus *Enterovirus* [178, 325]. However, if it were possible to identify "master" strains [352] that exhibit sufficient antigenic cross-reactivity to induce broad heterotypic responses against many other HRV strains, then an effective vaccine could still be possible. In fact, boosting host immunity to an HRV type by repeat infection does heighten immunity to one or more other types [44, 353]. The highest of these heterotypic antibody titers develop against those types with the highest preexisting antibody levels [352]. The first description of a unifying HRV numbering system recounted the appearance of minor serological cross-reactions, which were removed by modification of the technique [57]. Subsequently, cross-reactions were better defined during experimental inoculation when multiple HRV immunogens and antigens were used to deduce the extent of heterotypic responses [56, 210, 352, 354].

Less promising for HRV vaccinology was the description of antigenic variation within HRV types which suggested that immunity to one variant of the type might not protect against infection by other variants [355, 356]. The "prime strain" is a specific antigenic variant of a prototype HRV type that is neutralized to a lesser extent by antisera from the prototype, while yielding antisera that effectively neutralize both itself and the prototype [357]. Another form of this cross-neutralization is ascribed to the "intertypes," which are HRV isolates that share a lower-level serological relationship with a pair of HRV strains, which themselves share neutralizing reactivity, e.g., HRV-A36 and HRV-A58 [358]. The low-level reciprocal neutralizing activity was not equivalent in both directions; anti-HRV-A36 sera had a higher titer for HRV-A58 than anti-HRV-A58 sera did for HRV-A36 [358]. Over 40 strains were linked directly by such one- or two-way cross-reactions or indirectly through two or more strains. HRV-A67 and HRV-A28 are linked via HRV-A11, HRV-A13, and HRV-A32 (anti-A11 serum neutralizes HRV-A28, anti-A13 neutralizes HRV-A11, and anti-A32 neutralizes both HRV-A13 and HRVa-A67[358, 359]). A surrogate molecular method which provided insight into these interrelationships, perhaps expanding upon them to identify useful patterns for vaccine immunology purposes, would be most welcome.

In summary, heterotypic immunity and HRV intertypes might be exploitable features of HRV immunobiology that could confer maximum protection upon the host from the minimum number of HRV types [358].

8 Pathogenesis and Host Response

HRVs circulate in great numbers, and any specific roles for distinct HRV types in initiating disease remain to be defined. The relatively inconsequential common cold is the most frequent manifestation of viral infection in humans, with 30 to >80 % of colds positive for an HRV [69, 360, 361]. Furthermore, ARIs due to HRV infection can exacerbate or

result in a much greater burden of disease in those with asthma, COPD, or cystic fibrosis. Other complications include otitis media, pharyngitis, and wheeze in atopic people without asthma. The role of viruses in the origin of some of these diseases or their exacerbation is still unresolved. The LRT disease may mask the URT nature of the infection, favoring clinical diagnosis of an LRT illness. Interestingly, during the 2009 H1N1 pandemic, much of the parent-initiated healthcare visits from a birth cohort in the United States were not due to pandemic virus but HRV and HRSV [362]. There is no known natural murine rhinovirus on which to base a small animal model of HRV infection, and mice are not natural hosts for HRVs. A recently developed model of airway disease using mengovirus (a Picornavirus infecting rodents) may yield valuable in vivo airway infection and inflammation data [363].

HRVs are often detected in neonates and infants with LRT signs and symptoms because the very young have narrow, immature airways and are more significantly affected by airway swelling, excessive secretions, and smooth muscle contraction [364]. This may also be due to the relatively naive immunity of very young children. Much of the more severe disease in HRV-positive children occurs in the youngest of them. Some key examples are addressed below.

8.1 Common Cold

For the common cold, as for any illness, accurate epidemiology and burden of disease data underpin the prioritization of preventing, treating, and further researching the etiological agent. To assign funds for researching the agent, health policy makers also need to understand how efficacious and cost-effective the development of an intervention will be [365]. The host immune response to HRV replication is the main cause of the signs (quantifiable fever, rhinorrhea) and symptoms (feeling of fever, myalgia, headache, fatigue, and mood change) of a cold that the host experiences [321, 366, 367]. A feature of common colds is increased vascular permeability which, enhanced by kinins, results in increased plasma protein (albumin and immunoglobulin [Ig] G) levels in mucus, approaching the levels in serum [368]. Histamine levels do not rise in nasal secretions of otherwise healthy cold sufferers [368]. During the resolving phase of the ARI, glandular proteins (lysozyme, sIgA) predominate [346].

The common cold syndrome is also described as rhinosinusitis (the agglomeration of rhinitis and sinusitis since they frequently clinically coexist) [369, 370]. This consists of nasal discharge or rhinorrhea, nasal obstruction, sore throat, sinus pain, headache, sneezing, watery eyes, cough, fever, fatigue, muscle aches and pains, and mood changes [367, 371]. These are caused directly or indirectly by viral infection; cough is the result of vagus nerve irritation by mucus; sneeze results from trigeminal nerve irritation; sore throat is likely due to the action of prostaglandins and bradykinins; and fever, psychological effects, fatigue, and myalgia are mediated by cytokines [367]. Hypertrophic adenoids have also been found to have a high proportion of viral, especially HRV, occupation regardless of host symptomatic state [372]. Observation of natural culture-confirmed HRV colds in adults noted that cough usually started by day 1 and was more persistent up to 9 days later [264, 373]. Rhinorrhea, sneezing, and sore throat were reported by half or more of patients and headache by at least a quarter of cases [207, 373]. As neutrophils accumulate at the site of primary URT infection, the myeloperoxidase in their azurophilic granules creates the yellow-green coloration of nasal mucus that was once considered a sign of bacterial superinfection [271, 367]. A common cold caused by an HRV cannot be clinically distinguished from one that caused by any of the other respiratory viruses [207, 371]. As is likely for a single HRV type, once the host has been infected by an HMPV, HPIV, IFV, etc., a secondary exposure to that same virus type will produce less severe clinical outcomes due to pre-primed host immunity.

8.2 Asthma and Atopy

Asthma is a clinical diagnosis made on the basis of patient history, physical examination, assessment of airway obstruction or reversibility, and response to bronchodilators [374]. It is a complex chronic respiratory disease involving airway inflammation, airflow obstruction, and airway hyperresponsiveness, which manifests as recurrent reversible attacks with deteriorating asthma control that are generated by interactions between infectious agents and other environmental and genetic factors that remain incompletely characterized [375]. The mechanistic role for HRVs in asthma inception and exacerbation is not yet defined [364, 376] but is being revealed as the extremely complex interplay between inflammation due to virus versus that due to atopy is explored [315]. Possible virus-host interactions include (i) severe HRV infection of healthy infants which may result in subsequent development of asthma; (ii) HRVs may trigger asthma in children with a genetic predisposition toward atopy; (iii) repeated mild infections may protect against more asthmogenic/cytopathic viruses or the overdevelopment of the T_H2-type response; and (iv) HRVs may simply exacerbate that which already exists [377]. It is unclear if the risk of atopic asthma during infancy is increased by ARIs which affect the development of the immune system, or whether ARIs lead to asthma development in children with a genetic predisposition to more severe responses to infection [325, 343, 378], or a mix of both. In children with asthma, viruses have been detected in at least 77 % of exacerbations (65 % picornaviruses, probably HRVs [379]) and in 50 % of adults [380].

Acute wheezing episodes (including bronchiolitis and acute asthma) are a frequent, epidemic, and seasonal LRT manifestation of URT respiratory virus infection of children from all ages, especially during the first year of life [214, 381–385]. Bacteria are not major factors in wheezing exacerbations [386]. Wheezing is blamed for high socioeconomic and healthcare costs, overuse of antibiotics, being the primary cause of hospitalization among children, and, rarely, for death [109, 387, 388].

Traditionally HRSV infection has most often been the virus causally associated with expiratory wheezing, wheezy bronchitis, or asthma exacerbations because of the virus's well-known ability to infect the LRT, its more frequent detection in some studies [386], and the low perceived likelihood of URT viruses such as HRVs replicating in the warmer LRT. Nonetheless, periods of epidemic wheezing in the absence of high rates of HRSV detection are common [383, 389]. HRVs even predominated in some culture-based studies of wheeze [384, 390]. The COAST study used sampling criteria that were intentionally designed to investigate the role of HRSV in illness, but instead indicated that HRVs were the most important predictor of subsequent wheezing in early childhood, and this is supported worldwide [224, 391, 392].

The asthmatic airway is characterized by an infiltration of eosinophils and Th2-type T cells (Th2 cells) [393]. In those with an atopic background, eosinophilia was more common, and the virus isolation rate was higher than in the nonatopic group [394]. The cytokine and eosinophil activation profiles for HRSV-induced wheezing differ from those induced by HRV in which IL5 is significantly higher in serum and nasal aspirates than for HRSV [118]. IP10 was the only cytokine significantly elevated in all symptomatic wheezing groups [118]. Significantly higher rates of HRV detection with more obvious LRT symptoms are more common in children with asthma than in non-asthmatic populations [380, 388, 395, 396]. Exacerbations of asthma are often preceded by a symptomatic rather than asymptomatic HRV infection [17, 379, 388, 397–399] although, in some instances, an exacerbation is the only sign of infection [400]. Reduced peak expiratory volume in children is especially associated with detection of respiratory picornaviruses [379]. Severe "wheezy bronchitis," a historical term describing an acute illness with preceding ARI and characterized by cough, wheezing, breathlessness, and mucous production, was more often positive for a virus than mild disease [394]. Even the use of culture found that HRVs predominated in both URT and LRT (sputum containing BECs) or combined respiratory tract samples [394]. Bacteria were often present with IFV, but not with HRVs [394].

The airway epithelial cells form a physical barrier in addition to their roles in immune surveillance and regulatory control [393]. However, the asthmatic bronchial epithelium is compromised by incomplete tight junctions that are more sensitive to airborne pollutants [401] and most likely to allergens and respiratory viral infections. This is further specifically disturbed by HRV infection which reduces expression levels of tight and adherens junction proteins [402]. In those with asthma, the presence of an HRV can induce illness that, while often more severe than in non-asthmatics, has been associated with significantly different HRV load or duration of HRV RNA detection in people with asthma compared to those without [403]. HRV-C types are often detected in more serious clinical outcomes than HRV-A or -B [265] although hospitalizations may be fewer for HRV-Cs than the other species [404].

8.3 Acute Otitis Media

AOM is diagnosed by middle ear effusion (otorrhea) with simultaneous signs and symptoms of ARI including fever, earache, rhinitis, cough, sore throat, chest wheeze, nocturnal restlessness, irritability, poor appetite, diarrhea, and vomiting. Transient abnormal (negative) ear pressure upon tympanometry occurs in two-thirds to three quarters of uncomplicated colds among healthy children [405, 406]. AOM is a frequent reason for outpatient antibiotic therapy which can reduce the time to resolution of symptoms in infants and has been attributed to reducing the overall hospital burden of AOM [407–412]. Since a longitudinal day-care study in 1982, the association between AOM and viral URT infection has been coalescing, and it is now clear that AOM often occurs with or shortly after a viral ARI, most frequently in the young and occurring more often during winter than summer [413, 414]. The use of influenza vaccines reduced AOM occurrence by a third during an epidemic period [415], but the use of pneumococcal vaccine did not reduce the occurrence of AOM overall, just that relatively small fraction (6 %) due to the target bacteria [416]. The isolation by culture and PCR detection of viruses from middle ear fluids and the refractory nature of some AOM cases to antibiotic therapies confirmed that viruses play an important role in this illness [409, 414, 417]. Studies relying on underperforming culture-based techniques underestimated the role for viral ARIs [414, 418], but other studies using PCR techniques and including HRVs found them to be the most frequently detected virus in middle ear fluids and nasopharyngeal secretions [409, 417].

The use of PCR has identified respiratory viruses, most often HRVs, in nasal secretions of 50–70 % of children with AOM [66, 413]. Because virus is often detected in the nasopharynx at the same time as the middle ear fluid, the question of the relevance of a PCR positive is a valid one [419]. Picornaviruses have been detected in 30 % of nasopharyngeal swabs taken during cold season from AOM-prone

infants and young children, and large quantities of HRV RNA have been detected by in situ hybridization of adenoid tissues from 65 % of children with recurrent AOM and/or adenoid hyperplasia [259, 420]. In a cohort of children followed from 2 to 24 months and using culture-RT-PCR, HRVs in the URT were the second most frequent pathogens associated with AOM, after HRSV [66]. Viruses, most often HRVs (30.8 % of AOM with ARI), were also detected concurrently with non-ARI periods associated with AOM episodes (14 % of AOM without ARI)[418] suggesting that AOM may be the only manifestation of some HRV ARIs, just as wheezing sometimes is. In the United States, 180 subjects were enrolled and followed in a birth cohort until the first AOM episode or between 6 and 12 months of age 362]. HRVs accounted for 55 % of viruses detected and 69 % of specimens with a single virus detected. This dominance was maintained even through the 2009 H1N1 influenza pandemic [362].

In the day-care AOM study mentioned above, primary acquisition of *Streptococcus pneumoniae* or *Haemophilus influenzae* had minimal importance as an initiation factor for AOM with effusion, but nasopharyngeal colonization was important [413]. Animal studies have shown that virus-bacteria interactions have a role in nasopharyngeal colonization and AOM development [414]. Positive correlation has been made between HRV detection in AOM-prone children and *Moraxella catarrhalis* infection as well as a tendency toward the copresence of *Streptococcus pneumoniae* [259]. The presence of HRV-B14 was shown to increase adherence of *S. pneumoniae* in human tracheal epithelial cell cultures [421]. It is believed that these three bacterial pathogens can colonize without symptoms until a viral ARI shifts the balance toward a cytokine-mediated inflammatory state [419].

8.4 Other Diseases in Which HRVs Are Often Detected

8.4.1 Chronic Obstructive Pulmonary Disease

This disorder of older patients encompasses emphysema (alveolar destruction) and chronic bronchitis (large airway inflammation with chronic mucous production) and describes a long-term obstruction to airflow in the lung (compared to asthma which is a reversible obstruction with normal flow between exacerbations). While bacteria are found in half of all exacerbations, antibiotic therapies have often yielded poor outcomes [422]. HRV infections result in more COPD exacerbations (~66 % of cases [92]) than any other virus identified to date [422, 423]. An experimental human model of HRV infection in COPD provided preliminary evidence that HRVs cause exacerbations [286]. Viral culture associated symptomatic HRV infections with exacerbations among chronic bronchitics, including cases of isolation from sputum (LRT sample) in the absence of HRV in the URT [424]. Adding the measurement of an inflammatory marker in the serum, like IL-6, further improves the speed of predicting an infectious etiology for exacerbations of COPD [425].

8.4.2 Pneumonia

Pneumonia is a disease that often occurs early in life, is responsible for millions of deaths each year [426], and is caused by viral and/or bacterial infections. A diagnosis of pneumonia requires a radiologically confirmed inflammatory infiltration of the lung tissue. Childhood community-acquired pneumonia (CAP) is common in developing countries [427]. CAP also complicates existing chronic medical conditions and takes advantage of immunosenesence [428].

The role of HRVs in contributing to the development of bacterial pneumonia is likely underestimated [426, 429]. Determining an etiology is confounded by the rarity of obtaining LRT specimens, by short-term studies, and by the complex milieu of viruses and bacteria involved. Less invasive sampling of the URT permits more routine sampling and screening, and so convenience and reduced risk have led to the detection of putative pathogens in the URT with the general assumption that they account for LRT disease, especially in children under the age of 5 years [430]. Pneumonia studies are complicated by the lack of a suitable control group; sputum is not produced from the healthy lower airway and needle aspiration, while a gold standard is also a hospital procedure with some risk [431].

Studies that are comprehensive and use sensitive molecular testing are also rare for the study of CAP etiology. When used for CAP investigations, PCR methods almost double the microbiological diagnoses over conventional culture and serology techniques, especially improving the identification of mixed infections and fastidious viruses [432]. Rapid diagnosis aids management and helps make decisions about treatment, while prolonged searching for an etiological agent leads to further invasive testing [256, 433]. At least a quarter of clinical CAP cases remain unsupported by microbiological findings [241, 432].

Infections causing pneumonia vary with age and vaccination status [433]. Viruses can be detected in up to 90 % of infants (1–12 months of age) with pneumonia, and these cases follow a seasonal pattern [427, 433]. Bacteria can also be detected in over 90 % of infants and older children, the elderly, and those with severe CAP [432, 434]. Studies that predated the use of PCR pronounced HRSV, followed by HRVs, the major viral contributors to CAP, with viruses comprising 27–72 % of childhood pneumonia cases [429, 434]. In the PCR age, the role of HRVs has received increasing attention, and they are increasingly the major viral group detected from both URT and LRT (sputum) specimens of children with CAP. This holds true even when studies extend across 1 or more years, which presumably

would account for seasonal variation in virus prevalence [241, 256, 426, 434]. It is suspected that viruses such as HRV prepare the way for subsequent bacterial infection in some direct or indirect fashion [65, 259, 420, 435]. There are laboratory data which support this [436] as well as observational data showing a high proportion of HRV-bacterial co-detections [256, 437].

Mixed infections including viruses are a possible cause of antibacterial treatment failure and sometimes a puzzle for physicians. Mixed infections occur frequently in LRT diseases such as pneumonia, which is not surprising since new techniques make it clear that the lungs are not the sterile environments we once thought [427, 434, 438, 439]. Viral-bacterial coinfections can comprise 15 % of patients, while viral-viral (2–30 %) and bacterial-bacterial (1–7 %) are much less common [230, 241, 256, 434, 437]. HRSV or HRV is often co-detected with *S. pneumoniae* in URT samples [256, 437, 440]. HRV detections dominate in younger children with pneumonia during peak HRV seasons, although frequently in co-detections with other viruses [230].

8.4.3 Acute Bronchitis

Acute bronchitis (less than 4-week duration in children) is defined as a sudden cough that often results from large airway infection and frequently involves viruses. Croup or laryngotracheobronchitis (viral or recurrent [441]) is a common LRT illness in children that includes the trachea and larynx as well as the larger airways, resulting in a barking cough. Patients with croup most often have a viral infection with some role for HRVs, although the extent of this is unclear [441, 442]. Despite testing, a third of cases remain without a viral etiology [441]. Tracheobronchitis resulted from some HRV-A15 infection of volunteers [46]. Chest pain and cough have been reported in half or more of adults with HRV infection [207] as well as in children and adults with HRVs detected during exacerbations of bronchitis, with or without an associated ARI [443, 444].

8.4.4 Bronchiolitis

Bronchiolitis occurs seasonally, especially in winter, in infants (1–12 months of age), affecting the small peripheral bronchioles. Winter is the peak season for HRSV circulation, but not usually for HRV. Bronchiolitis is a clinical diagnosis encompassing various disease entities and is most often reported in association with detection of HRSV, a winter virus [445, 446]. However, HRVs make up the majority of HRSV-negative bronchiolitis cases [128], and HRVs are co-detected with HRSV for which hospitalization is prolonged compared to cases positive for either virus alone [447]. Those children positive for an HRV during a clinically diagnosed bout of bronchiolitis have a significantly higher risk of recurrent wheezing in the subsequent year than those in whom another virus is detected [446]. HRVs were reported in over fivefold more cases of bronchiolitis than HRSV among patients in a 2-year prospective cohort of very low birth weight infants in Buenos Aires, Argentina [448].

8.4.5 Sinusitis

After a viral ARI, some proportion of infections may be complicated by sinusitis (inflammation of the sinus mucosa), the extent of which may be underestimated in children if the ARI is mild and unattended by parents [11]. Symptoms may include sinus pain, headache, facial pain, discolored nasal discharge, postnasal drip, cough, sore throat, malaise, and sometimes fever (more so in children) [367, 449]. The precise role for viruses and bacteria in sinusitis is still unclear [450]. Sinusitis is a common comorbidity in those with asthma [451]. The in situ presence of HRV-B14 RNA in maxillary sinus epithelium was reported in seven of 14 adults with acute sinusitis [452]. HRVs were also detected by PCR in half of adults with acute maxillary sinusitis; half of the HRV positives were negative for any bacteria [65]. The common cold is often associated with computed tomographically confirmed sinus cavity occlusion or abnormality in adults with self-diagnosed ARIs [367, 453]. Magnetic resonance imaging identified reversible abnormalities of the paranasal sinuses in a third of healthy adult volunteers following challenge with HRV-A39 [454]. Further evidence of the tropism of HRVs for sinus tissue comes from it being, so far, the only successful host for in vitro HRV-C replication [63].

8.4.6 Cystic Fibrosis

Culture- and serology-based testing has shown that virus infections in cystic fibrosis (CF) patients occur with the same prevalence as the general community, but the consequences of infection are more obvious or severe. These include deterioration of lung function, cough, increased expectoration and weight loss, and a synergistic increase in bacterial growth or acquisition of new bacterial infections [107, 455–458]. The mechanism behind the acquisition of new bacteria is still unknown and not always observed [459], but may involve a reduction in the host's immune response or viral damage to the respiratory epithelium. There is circumstantial evidence that HRV infections have been associated with respiratory exacerbations in cystic fibrosis patients [459, 460], albeit in very low numbers by nonmolecular studies [461] and without a significantly different clinical outcome from non-HRV ARIs in these patients [107]. Molecular methods have not yet been applied regularly, thoroughly, and systematically, but they generally find HRVs to be prominent among CF children

with ARI-associated respiratory exacerbation and involved in mixed viral-bacterial infections [459].

9 Control and Prevention

Hand washing and disinfectant wipes have been shown to be effective methods of interrupting transfer from fomites to the nose or to conjunctivae [87, 288, 293]. However, with eye rubbing, face touching, and nose-picking occurring frequently [47, 290], self-inoculation often outpaces personal hygiene, particularly in the young.

Hand disinfection is frequently recommended for prevention of HRV infection but has not been supported by controlled clinical trials in a natural setting [462] despite good results in experimental tests [463]. Ethanol-containing disinfectants were more effective than simple hand washing with soap and water for removal of HRV-A39 inoculum, as assessed by culture, and the inclusion of organic acids afforded a residual antiviral effect [463–465]. However, continual hand washing with extra ingredients resulted in skin irritation [462]. The experimental testing [463, 464] may have been biased by short study periods, the absence of a mucus carrier to mimic natural surface deposition and overly stringent control over virus application/hand disinfection compared with the natural study. Additionally, the natural setting study used PCR [462] which detects HRVs more often than culture. The disparity between outcomes may also reflect the contribution of airborne HRV transmission.

Because of the absence of a vaccine or specific antiviral, the most popular method of intervention in uncomplicated HRV ARIs is treatment of the symptoms. This is achieved using analgesics, decongestants, antihistamines, and antitussives. Due to a lack of studies, data are limited on the effectiveness of over-the-counter common cold medications for children [466]. Anticholinergic agents have proven useful to reduce rhinorrhea [467]. For controlling symptoms in those with exacerbated asthma, most of which do not require hospitalization, bronchodilators and oral corticosteroids are the main treatments [468]. The interruption of proinflammatory immune responses or specific signaling pathways using steroids, or other novel therapeutics, may prove to be a more robust approach for treating HRV infections; they have not been successful for HRSV [325].

When initiated early in the illness, a combination of antiviral (IFN-α2b) and anti-inflammatory (chlorpheniramine) components showed promise for interrupting nasal viral replication and symptoms [469].

Antiviral agents (Table 29.3) require early application to effectively precede the pathogenic immune response to HRV infection [325], but they often fail to reproduce their in vitro successes in vivo. Most antirhinoviral drugs are based on capsid-binding agents (Fig. 29.11). Additionally, oral delivery can complicate drug safety because this route increases the risk of systemic side effects compared to a nasal or topical route, but these risks must be considered alongside the disease to be treated; drug side effects are disproportionately severe compared to a common cold than to a severe asthma exacerbation. A systemic route is beneficial if an effect is sought on HRV replication sites that are otherwise inaccessible, such as those not associated with respiratory tract illness [470].

10 Unresolved Questions and Problems

The recent discovery of the new species, HRV-C, has shone a bright light on how little was known about the HRVs. The HRV-Cs and also the newly discovered HRV-As and HRV-Bs are fastidious in culture, with a single report of HRV-C growth in primary sinus tissue, and the identity of a cellular receptor still unknown. Thus, it is difficult to proceed in many areas, including basic virology, seroepidemiology, immunobiology, and antiviral testing. Determination of the receptors for these new HRVs would aid the search for a more accessible culture system. There would be great interest in a vaccine for some or all of the HRVs, but with increasing evidence of the interactions between HRVs, their hosts, and other respiratory viruses, it may not be wise to interfere before we fully understand what the impact of losing a constantly circulating HRV challenge would be. Antivirals specifically targeting the HRVs may be a better bet, but routine HRV testing and genotyping will first need to be more widespread as surveillance for antiviral resistance will be an important component of monitoring the success of any intervention.

Studies to determine whether there are differences in clinical and immunobiological impact between the many different types are lacking but would greatly improve our ability to plan future routine testing, understand all the clinical responses to the diverse HRVs and to outbreaks of ARI, and improve HRV epidemiology. It is interesting to note that the HRV-Bs are significantly underrepresented in HRV detections. We do not yet know their niche or clinical impact. It may be possible that HRV-Bs are the most well adapted of the HRVs, causing little to no detectable clinical impact, or they may create a different impact than that which we expect, or they may be a species in decline.

The jury remains out on whether HRVs cause or are involved in the development of asthma or merely trigger exacerbations once asthma is established. With a very high healthcare impact from asthma around the world and atopic conditions that may be exacerbated by HRVs on the rise, this is an important area for further investigations.

Table 29.3 Preventive and therapeutic compounds affecting HRV infections

Therapeutic agent	Primary role	Effect	Evaluation	Reference
IFN-α	Elicit cellular antiviral effects	Decreased shedding if administered within 24 h	Toxicity	[471–475]
Pirodavir (R77975)	Capsid binder	Intranasal formulation useful against both HRV antiviral groups; three to six doses per day	Variable efficacy, irritation, and mucosal bleeding	[471, 476, 477]
WIN54954	Capsid binder	Broadly active in mice	Reduced efficacy in humans	[471, 478]
WIN56291	Capsid binder	Active against HRV-C15	Effect only in organ culture so far	[161, 479] [63]
Pleconaril (WIN63843)	Capsid binder	Resolved symptoms 1–2 days earlier than placebo. Some types are resistant	FDA issued "not approvable" letter because of side effects	[471, 477]
Vapendavir (BTA798)	Capsid binder	Potent binding in animal models	Good bioavailability and safety profile in animals. Phase IIa trial complete	[477, 480]
Rupintrivir (AG-7088)	3C protease inhibitor	Insignificant impact	Discontinued	[470, 471, 481, 482]
Enviroxime	Replication inhibitor	Potent anti-replicative activity in vitro	Side effects in vivo	[406, 472]
Tremacamra	Soluble ICAM-1 molecule	Could reduce experimental cold symptoms and the quantity of virus shed if administration occurs before or after inoculation but prior to the development of symptoms		[483]
Tiotropium	Anticholinergic agent (bronchodilator)	Reduced HRV-B14 viral load and RNA levels, decreased susceptibility of cells, reduced ICAM-1 mRNA levels and IL-1β, IL-6, and IL-8 protein levels in culture	No obvious change to cell viability in culture	[484]
Levofloxacin	Quinolone antibiotic	Reduced HRV-B14 and HRV-A15 viral load (major group HRVs; not the minor group virus, HRV-A2) and RNA levels, decreased susceptibility of cells, reduced ICAM-1 mRNA levels and IL1β, IL6, and IL8 protein levels in culture	No obvious cytotoxicity in culture	[485]
Pellino-1	Regulates IRAK-1	Controlled primary epithelial cell non-IFN response to HRV-A1; knockdown by siRNA reduced CXCL8 in primary BECs	Did not cause unwanted shutdown of an antiviral response. Target unknown	[486]
Azithromycin	Macrolide antibiotic	Significantly increased IFNs and ISG mRNA and proteins resulting from HRV-A1 and HRV-A16 infection in primary BECs. Reduced HRV replication and release	Modest effect in cell culture at relatively high concentration. Mechanism unknown	[487]

FDA US Food and Drug Administration, *ICAM-1* intercellular adhesion molecule 1, *IFN* interferon, *IRAK-1* interleukin-1 receptor-associated kinase-1, *BEC* bronchial epithelial cells

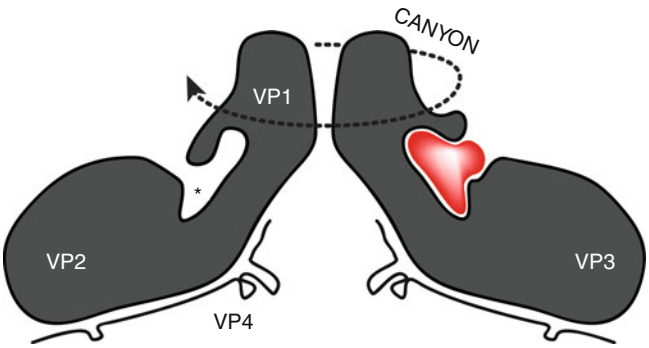

Fig. 29.11 A simplified depiction of two protomers in opposition on a cross section of a pentamer. The positions of the structural peptides are indicated as is the canyon that circumscribes the central axis of the pentamer. The pocket (*asterisked*) at the base of the canyon is shown without the pocket factor or occupied by a stylized capsid-binding molecule (*red*). *VP1-VP4* Viral protein (Adapted from McErlean et al. [61])

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Rotaviruses

30

Monica Malone McNeal and David I. Bernstein

1 Introduction

Before the use of rotavirus vaccines, rotaviruses were recognized as the single most important cause of severe infantile gastroenteritis worldwide and were estimated to be responsible for >600,000 deaths annually [1–3]. Today, rotavirus disease is much less common where the vaccines are available, but rotaviruses continue to cause disease and death in the most impoverished nations where vaccine is not yet available to most [4]. Transmission of rotaviruses occurs primarily by the fecal-oral route, providing a highly efficient mechanism for universal exposure that circumvents differences in cultural practices and public health standards [5]. Nearly all children less than 5 years of age have experienced at least one rotavirus infection [6–8].

The most common symptoms associated with rotavirus disease are diarrhea and vomiting accompanied by fever [8, 9]. Rotavirus illness can be mild and of short duration or produce severe dehydration leading to hospitalization and mortality if not treated. Severe disease occurs primarily in young children, most commonly between 4 and 24 months of age, and the treatment of rotavirus illness is largely limited to supportive measures such as oral or intravenous rehydration.

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2 History of Rotavirus

The first rotaviruses to be described, based on pathology and epidemiology, were murine strains which were classified under the general description as the agents responsible for "epizootic diarrhea of infant mice," i.e., EDIM [10, 11]. Murine rotaviruses were also among the first to be visualized by electron microscopy [12], along with viruses obtained from the rectal swabs of monkeys that contained viruses with comparable morphologic features [13]. These agents were described as 70-nm particles that had a wheel-like appearance and were later designated "rota" viruses from the Latin word for wheel [14, 15]. In 1969, Mebus and colleagues demonstrated the presence of these particles in stools of calves with diarrhea, thus associating these viruses with a diarrheal disease [16]. The correlation between these viruses and severe diarrhea in young children was reported first in 1973 by Bishop and colleagues, who used electron microscopy to examine biopsy specimens of duodenal mucosa from children with acute gastroenteritis [17, 18]. Within a short time, other investigators confirmed the association between the presence of rotavirus in feces and acute gastroenteritis.

In addition to their distinctive morphologic features, human rotaviruses, along with their animal counterparts, share a group antigen [19, 20] and are classified as members of the *Rotavirus* genus within the *Reoviridae* family [21]. In 1980, particles that were morphologically indistinguishable from established rotavirus strains but lacked the common group antigen were discovered in pigs [22, 23]. This finding led to the identification of rotaviruses belonging to six additional groups (B to G) based on common group antigens, with the original rotavirus strains classified as group A [24]. Recent sequencing of the gene responsible for the group antigen has led to additional groups (F to H) [25]. Only groups A to C have been associated with human diseases, and most known cases of rotavirus gastroenteritis are caused by group A strains. Although there have been large outbreaks associated with non-group A strains

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reported in China and Japan, particularly among adults [26–28], group A rotaviruses are the strains to which vaccine development has been directed.

3 Properties of the Virus

Rotavirus is a double-stranded RNA virus. When the genome of rotavirus is extracted from viral particles and separated by polyacrylamide gel electrophoresis, 11 bands are visualized by silver staining as shown in Fig. 30.1.

Each rotavirus strain has a characteristic RNA profile or electropherotype. The characteristic RNA electrophoretic pattern of group A rotaviruses consists of four size classes containing segments 1–4, 5 and 6, 7–9, and 10 and 11 [5]. A short pattern is seen when the segment 11 runs slower than segment 10.

The genome of rotavirus encodes six structural proteins—VP1 to VP4, VP6, and VP7—and six nonstructural proteins, NSP1 to NSP6 [29, 30]. Each segment encodes one rotavirus protein except segment 11 which encodes both NSP5 and NSP6 using alternative open reading frames [31]. The genome segments range in size from approximately 660–3,300 base pairs with a molecular weights of approximately 12,000–125,000 [29]. The known functions of these 12 proteins are briefly described in Table 30.1.

Computer-generated images of rotavirus particles obtained by cryoelectron microscopy show it is ca. 100 nm in diameter and is composed of three concentric protein layers depicted in Fig. 30.2 [32–34].

The outer layer contains 780 molecules or 260 trimers of the VP7 glycoprotein and 60 trimers of the VP4 protein [35–37] which forms spikelike projections that extend through and 11–12 nm beyond the VP7 layer [32, 33]. The VP4 protein is anchored to the intermediate layer of the particle composed of the VP6 protein, also arranged as trimers. There are 132 aqueous channels in the outer layer that are positioned over 132 channels within the perforated VP6 intermediate shell. Besides interactions between the two layers of VP7 and VP6, both proteins interact with VP4 in these aqueous channels [38]. The innermost layer contains 120 molecules of the VP2 protein that interact with 12 molecules each of the viral transcriptase (VP1) and the guanylyltransferase (VP3) along with the 11 segments of the double-stranded RNA genome [29, 39].

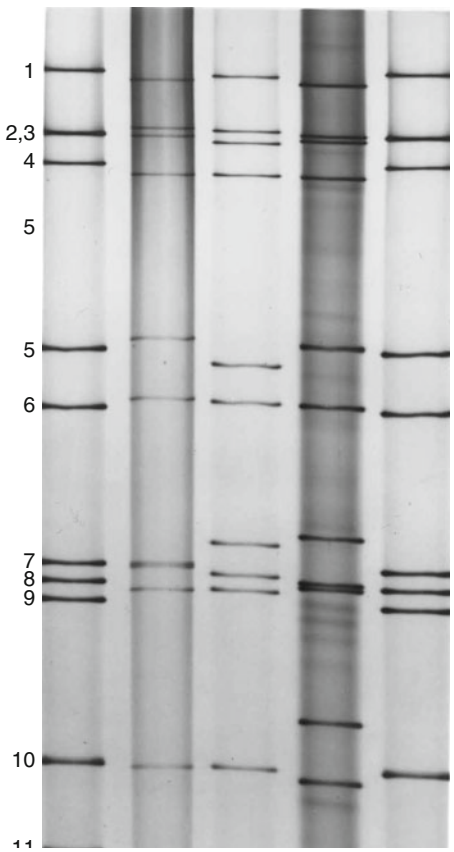

Fig. 30.1 Polyacrylamide gel electrophoretic patterns of genomic RNAs obtained from group A human rotaviruses and visualized by silver staining. The patterns demonstrate the characteristic four size classes of RNA separated into groups of 4, 2, 3, and 2 segments each. Human rotavirus strains included (from left to right) lane 1, Wa; lane 2, 248 strain; lane 3, 456 strain; lane 4, DS 1; lane 5, Wa.

4 Virus Entry and Replication

Rotaviruses must attach to and enter a cell prior to replication. The VP4 protein on the outer shell is involved in cell attachment. Cellular receptors have not completely been determined, but studies have indicated that sialic acid, heat shock cognate 70 protein (hsc70), ganglioside compounds, and integrins, including $\alpha 2\beta 1$, are probably involved in virus attachment and entry [40–45]. A recent study has shown that a rotavirus having a specific VP4 protein (P[14], see classification below) was able to bind to a glycan characteristic of A-type histo-blood group antigen (HBGA) [46]. Additional studies have shown that other rotavirus strains having different VP4 proteins, including strains that commonly infect humans, were able to bind to specific HBGAs found in saliva and milk [47, 48]. HBGAs have been shown to be important receptors for noroviruses. This finding may have importance to determining susceptibility to rotavirus infections in the human population.

Rotaviruses are activated by cleavage of the outer capsid VP4 protein by trypsin-like proteases into proteins termed VP5* and VP8* which remain associated with the virus. Studies to determine how rotavirus enters a cell after attachment have suggested endocytosis or a process similar to enveloped-virus fusion as the mechanism for viral internalization, but again, the process is not fully elucidated [43, 49, 50]. From a number of studies, it has been shown that the outer capsid proteins, VP5* and VP8*, undergo conformational changes that may also involve the VP7 protein during attachment and entry into the cell [49–52].

For replication to begin, the outer capsid proteins must be removed to yield a double-layered particle. The RNA-dependent

Table 30.1 Rotavirus gene segments and properties of the encoded proteins

RNA segment	Encoded protein	Properties of protein
1	VP1	Structural inner core protein RNA transcriptase and RNA binding
2	VP2	Structural inner core protein RNA binding
3	VP3	Structural inner core protein Complexes with VP1, RNA binding, guanylyltransferase, and methyltransferase activity
4	VP4	Outer capsid protein Receptor binding, hemagglutinin, and neutralization protein
5	NSP1	Nonstructural protein RNA binding, possible suppression of host innate antiviral response
6	VP6	Intermediate capsid protein Group and subgroup antigen, necessary for transcription
7	NSP3	Nonstructural protein RNA binding and control of viral translation, inhibits host translation
8	NSP2	Nonstructural protein Multifunctional enzyme activity, interacts with NSP5 in formation of viroplasm
9	VP7	Outer capsid protein Neutralization protein
10	NSP4	Nonstructural protein Multifunctional protein, essential for replication, transcription and morphogenesis, enterotoxin
11	NSP5	Nonstructural protein Binding to NSP2 to form viroplasm
	NSP6	Nonstructural protein Not encoded by all rotavirus strains, RNA binding, role not clearly determined

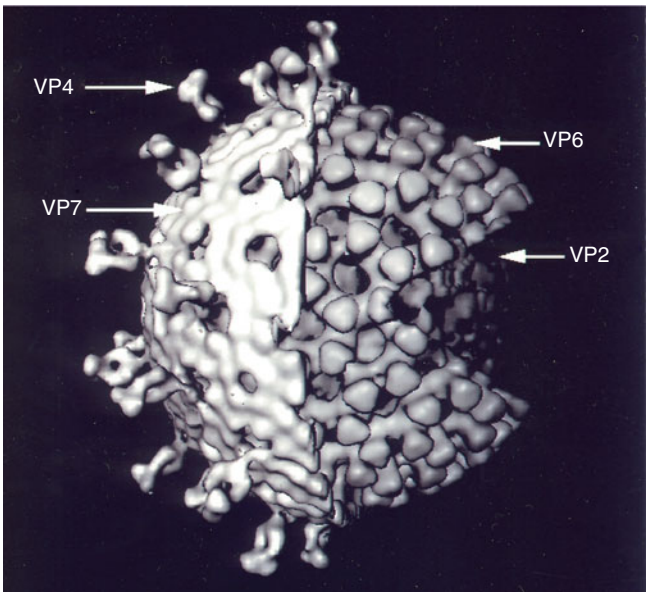

Fig. 30.2 Computer generated image of the triple shelled rotavirus particle obtained by cryoelectron microscopy. The cut away diagram shows the outer capsid composed of VP4 spikes and VP7 shell, intermediate VP6 shell, and inner VP2 shell surrounding the core containing the 11 double stranded RNA segments and VP1 and VP3 proteins. (Courtesy of Dr. B. V. V. Prasad, Baylor College of Medicine, Houston, TX.)

RNA polymerase (i.e., the VP1 transcriptase) associated with the inner shell is then stimulated to synthesize the 11 viral mRNAs that are capped by VP3 [53–55]. The mRNAs are extruded from the virus cores through channels in the VP2 and VP6 protein layers at the 12 vertices of the viral particles and subsequently translated into viral proteins [56, 57]. A number of studies using the technique of adding "short interfering RNAs" (siRNAs) have helped to elucidate the various steps of viral replication and how the different viral proteins are involved [58]. Two nonstructural proteins, NSP2 and NSP5, have been found to be involved in the formation of large inclusions or viroplasms [59]. Particle assembly may be initiated by the formation of complexes within the viroplasm that contain plus-strand RNAs from the 11 genome segments along with VP1 and VP3. Although the mechanism is not fully known, the virus assembles and packages one of each of the plus-strand RNAs within individual precursor viral complexes. Upon interactions with VP2, the minus-strand RNA is synthesized and the 11 ds RNA genome segments are produced [60]. VP6 trimers interact with the VP2 core and a double-layered particle is completed [61–63]. The double-layered particles then bud into the rough endoplasmic reticulum (ER) after their association with the NSP4 transmembrane glycoprotein [64]. VP4 is either added prior to entry into the ER or sometime thereafter. The other rotavirus

glycoprotein VP7, which becomes sequestered within the rough ER, is added to complete the formation of mature viral particles [65, 66]. These mature viruses accumulate within the lumen of the rough ER until cell lysis occurs.

5 Serotypes

The two outer capsid proteins, VP7 and VP4, contain the only neutralization epitopes for this virus [67, 68]. Early studies using sera from hyperimmunized animals in cross-neutralization assays described a number of different serotypes from infected humans and animals based on the VP7 protein [68, 69]. Viruses were given a G type referring to the glycosylated structure of this protein. When animals were hyperimmunized, most of the neutralizing antibody was directed against the VP7. However, after oral infection, VP4 appeared to be the dominant neutralization protein [70–73]. A dual serotyping scheme for both VP7 and VP4 was therefore developed.

VP7 serotypes are determined by cross-neutralization and by using panels of monoclonal antibodies [74–76]. VP4 serotype determination, however, is more difficult [77–79]. Two numeric systems were established to classify the VP4 protein, or P type referring to the protease sensitivity of this protein. The P serotype is based on neutralization assays using antisera against recombinant-expressed VP4 proteins or viruses with specific VP4 genes [80, 81]. A second classification system, one that is now more widely used to characterize rotavirus isolates, is based on sequence analyses, and rotaviruses are described as different genotypes [82, 83]. The P serotype is indicated by an open number and the genotype is noted with a bracketed number. For example, the most common P type worldwide belongs to serotype P1A and genotype 8 and is therefore designated P1A [8]. Currently, rotaviruses are most often only classified by genotyping for the P protein, i.e., P [8]. To date, 27 G types and 35 P types have been identified on the basis of sequence analyses from animal and human isolates [84–86]. Recently, all 11 gene segments of rotavirus isolates have been sequenced. The data from this analysis have importance in determining the evolution of different rotavirus strains [87]. However, as discussed below, only a few G- and P-type combinations account for the vast majority of human infections.

Rotaviruses, similar to other viruses with segmented genomes, have the ability to form reassortants. This ability to reassort has contributed to the diversity of G and P types found throughout the world. In addition, as will be discussed, a number of reassortant viruses have been used as vaccine candidates including the currently licensed vaccine, RotaTeq™. During replication, if a coinfection has occurred, gene segments from each parent virus can be incorporated into new progeny viruses. The gene segments from different viruses can segregate independently, and the new viruses produced can contain genes from either parent. Although numerous human isolates appear to be animal strains or animal-human rotavirus reassortants as determined by sequence analyses, the number of cross-species infections appears to be low [88–91].

6 Epidemiology of Rotavirus

6.1 Disease Burden

6.1.1 United States

Before the use of vaccines, nearly every child was infected with rotavirus by the age of 5, and rotavirus illness resulted in 20–70 deaths annually in the United States [92–94]. Infection with rotavirus often resulted in more severe illness than other pathogens, and therefore, rotavirus accounted for a higher percentage of gastroenteritis episodes requiring medical intervention [95, 96]. During peak rotavirus seasons, 70 % of all gastrointestinal hospitalizations were due to rotavirus-associated gastroenteritis [97]. It is estimated that 1 child in 7 required a doctor's visit at a clinic or emergency room, and about 1 in 75 were hospitalized because of a rotavirus illness [93, 98]. Based on these estimates, rotaviruses were responsible for 5–10 % of all gastroenteritis episodes in children under 5 years of age leading to over 50,000 hospitalizations. Depending on how hospital surveillance studies were conducted, these estimates may be lower than the actual numbers of rotavirus-related hospitalizations [92, 99, 100]. Similar estimates of the disease burden due to rotavirus have been reported in studies conducted in European countries [101–103].

6.1.2 Worldwide

Globally, rotavirus disease has an even more dramatic and significant impact on infant health. Although rates of rotavirus illness among children are similar throughout the world, the resulting mortality differs substantially. It is estimated that rotavirus illness is responsible for over 600,000 deaths annually, representing 5 % of all deaths in children younger than 5 years of age worldwide [2]. More than 90 % of these deaths occur in Africa and Asia (Fig. 30.3). More than 100,000 deaths occur in India and sub-Saharan Africa and 35,000 occur in China [2, 104, 105].

6.2 Distribution of Human Rotavirus Serotypes

Early studies using cross-neutralization assays and monoclonal antibodies to determine serotypes found that four main G types (G1, G2, G3, and G4) accounted for 90 % of the strains isolated from humans. However, with the development of

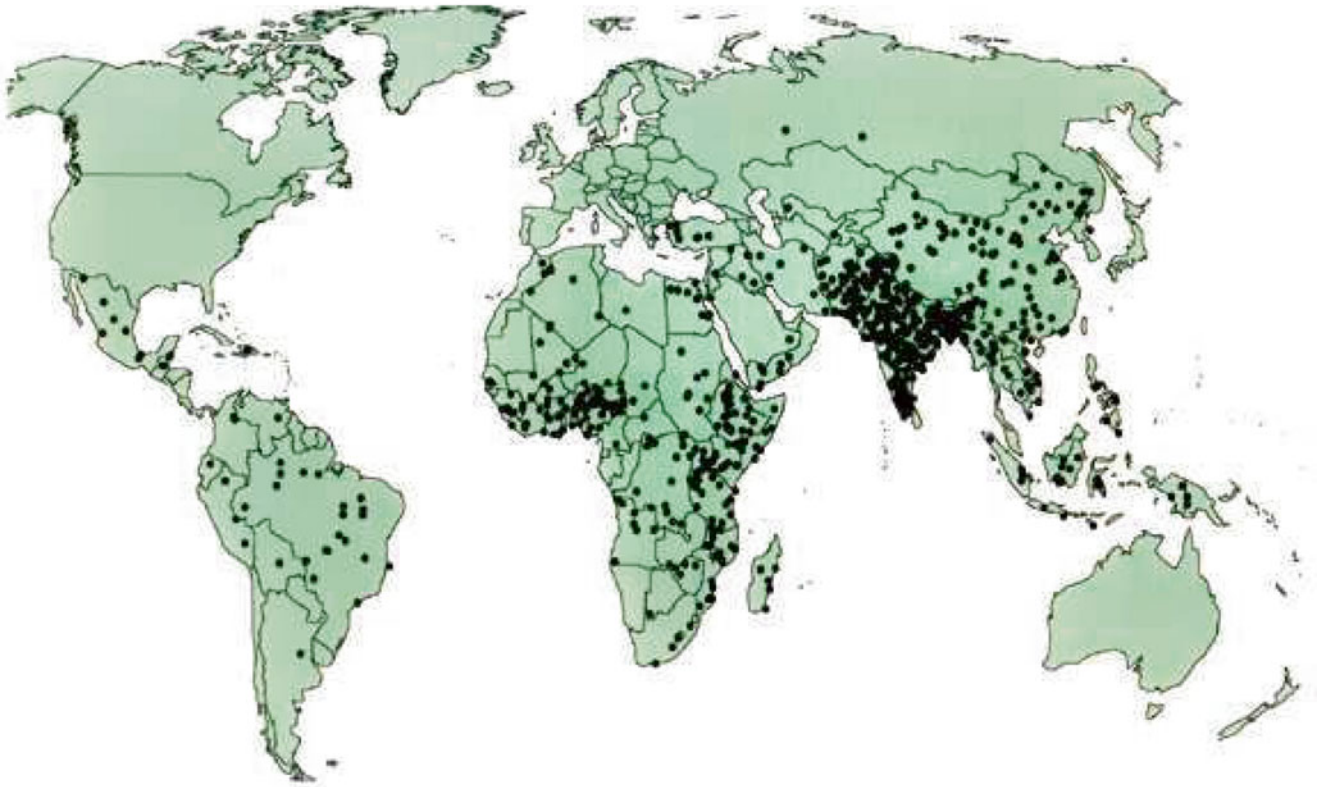

Fig. 30.3 Each dot on this figure represents 1,000 deaths. It is estimated that there are over 600,000 deaths worldwide. Edited from Parashar et. al. Emerg Infect Dis. 2003;9:565–72

surveillance studies using reverse transcription-polymerase chain reaction (RT-PCR) genotyping and automated nucleotide sequencing of VP7, for G type, and VP4, for P type, more strains have been identified and genotyped. The global distribution of G and P serotypes can vary greatly by location and time or, as was found in some studies, vary little over sequential seasons [74, 106]. Over 42 different P-G combinations of human strains containing 10 different G types and 11 different P types have been described [85].

In North America, Europe, and Australia, the G1P [8] strain has been responsible for over 70 % of rotavirus infections. In European countries, G1, G2, G3, G4, and G9 were the predominant G serotypes found, although the incidence varied by country [107]. Similar findings were reported from Central and South-Eastern Europe [108]. In the United States, the most prevalent strain in the pre-vaccine era was G1P[8] (78.5 %) followed by G2P[4] (9.2 %), G9P[8] (3.6 %), G3P[8] (1.7 %), and G4P[8] (0.8 %) [109]. Rarer genotypes included G2P[6], G6P[9], and G3P[9]. These studies also demonstrate that strain circulation can vary greatly in the same location over time.

A more global perspective has been described in two reviews. A review by Santos and Hoshino [84] summarized rotavirus serotypes from 124 studies performed between 1989 and 2004 and included data on 45,571 strains collected from 52 countries on five continents. The four common G types, G1, G2, G3, and G4, with either P[8] or P[4], represented over 88 % of the strains identified worldwide during this period. Serotype G9, containing either P[8] or P[6], was found to be an emerging strain in a number of different locations. In the second review by Gentsch et al. [85], data from studies conducted in 35 countries, involving over 20,000 strains, were analyzed. In this study the four common strains, G1P[8], G2P[4], G3P[8], and G4P[8], represented 72 % of all strains with G9 containing either P[6] or P[8] identified in over 2 % of the isolates. In South America and Asia, the incidence of G1P[8], about 30 %, is lower than in the United States and Europe, while in Africa, it was even lower (20 %). In Latin America [110], the most common G types were G1 (34.2 %), G9 (14.6 %), and G2 (14.4 %), while the most common P types were P[8] (56.2 %), P[4] (22.1 %), and P[1] (5.4 %). A recent review of rotavirus infections in Africa suggested that strain diversity was increasing and identified a total of 24 P/G combinations [111]. A review of studies from China showed that G1 was the overall predominate strain but that G3 viruses were becoming more common [112, 113].

In summary, although the most common G types still remain, G1 through G4, common strains now also include G5, G8, G9, G10, and G12 (especially G9) in various regions around the world [109–111, 114, 115]. The most common

types for the P protein are P[8] and P[4] but also include the more recently described P[6] and P[1] as well as P[9] and P[14]. The effect of rotavirus vaccine introduction in countries around the world on the diversity of circulating rotavirus strains remains to be determined. Surveillance programs are important to monitor the changes in rotavirus strains.

7 Effect of Age and Seasonality

Severe human rotavirus disease occurs most commonly between 4 and 24 months of age [7, 116], but the age may be lower in less developed countries [117, 118]. Neonatal rotavirus infections, which are often asymptomatic, occur and appear to be endemic in some newborn nurseries [119, 120]. The asymptomatic nature of neonatal rotavirus infections is due, at least partially, to protection from transplacental antibody that can persist for the first months of life. The onset of rotavirus disease in infants has been reported to coincide with the decline of maternal IgG antibody [121].

Older children and adults, including elderly patients, are susceptible to reinfections with rotavirus. Infections typically cause mild disease but rarely can be severe and require hospitalization [122–126]. The reduced severity of rotavirus disease in older children and adults is due primarily to the immunity induced by previous rotavirus infections. One of the interesting and somewhat unanticipated results of the introduction of rotavirus vaccines has been the impact on disease in older children and even adults [127].

As with other respiratory and enteric viruses, distinct seasonality is associated with rotavirus disease [29, 128–131]. This seasonality is particularly evident in temperate climates, where rotaviruses are responsible for the large increase in hospitalizations and deaths due to diarrheal diseases found during the winter season [97]. The seasonality of rotavirus disease is less apparent in tropical climates, but disease is still more prevalent in the drier, cooler months [129]. The cause for the seasonality of rotavirus disease remains unknown.

8 Clinical Disease and Treatment

Following an incubation period of 1–3 days [132], the onset of rotavirus disease is often abrupt with fever and vomiting followed by explosive, watery diarrhea. About two-thirds of children with rotavirus disease have diarrhea, vomiting, and fever [133–135], but children can also present with any two or only one of these symptoms [9]. Stools are non-bloody and generally lack fecal leukocytes, but mucus may be found. Rotavirus infections are typically more severe than those caused by other viral gastrointestinal agents and therefore more likely to result in hospitalization [134, 135]. In one study [134] vomiting and diarrhea lasted longer in patients infected with rotavirus than other causes of gastroenteritis, while in another study, the severity of rotavirus diarrhea was almost twice that of other causes of diarrhea [136]. The disease lasts about 4–8 days, with a range between 2 and 22 days [137], while virus shedding has ranged from 4 to 57 days with a peak at about day 3 [138, 139].

Virus replication was initially thought to be limited to the intestine, but it is now known that both antigenemia and viremia are common [140–145]. The dissemination of virus may be responsible [142] for other clinical manifestations including encephalitis and meningitis [146, 147], upper and lower respiratory infections including otitis media and pneumonia [134, 148, 149], and mild hepatitis [150]. Recently, cases of encephalitis have been confirmed by identification of rotavirus RNA in the cerebrospinal fluid using reverse transcription-polymerase chain reaction (RT-PCR) analysis [147]. Rotavirus infections are also often more severe in immunosuppressed persons, including bone marrow transplant recipients, patients infected with human immunodeficiency virus, and those who are malnourished.

The treatment of rotavirus gastroenteritis is mainly supportive, aiming to restore fluid balance in dehydrated patients. Emphasis has shifted away from intravenous rehydration to oral rehydration with glucose-electrolyte solutions. However, should oral rehydration efforts fail, or in cases of severe dehydration and shock, fluids are administered intravenously. Other experimental approaches to treatment include bismuth subsalicylate [151, 152] and probiotics (reviewed in [153, 154]). Racecadotril (acetorphan), an enkephalinase inhibitor with antisecretory and antidiarrheal properties, has been shown to be effective in the treatment of diarrhea in adults and children [155–157], while another drug, nitazoxanide, licensed to treat *Giardia intestinalis* and *Cryptosporidium parvum* infections [158, 159], has shown some effectiveness in the treatment of rotavirus infection.

9 Pathogenesis

The incubation period for rotavirus is approximately 1–3 days [132]. In children with symptoms, the onset is often abrupt, with fever and vomiting followed by explosive, watery diarrhea. Vomiting may precede the diarrhea in approximately half the cases [160]. Fever occurs commonly during rotavirus illness, with estimates of between 45 and 84 % of patients [134, 135]. The disease is usually self-limited, lasting 4–8 days, although the duration of symptoms ranged between 2 and 22 days in a Guatemalan study [137]. In a study conducted in the United States, 63 % of hospitalized children with rotavirus infection had fever, vomiting, and diarrhea at presentation, but children also presented with combinations of only one or two of these symptoms [9].

After fecal-oral transmission of rotavirus, infection is initiated in the upper intestine and typically leads to a series of histologic and physiologic changes. Studies in calves revealed that rotavirus infection caused the villus epithelium to change from columnar to cuboidal, which resulted in a shortening and stunting of the villi. The cells at the tips of the villi became denuded, while in the underlying lamina propria, the number of reticulum-like cells increased and mononuclear cell infiltration was observed. The infection appears to start at the proximal end of the small intestine and then advances distally [161, 162]. From the few studies examining the pathologic changes in the intestines of humans, the changes appeared to be similar to those found in animals [163, 164].

Although rotaviruses cause severe diarrhea in numerous species, including humans, the mechanisms responsible for the illness have not been completely determined but are due to multiple factors (reviewed in [165]). From various studies in animals, malabsorption of carbohydrates, sodium and calcium fluxes, retarded differentiation of uninfected enterocytes, activation of the enteric nervous system, and toxin (NSP4) have been found to be involved in causing diarrhea. It is clear that infection of mature enterocytes of the small intestines leads to damage and functional abnormalities, but the degree of damage does not appear to correlate with the severity of diarrhea. Further, diarrhea can occur before evidence of intestinal pathology. Thus, although damage to the mature enterocytes leads to loss of the enzymatic activities of the brush border leading to malabsorption of electrolytes and glucose/amino acids, other mechanisms are also involved.

Diarrhea has been induced in infant mice and rats by intraperitoneal inoculation with the rotavirus NSP4 protein as well as with a 22-amino acid peptide derived from this protein [166, 167]. NSP4 possesses membrane destabilization activity that may result from increased intracellular calcium concentrations, resulting in cytoskeleton disorganization and cell death [168–170]. NSP4 is also an enterotoxin that binds to surface receptors and initiates signal transduction pathways that leads to mobilization of intracellular Ca and chloride secretion. NSP4 increases the levels of intracellular calcium [171] by activating a calcium-dependent signal transduction pathway that mobilizes transport of this ion from the endoplasmic reticulum [172, 173].

Another factor that may have a role in rotavirus-induced diarrhea is the enteric nervous system underlying the villus epithelium. It has been reported that rotavirus infection can activate this system in mice and drugs that block nerve activity, attenuate rotavirus-induced fluid secretion in vitro, and attenuate diarrhea in vivo [174]. Whether this is a major mechanism of diarrhea occurring after rotavirus infection in humans remains to be determined.

The tissue tropism of rotavirus infection in humans was thought initially to be restricted to the villi of the small intestine. Because no consistent evidence of extraintestinal replication of rotavirus had been shown, the general assumption was that rotavirus pathology is strictly intestinal. However, instances of non-gastrointestinal rotavirus-associated disease, including the association with abnormal liver function, as well as respiratory and nervous system involvement were reported [175–180]. This spread was explained when it was reported that both antigenemia (presence of rotavirus protein in the blood) and viremia (presence of live rotavirus in the blood) were found during rotavirus infection in several strains of animals and in humans [140]. Since that time, this observation has been confirmed by a number of studies in humans [144, 145, 181–184].

10 Immunity

After more than 30 years of research involving studies of natural rotavirus infection, vaccine studies, and animal studies, the mechanisms of immunity to rotavirus infection remain incompletely understood. Humoral antibody responses include an early IgM response followed by the production of rotavirus IgG and IgA [185, 186]. Infection also induces local, intestinal antibodies that are predominantly IgA [187–190]. T cell responses have been difficult to measure in humans, but several rotavirus proteins have been identified that contain T cell epitopes and have been used to measure T cell responses after rotavirus infection [191–193].

As stated before, virus neutralization epitopes are found on the VP4 and VP7 proteins located on the surface of the virion, and neutralizing antibody to the infecting virus can be detected after infection or vaccination. However, VP6 appears to be the most immunogenic protein, although antibody made against VP6 is not neutralizing. One of the important questions concerning the development of immunity to rotavirus infection is whether or not neutralizing or serotype-specific antibody is necessary for protection. Studies evaluating natural rotavirus infections were used initially to understand rotavirus immunity and the role of serotype-specific antibodies. Many investigators reported that natural rotavirus infections produce incomplete protection, but most showed that previous infections protected against severe disease [194–199]. In a large study conducted in Mexico, protection from both rotavirus reinfection and rotavirus diarrhea increased with each new infection [198]. However, sequential infections even with the same serotype have been reported. In an early study reporting rotavirus disease with reinfection by the same serotype, the investigators noted that protection of young children in a Japanese orphanage lasted 6 months then declined after 1 year. This study noted a close correlation between titers of serotype-specific antibody and protection [200]. This study is often cited to support the idea that neutralizing antibody is necessary for protection. While it is generally accepted that if present at a

sufficient titer, neutralizing antibody will protect, it is still unclear whether only neutralizing antibody is necessary for protection.

Although reinfections with rotavirus are common, other studies have shown that protection lasts at least 1 year. Neonates infected within the first 2 weeks of life were protected against severe disease but not against reinfection in one study [119]. In another study, infants who developed a symptomatic or an asymptomatic rotavirus infection during the first year of the study were protected against contracting a subsequent rotavirus illness or even an asymptomatic reinfection during the following year [195]. Similarly, in another study, a natural rotavirus infection in the first year was found to be 93 % protective against a symptomatic reinfection in the second year. This protection occurred even though the G1 strains that circulated during the first year were responsible for only 66 % of rotavirus disease in the second year [197]. In a recent cohort study conducted in India, it was found that rotavirus infections generally occurred early in life with 56 % of children infected by 6 months of age. Reinfections were common, and protection against moderate or severe disease increased with the order of infection. However, protection was only 79 % after three infections and there was no evidence of homotypic protection [201]. Protection from the current vaccines appear to last at least 2–3 years (discussed below).

Correlations of serotype-specific neutralizing antibody and protection have not been supported in other larger studies. In the largest study, conducted in Bangladesh during a 2-year period when the four major G serotypes circulated, the titers of both preexisting homologous and heterologous neutralizing antibody were significantly lower in patients with acute rotavirus disease than in matched control subjects. Further analysis, however, could not find a correlation with serotype-specific neutralizing antibody, and protection seemed to correlate better with the magnitude of the response rather than with specific neutralizing responses [202]. Differences in the studies evaluating the role of serotype-specific antibody and the protection provided by natural infection may be due to many factors including the viral strains circulating, overall health of the child, or duration of protection. Studies of the monovalent G1P[8] vaccine, Rotarix™, discussed later, provide strong support for cross-protection between serotypes.

Due to the difficulties associated with human studies, animal models using mice, rabbits, and gnotobiotic pigs have been developed [203–205]. Early studies in mice showed that while CD8 T cells are involved in the resolution of an infection, antibody is necessary for protection against reinfection [206–208]. In one study, mice given an oral immunization of a fully heterotypic rotavirus were almost completely protected from shedding when later challenged, and this protection was dependent on the ability of rotavirus-specific IgA to be transported through intestinal epithelial cells or be present at the intestinal mucosa [209]. In a recent study using mice deficient in IgA, shedding of murine rotavirus after primary infection was prolonged, and mice were not protected against subsequent infection with the same strain of virus [210]. The role of rotavirus-specific serum IgG is not clearly defined but has been shown in some animal models, using passive transfer of IgG antibodies, to have some protective effect [211]. If these animal studies mirror what is needed in humans, then local antibody may provide protection and may involve antibody that is not serotype specific.

Serum levels of rotavirus-specific IgA appear to be a good correlate of protection, because it appears to parallel antibody levels in the gut [190, 212]. However, studies in vaccinated infants showed only a low correlation between protection from disease and circulating rotavirus-specific memory B cells expressing α4β7 intestinal homing phenotype [190, 212, 213].

11 Control and Prevention

11.1 Rotavirus Vaccines (Table 30.2)

Public health institutions around the world made the development of an effective rotavirus vaccine a high priority [214].

Table 30.2 Selected rotavirus vaccines used in clinical trials

Candidate	Origin	G and P type	Comments
RIT4237	Bovine	G6P[1]	In small trials, safe but variable results. No efficacy in developing countries. Discontinued development
WC3	Bovine	G6P[5]	Safe but variable results. Virus used to make reassortants containing human genes
MU18006 (RRV)	Simian	G3P[3]	Some incidence of fever in vaccinees. Inconsistent protection. Virus used to make reassortants containing human genes
LLR	Lamb	G10P[12]	Used in China, limited efficacy data
RRV-TV	Simian	G1,G2,G3,G4,P[3]	Licensed as Rotashield™ (Wyeth) and then withdrawn because of association with intussusception
RV5	Bovine	G1, G2, G3, G4, P[8]	Currently licensed as RotaTeq™ (Merck)
RV1	Human	G1, P[8]	Currently licensed as Rotarix™ (GlaxoSmithKline)
116 E	Human	G9, P[11]	

Numerous cost-effectiveness analyses in many countries have documented the need and benefit of a rotavirus vaccine [105, 215–217]. Because natural rotavirus infections can induce excellent protection, at least against severe rotavirus disease, vaccine efforts have been directed mostly at the development of live attenuated rotavirus vaccines given orally. The introduction of rotavirus vaccines has profoundly altered the burden of rotavirus disease in the United States [218–221] and wherever it has become available [222–224].

Early efforts concentrated on the use of animal rotavirus strains, labeled the Jennerian approach because it relies on the natural attenuation of animal viruses in humans for safety and largely heterotypic immune responses for protection. The initial efforts with animal rotavirus vaccines yielded inconsistent results. Therefore, in an attempt to make the vaccines more closely related to human strains, human rotavirus genes coding for the proteins that induce neutralizing antibody, VP4 and VP7, were introduced into these animal strains by creating reassortant viruses as described earlier. Another approach was the use of attenuated human rotaviruses, either isolated from asymptomatic infections in human neonates or attenuated by cell-culture adaptation and passage.

11.1.1 Animal Strains

The first vaccine trial was conducted 10 years after the identification of rotavirus as an agent of severe diarrhea. This vaccine candidate was based on a bovine rotavirus strain, RIT4237, which is a G6P[1] virus [225]. The initial studies of the RIT4237 vaccine produced variable results. The vaccine was safe and effective in Finland, thus supporting the hypothesis that heterotypic protection was possible since this virus shared neither G nor P serotypes with the predominant human types and also suggested that protection may be greater than immunogenicity would indicate [226, 227]. However, later studies in developing countries and in a Navajo reservation were disappointing [228–231], and ultimately, the development of this vaccine was discontinued.

The initial studies of another bovine vaccine, WC3, conducted in Philadelphia appeared promising, during a season that had predominantly G1 strains [232]. Unfortunately, later trials in Cincinnati [233] and in less developed Central African countries [234] showed no significant protection. This virus was later used to develop reassortant vaccines and is the backbone of the RotaTeq™ vaccine now used around the world.

The next major vaccine candidate was a simian (rhesus monkey) termed MMU18006 or more commonly known as RRV. This vaccine is a G3P[3] strain and thus shared the G type with a human strain. RRV vaccination was associated with mild side effects, including low-grade fever and mild diarrhea, especially when it was given to older children who had lost maternal antibodies [235–237]. Protection with this vaccine was also inconsistent, ranging from greater than 50 %, even in developing countries, to moderate (20–50 %) to nonexistent [238]. This virus was later used to make the reassortants containing human rotavirus VP7 genes that became Rotashield™ vaccine, the first licensed rotavirus vaccine that was subsequently withdrawn from the market (see below).

One additional animal strain that has been developed into a vaccine is a G10P[12] strain isolated from a lamb, designated LLR. This vaccine produced by the Lanzhou Institute of Biological Products was introduced in China [239] but was only recently demonstrated to be effective [240].

11.1.2 Reassortant Vaccines

Because of the belief that homotypic immunity, or the development of serotype-specific antibody responses, might increase the protection seen with rotavirus vaccines and because of a lack of consistent protection after vaccination with heterotypic animal strains, the next vaccines developed were reassortant vaccines. These vaccines contained the genes encoding VP7 or the VP7 and VP4 proteins of human rotavirus strains, with the remainder of the genes from an animal strain. As discussed earlier, these proteins were chosen because they induce neutralizing antibody.

Rotashield™

The simian strain, RRV, was used as the background to make three different reassortant viruses containing the VP7 gene from human strains D (G1 serotype), DS-1 (G2 serotype), and ST-3 (G4 serotype) with the G3 serotype represented by RRV itself. The initial trials with monovalent viruses yielded varying results [241]; therefore, all four strains were incorporated into a tetravalent vaccine, RRV-TV, later licensed as Rotashield™. Extensive evaluations of the RRV-TV were completed before licensure. In two large trials conducted at centers across the United States, the RRV-TV vaccine was found to be safe, with the subjects developing a slight increase in temperature after the first dose. Protection against severe disease was 80 % [241, 242]. Similar results were reported from Finnish studies except that fever occurred somewhat more commonly and efficacy was somewhat enhanced [243]. Protection similar to that seen in the US studies was also demonstrated when it was evaluated in a less developed country, Venezuela [244].

As a result of these studies, RRV-TV or Rotashield™ was licensed in the United States in 1998 and recommended for general use in all infants [245]. However, less than 1 year after licensing, 15 cases of intussusception that occurred shortly after vaccination were reported to the Vaccine Adverse Events Reporting System (VAERS). Intussusception is a form of intestinal blockage caused when a segment of the bowel prolapses into a more distal segment of the intestine [246]. Initial publications reported an increased risk of the development of intussusception from 3 to 14 days after

receipt of the first dose of Rotashield™ and a smaller risk after administration of the second dose [247–249]. The initial estimate that one case of intussusception attributable to vaccination with RRV would occur for every 4,670–9,474 infants vaccinated was later revised to approximately 1 in 10,000 [250]. The risk of intussusception appears to be age related and increased substantially in children greater than 90 days of age [251, 252]. Based on these findings, the use of Rotashield™ was discontinued. Because of these findings, the FDA also recommended that future trials using live oral rotavirus vaccines should be conducted with more than 60,000 children to ensure that the risk of intussusception from a new vaccine would be less than was attributed to Rotashield™ [253].

The pathogenic mechanism underlying the association of the Rotashield™ vaccine and intussusception has not been defined [254]. Several studies have investigated the possible infectious etiology of intussusception, but these studies have had a small sample size, and several different pathogens, including adenoviruses, have been identified [255, 256]. It is unclear if wild-type rotavirus causes intussusception as there is a lack of seasonal variation for intussusception that might be expected to occur if rotavirus was a major cause of intussusception [257–259].

RotaTeq™

Initially, a monovalent vaccine containing the VP7 gene of a human G1 rotavirus (WI79-9) and the remainder of the genes from WC3 was developed after the inconsistent results with WC3. This vaccine was reported to be effective [70, 260], but because of the idea that serotype-specific protection was necessary, a quadrivalent vaccine containing three viruses with gene substitutions of the VP7 gene with human G1, G2, or G3 and one virus containing a VP4 gene substitution with a human P[8] was developed and shown to be safe and effective [261]. Finally, by adding a reassortant virus containing a human G4 VP7 to the above four viruses, a pentavalent vaccine, RotaTeq™, was developed by Merck Research Company [262].

Because of the association of Rotashield™ with intussusception, the large pivotal trial of RotaTeq™ included over 70,000 infants from 11 countries. The vaccine was shown to be safe and, unlike Rotashield™, it did not induce fever [263]. Most importantly, there was no association with intussusception. The vaccine was highly effective, reducing all G1–G4 rotavirus gastroenteritis by 74.0 %, severe rotavirus gastroenteritis by 98.0 %, and hospitalizations and emergency room visits by 94.5 % [263]. RotaTeq™ was licensed in 2006 by the US Food and Drug Administration (FDA) for use in infants.

Since the pivotal trial was published, there have been multiple trials of RotaTeq™ conducted around the world [264–268]. Efficacy has been excellent, but as appears to be true for all rotavirus vaccines, the vaccine appears to less effective in less developed areas of the world [269, 270]. In the poorest settings such as Africa and Asia, efficacy has only been 40–50 % [265, 271, 272]. In general this correlates with the lower immune response in these settings [273, 274]. The reasons for this decrease are not well understood. Possible contributing factors include higher levels of transplacental and breast milk antibody that could inhibit vaccine virus replication, malnutrition, micronutrient deficiencies, interfering gut flora, and differences in the epidemiology [275].

Post-licensure evaluations of the vaccine have shown substantial decreases in rotavirus diseases wherever evaluated [224, 276, 277]. Surveillance studies and laboratory monitoring conducted in the United States have shown remarkable reductions in rotavirus disease every year since vaccine introduction [219, 278]. Interestingly this has included not only those immunized but older children and adults suggesting a high level of herd immunity [127, 219, 278]. Safety has also been monitored. Except for a small increase in the rates of intussusception identified in Australia (relative risk 5.3 and 3.5 for the 1–7 and 1–21 days after the first dose of RotaTeq™, respectively, in infants 1 to <3 months of age), the vaccine has been shown to be safe [279]. The increased risk detected in Australia has not been confirmed in studies conducted in the United States [280].

Bovine UK Reassortant Vaccine

The bovine UK strain, which is also a G6P [5], similar to WC3, was used to make a reassortant with G1, G2, G3, or G4 human rotaviruses on a 10-gene UK background. The tetravalent UK vaccine appears to be safe and immunogenic [281]. Clinical trials are ongoing to evaluate this UK reassortant vaccine. There has been additional work done to develop UK-based reassortants containing additional VP7 genes for some of the emerging viruses such as G8 or G9 strains, creating a hexavalent reassortant vaccine [282].

11.1.3 Human Strains

The use of human strains for vaccine candidates is based on the data that show that natural infection can provide protection against subsequent infection or disease. The human strains considered as vaccine candidates are either naturally attenuated, as thought to be the case with neonatal strains, or attenuated by culture adaptation and multiple passage. The candidates discussed below are each composed of one strain and, therefore, rely on homotypic and heterotypic mechanisms of protection.

Neonatal Strains

The first natural history study showed that asymptomatically infected neonates had a subsequent reduction in the frequency and severity of rotavirus diarrhea, thereby generating

interest in the use of neonatal strains as vaccine candidates [119]. One of the first human strains to be tested as a rotavirus vaccine was a neonatal, G1P[6] strain identified as M37. Several small studies showed that the vaccine was safe but only moderately immunogenic, inducing neutralizing antibody responses to the M37 strain but not to other G1 strains [283]. In an efficacy study of Finnish infants, there was no protection against predominantly G1 strains, and this vaccine candidate was not pursued [284].

RV3, another vaccine candidate, is a G3P[6] human rotavirus that was isolated in a nursery in Melbourne, Australia, where it caused endemic, asymptomatic infections in newborn infants in the 1970s. Neonates infected with this virus were 100 % protected against severe rotavirus disease, caused primarily by heterotypic G2P[4] strains, for their first 3 years of life [119]. The vaccine was moderately immunogenic and protective in later studies [285, 286]. This vaccine is also being evaluated in clinical trials.

Two other more recent vaccine candidates were derived from two neonatal strains isolated from asymptomatically infected neonates in India in the mid-1980s: one from New Delhi and the other from the Bangalore region of India. Both of these strains are natural reassortants between bovine and human strains. The strain from New Delhi, 116E, a G9P[11] strain, contains a VP4 gene segment from a bovine rotavirus and the other ten genes are from a human strain [89]. The other strain, I321, is a G10P[11] strain and contains nine genes of bovine origin and only gene segments for two nonstructural proteins from a human strain [287]. Both I321 and 116E vaccine candidates were tested in a small, placebo-controlled trial, which showed only the 116E strain elicited significant immune responses [288]. Therefore, only the 116E strain is being pursued as a vaccine candidate in India. The most recent trials showed good immunogenicity for this vaccine candidate [289], and efficacy trials are under way.

Rotarix®

This vaccine was first licensed in Mexico in 2005 and has since been approved in countries around the world including developed, less developed, and developing nations [223, 290]. The product is based on the attenuated human strain, 89–12 obtained from an infant with rotavirus gastroenteritis in Cincinnati, Ohio, and is a G1P[8] strain, representing the most common strain worldwide. The isolate was attenuated by multiple passages in tissue culture. Results of a multicenter efficacy trial showed that two doses of this vaccine provided 89 % protection against any rotavirus disease and 100 % protection from severe disease [291]. The 89–12 strain was further purified by limiting dilution and passaged in tissue culture by GlaxoSmithKline. The final product was called RIX4414 and is now marketed as Rotarix® (GlaxoSmithKline), a two-dose oral vaccine. Initial testing showed that the vaccine was safe, immunogenic [292–294], and did not interfere with other concomitantly administered childhood vaccines [295, 296]. In the initial efficacy trial conducted in Finland, the vaccine was 73 % protective against all rotavirus gastroenteritis and 90 % protective against severe illness even though a relatively low dose was used [297].

Similar to RotaTeq™, the pivotal vaccine trial with RIX4414 involved over 63,000 infants but was performed in Latin America and Finland [298]. The RIX4414 vaccine ($10^{6.5}$ median cell-culture infective dose) given in a two-dose schedule at approximately 2 and 4 months of age was safe, did not induce fever, and again, most importantly, was not associated with intussusception. During the entire study, there were 25 cases of intussusception, 16 in the placebo group and 9 in the vaccine group. Efficacy data from a subset of 20,000 infants from this trial showed 85 % protection against severe rotavirus diarrhea and hospitalization. Protection against more severe gastroenteritis was 100 %. It was also demonstrated that protection was high (86 %) not only against severe rotavirus diarrhea caused by G1P[8] strains but also against G3P[8], G4P[8], and G9P[8] strains which all shared the VP4 P[8] genotype. Efficacy against the few G2P[4] infections (a strain that is not matched for either VP4 or VP7) was 41 %. However, in an integrated analysis, efficacy against G2[P4] was 71.4 % against severe disease and 81.0 % against disease of any severity [299]. More recently a study in Mexico showed efficacy of over 90 % against a fully heterotypic G9P[4] rotavirus strain in Mexico [300].

In a subsequent trial of RIX4414 conducted in six European countries, protection was 87 % against any rotavirus gastroenteritis, 96 % against severe disease, and 100 % against hospitalization due to rotavirus. In this study, efficacy against G3, G4, and G9 strains was similar to that against G1 strains and was over 95 %. Efficacy against the unrelated G2 strains was 85 %, suggesting that heterotypic or nonneutralizing antibody responses are also involved in protection [301, 302]. Similar to other rotavirus vaccines, efficacy in less developed countries is decreased. In a study of African infants, efficacy against severe disease was 61.2 % and was even lower in Malawi infants, 49.4 % compared to 76.9 % in South Africa [303]. Despite this lower efficacy, it is important to understand that, because of the higher mortality rates, more lives will be saved in counties like Malawi compared to those with lower mortality despite the lower efficacy with either vaccine.

The post-licensure effectiveness of Rotarix® has been verified in several studies (reviewed in [223, 264]). Perhaps, most importantly, vaccination has been associated with reduced mortality from diarrheal-associated disease in Mexico [304] and in Brazil [305].

Similar to RotaTeq™, post-licensure safety of Rotarix® is being monitored. Studies conducted in Mexico and Brazil

[307] revealed an increased rate of intussusception after the first dose in Mexico (1 per 51,000 infants) and a small increase only after the second dose in Brazil (1 per 68,000 infants). In an Australian study that evaluated both RotaTeq™ and Rotarix®, there was an increased relative risk for intussusception of 3.5 and 1.5 for the 1–7 and 1–21 days, respectively, after Rotarix immunization in infants 1 to <3 months of age [279].

12 Nonliving Rotavirus Vaccine Candidates

In addition to live oral rotavirus vaccines, a number of nonliving vaccine candidates have been developed and evaluated in animal models. Nonliving vaccine candidates were developed in an attempt to formulate a vaccine that would be more effective than live oral vaccines, since even natural infection does not always result in 100 % protection from reinfection or to be safer. In addition, these candidates were often studied in an attempt to better define mechanisms of protection, including determinations regarding the protective efficacy of various viral proteins. Finally, after the association of Rotashield™ with intussusception, possible safety concerns with live virus vaccines added to the possible need for nonliving vaccine candidates. The nonliving candidates studied include DNA vaccines [307, 308], inactivated purified triple- and double-layered virus particles [309, 310], recombinant virus-like particles (VLPs) containing VP2 and VP6, with or without VP4 and VP7 [311], inactivated virus [312], and recombinant-expressed proteins including VP6 and portions of VP8 protein [313, 314]. To date none of these candidates have been tested in humans.

Using animal models, each of the above candidates have shown significant levels of protection, especially in the adult mouse model which uses reduction of viral shedding as its measure of protection. Of note, VLPs did not induce protection from illness when used in the gnotobiotic pig model, currently the only illness animal model [315]. Except for DNA-based vaccines, the other candidates often require the use of an effective adjuvant, usually recombinant formulations of bacterial toxins, to induce protection [316, 317]. The use of this type of adjuvant in humans greatly increases the safety concerns associated with these vaccine approaches. The immunogenicity and protection of a candidate inactivated rotavirus vaccine, human strain CDC-9 (G1P[8]) formulated with aluminum phosphate, was recently evaluated in gnotobiotic piglets. The vaccine was immunogenic and protected animals following oral challenge with a homologous virulent human strain Wa (G1P[8]) [318]. It remains to be seen whether these strategies will have a role as alternative approaches to rotavirus immunization. If the present licensed live oral vaccines, RotaTeq™ and Rotarix®, or other live oral rotavirus vaccines prove to be ineffective or have safety issues, nonliving vaccines may be further developed.

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Rubella Virus

31

Walter Orenstein and Susan E. Reef

1 Introduction

Rubella (German measles) is usually a mild febrile viral rash illness in children and adults. However, infection early in pregnancy, particularly during the first 16 weeks, can result in miscarriage, stillbirth, or an infant born with birth defects known as congenital rubella syndrome.

2 Historical Background

German physicians, in the mid-eighteenth century, were the first researchers to distinguish the rubella disease from other exanthems. Even though they named it Rotheln, rubella is recognized today by its common English language eponym German measles [1]. In 1841, a British physician reported an outbreak in a boys' school in India and coined the term rubella, a Latin diminutive meaning "little red" [2]. Even though, in the late nineteenth century, rubella was considered different from measles or scarlet fever [3], not until 1941 the significance of rubella was noted. In 1941, Norman McAlister Gregg, an Australian ophthalmologist, linked congenital cataracts to maternal rubella. In his practice, Gregg had noticed an unusual number of infants with cataracts [4]. It is noted that a crucial clue was a conversation he overheard in his waiting room between 2 mothers who were discussing the rubella they both had sustained in pregnancy during the Australian outbreak of 1940 [5]. Gregg's original observation was followed by reports of Australian [6], Swedish [7], American [8], and British [9] epidemiologists and teratologists confirming his observations and also noting infants presented with heart disease and deafness. Thus, the characteristic congenital rubella triad was established.

After Gregg's discovery, it was 20 years before the isolation of the causative agent, rubella virus. During this time, various estimates of the risk of fetal disease after maternal rubella were made. However, the wide range of estimates stemmed from the absence of a definitive diagnostic test and consequent misdiagnosis of rubella in the mother. In late 1962, the rubella virus was isolated by two different groups: Weller and Neva [10] in Boston and by Parkman, Beuscher, and Artenstein [11] in Washington, DC.

In 1962–1965 a worldwide rubella epidemic started in Europe and spread to the United States. In the United States an estimated 12.5 million cases of rubella occurred in the United States, resulting in 2,000 cases of encephalitis, 11,250 fetal deaths, 2,100 neonatal deaths, and 20,000 infants born with CRS, a constellation of birth defects that often includes blindness, deafness, and congenital heart defects. The economic impact of this epidemic in the United States was estimated at $1.5 billion [12, 13]. The pandemic led to the recognition of an expanded congenital rubella syndrome (CRS), which added hepatitis, splenomegaly, thrombocytopenia, encephalitis, mental retardation, and numerous other anomalies to the already described deafness, cataracts, and heart disease [14, 15]. The pandemic also made it obvious that a vaccine was needed, and many groups set to work.

The global epidemic spurred development of rubella vaccines and emphasized the need to develop and implement strategies for using these vaccines to prevent this devastating health burden [3]. Between 1965 and 1967, several attenuated rubella strains were developed and reached clinical trials [16–18]. In 1969 and 1970, rubella vaccine was introduced in Europe and North America. Since the late 1970s, vaccination has had a major impact on the epidemiology of rubella and CRS resulting in the interruption of endemic rubella virus transmission in 2001 [19].

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3 Methodology

3.1 Mortality Data

Deaths associated with rubella are rare enough that they have no impact on the epidemiology of the rubella.

3.2 Morbidity Data

In the United States, rubella and CRS became nationally reportable to the National Notifiable Disease Surveillance System (NNDSS) in 1966. In 1969, Centers for Disease Control and Prevention established the National Congenital Rubella Syndrome Registry (NCRSR). The reporting efficiency for clinical cases is estimated to be only 10–20 %. As part of the process for documenting the elimination of rubella and CRS in 2004, the adequacy of surveillance was evaluated by reviewing five different sources. The conclusion was that the surveillance system was adequate and was able to support the elimination of endemic rubella transmission [20].

3.3 Serological Surveys

Serological surveys of healthy population groups have been of major importance for understanding the pre-vaccine rubella epidemiology including age specific to identify the target age groups and strategy for vaccine introduction [21]. In addition, serosurveys are used to monitor the impact of the vaccination program, provide evidence for modifying the vaccination strategy, and support the documentation of elimination of rubella/CRS [22].

Prior to the licensure and introduction of the rubella vaccine, the World Health Organization sponsored collaborative serosurveys in 1967–1968 assessing the rubella seropositivity in five continents; however, most of the studies were conducted in the Americas and Europe. In 12 of the 25 studies conducted, the seropositivity rate was 80 % or greater among women aged 17–22 years of age [23]. In the United States, serological surveys conducted in the pre-vaccine era showed seropositivity ranging between 80 and 92 %.

In some regions and countries, post-vaccination serosurveys are used to monitor the vaccination program. However, interpretation of serological studies can be complicated due to variations in the sensitivity of the assays. In the European Region, European Sero-epidemiology Network, the aim is to standardize the serological survey of eight vaccine preventable diseases in 22 countries [24]. By standardizing the methodology, international comparisons can be made to allow to evaluate the effectiveness of different immunization programs and to coordinate vaccine policy to ensure that adequate levels of immunity exist throughout Europe.

In the United States, the most recent use of post-vaccination serosurveys was to support the documentation of elimination of rubella and CRS [25]. Two nationwide seroprevalence studies were conducted through the population-based National Health and Nutrition Examination Survey. Sera were tested for rubella immunoglobulin G antibodies during 1988–1994 and 1999–2004. From the earlier to the later period, the overall age-adjusted rubella seroprevalence in the US population 6–49 years of age rose from 88.1 to 91.3 % was statistically a significant increase. Additional analyses showed that seroprevalence either remained at the same level or higher for the groups (i.e., children of both sexes, women of childbearing age) that were targeted for vaccination.

3.4 Laboratory Methods

3.4.1 Virus Isolation/Detection

In persons with acquired rubella, the rubella virus can be isolated from the blood and nasopharynx during the prodromal period and from the nasopharynx for as long as 2 weeks after eruption. However, the likelihood of virus recovery is sharply reduced by 4 days after the rash. Rubella virus can be isolated using several different cell lines: Vero cell line, primary African green monkey kidney cells, or the RK13 cell line. Through the World Health Organization Global Laboratory Network, laboratories are trained to use either Vero/SLAM cells or Vero cells to isolate rubella virus [26, 27]. The method for detection of the rubella E1 glycoprotein is by using monoclonal antibodies in either an immunofluorescent or an immunocolorimetric assay.

Another recently developed method for virus detection is the polymerase chain reaction (PCR). The most important roles of RT-PCR in rubella control are to characterize the virus genetically and to detect inactivated virus. RT-PCR can be used to detect virus before the IgM is positive and used to detect inactivated particles when the person is shedding only small amounts of virus. The polymerase chain reaction (PCR) has been adapted to the detection of rubella RNA by reverse transcription and amplification.

3.4.2 Antibody Tests

Sera

After the isolation of the rubella, laboratory confirmation of rubella infection was available. Over the last 40 years, several different testing methodologies were developed (Fig. 31.1). These include the following: neutralization assays (NT), hemagglutination-inhibition (HI) assays, enzyme immunoassay (EIA), single radial hemolysis (SRH), and latex agglutination. NT assays were the first to be developed [28], but they are seldom used today, as they are demanding and require use of cell cultures. HI assays were

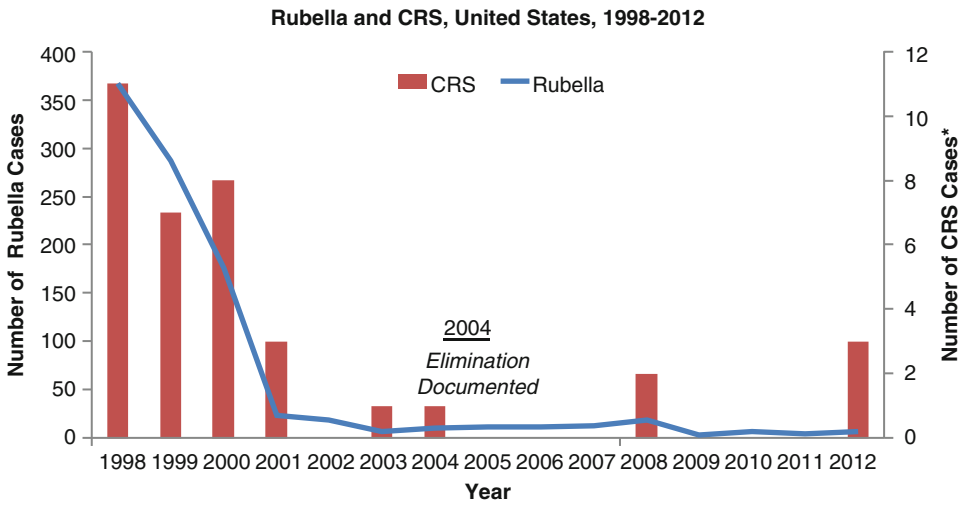

Fig. 31.1 Rubella and CRS, United States, 1998–2012 (*By year of birth)

developed in 1967 [29] and results were shown to correlate well with NT. However, HI is also labor intensive. HI is no longer recommended for routine testing; however, the HI is used as a reference method to establish a calibration standard for other rubella assays. Other more recent assays developed included enzyme immunoassay (EIA), single radial hemolysis (SRH), and latex agglutination, which have been used extensively for rubella antibody screening [30].

Nowadays, EIA is the most frequently used test for rubella antibody screening and diagnosis, as it is a sensitive and an adaptable technique and is readily automated. EIA can also be adapted to detect class-specific antibodies (e.g., IgM, IgG1, IgG3, IgA) and is the method of choice for detection of rubella-specific IgM [31–33]. Indirect and M-antibody capture EIAs are available commercially for detection of rubella IgM. Another adaptation of EIAs is the IgG1 avidity assay. The avidity assays are useful for diagnostic purposes, particularly to distinguish primary rubella from rubella reinfection and to identify persistent IgM responses and nonspecific IgM [34]. The most common diagnostic method employs a denaturating agent (6–8 M urea or DEA) to elute low avidity antibody from antigen-antibody complexes in an EIA (reviewed by Thomas et al. [34]; Best and Enders [30]). Depending upon the strength of this binding, the complex formed may or may not be easily dissociated. Antibody avidity is low after primary antigenic challenge, becomes higher with time, and usually involves IgG antibodies [51, 52]. However, IgG avidity assays can be difficult to establish, standardize, quality control, and interpret; they are therefore recommended only for laboratories experienced in using these assays [35].

Oral Fluids

Since the early 1990s, the use of oral fluid sampling has been used successfully for the detection of rubella antibodies as part of the surveillance system in the United Kingdom [35] Oral fluid sampling can also be used for detecting RNA. In a recent study, the use of oral fluids for detection of RNA was superior to testing of sera or oral fluid for rubella IgM within the first few days after rash onset [36]. Oral fluid samples are easy to collect, noninvasive, and more acceptable to the population. Its use enables field workers to obtain more complete sampling of suspected cases.

4 Biological Characteristics of the Vaccine

Rubella virus is a member of the *Togaviridae* family and the genus *Rubivirus*. Rubella virus is a single-stranded enveloped RNA with a single antigenic type. It measures 50–70 nm in diameter and has two envelope proteins (E1,E2) and a core protein (c). The core protein is surrounded by a single-layer lipoprotein enveloped with spike-like projections that contain the two glycoproteins, E1 and E2. Humans are the only known reservoir.

The rubella virus is relatively temperature labile but is more heat stable than measles virus; it is inactivated after 30 min at 56 °C, 4 min at 70 °C, and 2 min at 100 °C. It degrades rapidly with conventional freezing at −20°, but the virus is stable at −60 °C and below and when freeze-dried with stabilizers. When stabilized with protein it can be repeatedly frozen and thawed without loss of titer. Lipid solvents, weak acids and alkalis, and UV light inactivate the rubella virus. It is also susceptible to a wide range of disinfectants and is inactivated by 1 % sodium hypochlorite, 70 % ethanol, and formaldehyde [37].

Over the last 15–20 years, the study of molecular epidemiology has evolved. In 2005, a systematic nomenclature was adopted by the WHO [38, 39]. The genetic characterization of rubella virus has identified two clades that differ by 8–10 % at the nucleotide level. Clade 1 is divided into 10

genotypes (1a, 1B, 1C, 1D, 1E, 1 F, 1G, 1 h, 1i, and 1j), of which 6 are recognized and 4 are provisional (designated by lowercase letters). Clade 2 contains 3 genotypes (2A, 2B, and 2C) [40]. For rubella isolates collected before 2000, isolates from North America, Europe, and Japan are closely related to each other and form clade I, whereas clade II comprises some strains from China, Korea, and India [41]. However, in China between 2001 and 2007, there was a shift of genotypes toward predominance of IE and 1F [42]. Of rubella isolates collected between 2005 and 2010, three genotypes (1E, 1G, 2B) had wide geographic distribution whereas others occurred sporadically or were geographically restricted [40].

The genetic differences between clades do not appear to translate into antigenic differences, despite amino acid changes of 3–6 % in viral proteins. Isolates from CRS cases are not genetically distinct from isolates from acquired rubella.

5 Descriptive Epidemiology

5.1 Incidence and Prevalence

Our understanding of pre-vaccine era epidemiology of rubella in the United States is from surveillance conducted from 1928 to 1983 in 10 selected areas in the United States. During the 1962–1965 the United States experienced an epidemic with an estimated 12.5 million cases of rubella, resulting in 2,000 cases of encephalitis, 11,250 fetal deaths, 2,100 neonatal deaths, and 20,000 infants born with CRS, a constellation of birth defects that often includes blindness, deafness, and congenital heart defects.

In 1969, live-attenuated rubella vaccines were first licensed in the United States [43], and a vaccination program was established with the goal of preventing congenital infections, including CRS. Before the introduction of vaccine, rubella incidence was highest among children aged <9 years [44]. The new rubella vaccination program targeted a dose of vaccine to children aged 1 year to puberty [45]. To increase coverage among school-aged children rapidly, mass campaigns were conducted, particularly in schools. By 1977, reported vaccination levels were approximately 60 % for children aged 1–4 years, 71 % for those aged 5–9 years, and 64 % for those aged 10–14 years [46]. The number of reported rubella cases declined 78 %, from 57,686 cases in 1969 to 12,491 cases in 1976. As anticipated, the greatest decreases in rubella occurred among persons aged <15 years; however, incidence declined in all age groups, including adults. This decrease in rubella also resulted in a decline in the number of reported CRS cases, from 68 cases reported in 1970 to 23 reported in 1976 [47]. The total number of rubella cases continued to decline overall during the late 1970s; however, in subsequent years a resurgence of rubella occurred among older adolescents and young adults, with outbreaks occurring among students in high schools, colleges, and universities and among persons on military bases and workers in hospitals. Rubella incidence was highest among young adults [48]. In addition, the number of reported CRS cases increased, from 23 in 1976 to 57 in 1979; however, the annual number of CRS cases never reached the level reported during the 1960s in the pre-vaccine era. Serologic studies at that time suggested that 10–20 % of adults remained susceptible to rubella [49].

The resurgence of rubella and its increased incidence among young adults focused attention on the need for additional strategies. In 1978, the changing epidemiology of rubella prompted the Advisory Committee on Immunization Practices (ACIP) to additionally recommend that rubella vaccine be targeted to susceptible postpubertal females, in addition to adolescents, persons in military service, college students, and persons in certain work settings (e.g., hospitals) [50]. Efforts to increase overall childhood vaccination coverage to greater than 90 % for all vaccine-preventable diseases, including rubella, had begun in 1977, with the first National Childhood Immunization Initiative [51]. In 1978, a program was undertaken to eliminate indigenous measles in the United States; the use of combined vaccines, either measles-rubella (MR) vaccine or measles-mumps-rubella (MMR) vaccine, was encouraged. These efforts to increase immunity among selected adults and children resulted in substantial decreases in the numbers of both rubella and CRS cases. During 1977–1981, reported rubella cases declined from 20,395 to 2,077. During 1979–1981, reported CRS cases decreased from 57 to 10 [52]. For the 1981–1982 school year, rubella vaccination coverage was 96 % for children entering school (i.e., into kindergarten or first grade) in the 50 states and the District of Columbia [53]. Efforts to maintain high coverage through enforcement of school immunization laws produced a continuing decrease in reported rubella cases.

In 1979, a new formulation of live-attenuated rubella vaccine (RA 27/3) replaced the previous rubella vaccines in the United States. RA 27/3 vaccine had been determined to induce higher antibody titers and produce an immune response more closely paralleling natural infection than previous vaccines [54].

By 1979, rubella vaccination had eliminated the characteristic 6–9-year epidemic cycle of rubella in the United States [52]. In 1980, national health objectives for 1990 were established for rubella and CRS, calling for reductions in the annual number of rubella cases to fewer than 1,000 and CRS cases to fewer than 10 [55]. During the 1980s, the number of reported rubella cases continued to decline steadily, and overall incidence continued to decrease in all age groups. By 1983, the 1990 objectives already had been achieved, with 970 rubella cases and four CRS cases reported. During the early 1980s, outbreaks continued to be reported in health-care settings, universities,

workplaces, and prisons. In 1981, ACIP recommendations increased emphasis on targeting these settings to ensure vaccination coverage among students and staff members [56].

In 1988, state health departments reported an all-time low of 225 cases of rubella; however, in 1989, a total of 396 cases were reported, and in 1990, the number increased to 1,125 [57]. Most cases were associated with outbreaks that occurred in settings where unvaccinated adults congregated, including colleges, workplaces, prisons, and in religious communities that did not accept vaccination. Outbreaks among these populations accounted for 56 % of CRS cases in the 1990s. In 1989, a goal was established to eliminate indigenous rubella transmission and CRS in the United States by 2000 [58]. With establishment of the 1993 Childhood Immunization Initiative, the number of annual rubella cases continued to decline in the mid-1990s. Outbreaks continued to be associated with settings where adults had close contact; however, the demographic characteristics of rubella patients changed. Before 1995, most persons with rubella were non-Hispanic; beginning in 1995, most were Hispanic [59]. Beginning in 1998, data on country of origin were collected for rubella patients. These data revealed that, during 1998 and 1999, approximately 79 and 65 % of patients whose country of origin was known were foreign born. Of these, 91 % in 1998 and 98 % in 1999 were born in the Western Hemisphere, and 43 % in 1998 and 81 % in 1999 were born in Mexico. These persons were either unvaccinated or their vaccination status was unknown. During 1998–2000, a total of 23 CRS cases were reported to CDC. The infants in 22 (96 %) of these cases were born to Hispanic women, and 22 of the mothers with known country of birth were born outside the United States. The countries of origin of these mothers were Mexico (14 mothers), Dominican Republic (four), Honduras (two), Colombia (one), and Philippines (one). Since 2001, the annual numbers of rubella cases have been the lowest ever recorded in the United States: 23 in 2001, 18 in 2002, seven in 2003, and nine in 2004. Approximately half of these cases have occurred among persons born outside the United States, of whom most were born outside the Western Hemisphere. During 2001–2004, four CRS cases were reported to CDC; the mothers of three of the children were born outside the United States. In 2004, the panel convened by CDC concluded that sustained transmission of rubella has been interrupted. Since 2004, the United States has maintained elimination of rubella and CRS. From 2005 to 2011, a median of 11 rubella cases was reported each year in the United States (range: 4–18). In addition, two rubella outbreaks involving three cases, as well as four total CRS cases, were reported [60]. In 2012, as part of the documentation process for PAHO, the United States convened an independent external panel to evaluate if elimination of measles, rubella, and CRS had been maintained.

The incidence of CRS has been evaluated mainly in developed countries over the last 60 years. Initially in the United States, the incidence of CRS roughly paralleled the incidence of rubella in individuals over 15 years; however, with the interruption of endemic rubella virus transmission, CRS cases became very rare and occurred mainly among mothers who are foreign born [61].

In most developing countries, there is little documentation to illuminate the epidemiology of either rubella or CRS. The epidemic pattern for developing countries is similar to the developed countries with cycles 3–7 years. Globally, it is estimated that approximately 103,000 infants with CRS were born in 2010 with the greatest burden in regions where rubella vaccine uptake is limited. A review of worldwide data concerning CRS revealed rates in developing countries varying between 0.6 and 2.2 per 1,000 live births, similar to rates seen in developed countries before universal vaccination [62, 63]. It has been estimated that the incidence of CRS is 0.1–0.2 per 1,000 live births during endemic periods and 1–4 per 1,000 live births during epidemic periods [64]. Where rubella virus is circulating and women of childbearing age are susceptible, CRS cases will continue to occur.

5.2 Epidemic Behavior

Rubella usually occurs in a seasonal pattern, with epidemics every 5–9 years. However, the extent and periodicity of rubella epidemics is highly variable in both industrialized and developing countries. From published literature, epidemics have been reported every 6–7 years in Hong Kong [65] and São Paulo, Brazil [66]; every 4–5 years in Panama [67]; and every 4–7 years in Argentina [68] and Bangkok, Thailand [69–71].

5.3 Geographic Distribution

Prior to the establishment of the rubella and CRS elimination goal in the Region of Americas, rubella had a worldwide distribution. However, in 2009, the last endemic rubella case in the Region of the Americas was documented in Argentina [72]. Rubella continues to circulate in the Eastern Hemisphere. In 2012–2013, rubella epidemics have been documented in several countries (i.e., Romania [73], Poland [74], Japan [75], Ethiopia) in three different continents.

5.4 Temporal Distribution

Prior to the elimination of endemic rubella virus in the United States, the largest number of rubella cases occurred in late winter and spring, in both high and low incidence. Because

of the acceleration of measles control, the understanding of rubella seasonality can be documented in developing countries. Using the measles case-based surveillance in African region, rubella seasonality could be detected with variation of seasonality by subregion [76]. In the West subregion, during 2003–2009, marked seasonality of rubella occurred each year with sharp increases in reporting during January with peaks in March–April followed by sharp declines in May, leading to troughs during October– December each year. However, in the South subregion, a distinct annual seasonality was observed with consistently few cases reported during January–June each year, followed by gradual increases in June–July and peaks in September–October.

5.5 Age and Sex

In the pre-vaccine era in the United States, rubella was primarily a disease of school-aged children; however, rubella occurred also in preschool children. In many countries, this is the pattern for rubella infection. However, in other countries such as Caribbean islands and Southeast Asia, young adult females show high susceptibility, which can result in cases among pregnant women with subsequent CRS.

In the pre-vaccine era, there were no differences in attack rates by sex for children. In the post-vaccination era, in countries where adolescent girls were targeted for vaccination, outbreaks among adolescent and adult males have been documented [71]. However, in countries that have not targeted females only in vaccination, attack rates in males and females are similar.

6 Mechanisms and Routes of Transmission

Rubella virus is spread from person to person via respiratory droplets. Individuals with acquired rubella may shed virus from 7 days before rash onset to ~5–7 days thereafter. Both clinical and subclinical infections are considered contagious.

After primary implantation and replication, subsequent viremia occurs, which in pregnant women often results in infection of the placenta. Placental virus replication may lead to infection of fetal organs. Infants with CRS may shed large quantities of virus from bodily secretions, particularly from the throat and in the urine, up to 1 year of age. Outbreaks of rubella, including some in nosocomial settings, have originated with index cases of CRS. Thus only individuals immune to rubella should have contact with infants who have CRS or who are congenitally infected with rubella virus but are not showing signs of CRS.

7 Pathogenesis and Immunity

Although the pathogenesis of postnatal (acquired) rubella has been well documented, data on pathology are limited because of the mildness of the disease. Primary implantation and replication in the nasopharynx are followed by spread to the lymph nodes. This is followed by viremia and shedding of virus from the throat.

For acquired rubella, the rubella virus induces both circulating and cell-mediated immune (CMI) responses. HI and NT antibodies develop very rapidly and may be detectable, while the rash is still specific antibodies, rubella-specific IgM appears first and is closely followed by IgG1, IgG3, and IgA [31]. IgM is transient; it peaks on about day 7 and persists for 4–12 weeks after illness and occasionally for about a year [77].

The role of CMI in protection from rubella has not been determined. Rubella infection induces a fall in total leukocytes, T cells, and neutrophils, and a transient depression in lymphocyte responses to mitogens and antigens (e.g., purified protein derivative, PPD), but the mechanism responsible for the mild immunosuppression has not been elucidated. Studies of cytokine secretion demonstrate the strongest responses in persons with a recent history of rubella [78, 79]. Lymphoproliferative assays show that CMI responses develop a few days after onset of rash and persist at low levels for many years.

The pathology of CRS in the infected fetus is well defined, with almost all organs found to be infected; however, the pathogenesis of CRS is only poorly delineated. In tissue, infections with rubella virus have diverse effects, ranging from no obvious impact to cell destruction. The hallmark of fetal infection is chronicity, with persistence throughout fetal development in utero and for up to 1 year after birth.

The immune response to the intrauterine rubella infection starts while in pregnancy. However, the development of the fetal humoral immune system appears to be too late to limit the effects of the virus. Cells with membrane-bound immunoglobulins of all three major classes—IgM, IgG, and IgA—appear in the fetus as early as 9–11 weeks gestation [80]. However, circulating fetal antibody levels remain low until mid-gestation, despite the presence of high titers of virus. At this time, levels of fetal antibody increase, with IgM antibody predominating [81]. As in the case with other chronic intrauterine infections, congenital rubella infection may lead to an increase in total IgM antibody levels [82]. At the time of delivery of infected infants, levels of IgG rubella antibodies in cord sera are equal to or greater than those in maternal sera, even if the infant is born prematurely. IgG is the dominant antibody present at delivery in rubella-infected infants and is mainly maternal in origin. In contrast, the IgM levels are lower but are totally fetus derived.

8 Patterns of Host Response

In acquired rubella, the ratio of inapparent to apparent infections has been estimated to be from 1:1 to as high as 6:1 [83, 84]. Age probably influences the clinical expression of infection. Children usually develop few or no constitutional symptoms.

8.1 Clinical Manifestations

8.1.1 Acquired Infection

The average incubation period is 14 days with a range of 12–23 days. During the first week after exposure, there are no symptoms. During the second week after exposure, there may be a prodromal illness consisting of low-grade fever (<39.0 °C), malaise, mild coryza, and mild conjunctivitis, which is more common in adults. Postauricular, occipital, and posterior cervical lymphadenopathy is characteristic and typically precedes the rash by 5–10 days. Children usually develop few or no constitutional symptoms. Rarely, rubella may mimic measles in its severity of fever and constitutional symptoms, but Koplik's spots are absent.

At the end of the incubation period, a maculopapular erythematous rash appears on the face and neck. The rubella rash occurs in 50–80 % of rubella-infected persons and is sometimes misclassified as measles or scarlet fever. The maculopapular erythematous rash of rubella starts on the face and neck and progresses down the body. The rash, which may be pruritic, usually lasts between 1 and 3 days. The rash is fainter than measles rash and does not coalesce, and it may be difficult to detect, particularly on pigmented skin.

Rubella disease is usually mild, resulting in very few complications apart from the serious consequences of congenital rubella infection. Transient joint symptoms (e.g., arthritis, arthralgias) may occur in up to 70 % of adult women with rubella. They usually begin within 1 week after rash onset and typically last for 3–10 days, although occasionally they may last for up to 1 month. Other complications include thrombocytopenic purpura (1 in 3,000 rubella cases) and encephalitis (1 in 6,000 rubella cases). In outbreaks in the Kingdom of Tonga (2002), the Independent State of Samoa (2003), and Tunisia [85], encephalitis was seen more commonly, with an estimated rate of 1 in 300 to 1 in 1,500 cases. Long-term sequelae with such progressive rubella panencephalitis (PRP) are rare. PRP has similarities to subacute sclerosing panencephalitis (SSPE) caused by measles.

8.1.2 Congenital Rubella Infection

The risk of congenital infection is related to the gestational age at the time of maternal infection. The outcome of a primary rubella infection during pregnancy includes the following: spontaneous abortion, stillbirth/fetal death, infant born with CRS, infant born with congenital rubella infection without congenital defects, and birth of a normal infant.

The most common defects of CRS are hearing impairment (unilateral or bilateral sensorineural), eye defects (e.g., cataracts, congenital glaucoma, or pigmentary retinopathy), and cardiac defects (e.g., patent ductus arteriosus or peripheral pulmonic stenosis). Other clinical manifestations may include microcephaly, developmental delay, purpura, meningoencephalitis, hepatosplenomegaly, low birth weight, and radiolucent bone disease (Table 31.1).

Children with CRS may develop late-onset manifestations including endocrine abnormalities (e.g., diabetes mellitus, thyroid dysfunction), visual abnormalities (e.g., glaucoma, keratitic precipitates), and neurological abnormalities (e.g., progressive panencephalitis), in addition to developmental manifestations which include autism [86].

When pregnant women are infected with rubella during the first 11 weeks of gestation, up to 90 % of live-born infants will have CRS; thereafter the rate of CRS declines until 17–18 weeks' gestation when deafness is the rare and only consequence. Reinfection with rubella may occur, but if this occurs early in pregnancy, transmission to the fetus is rare, and the risk of congenital rubella defects is probably less than 5 %.

8.2 Serological Responses

Antibodies to rubella virus develop promptly and can sometimes be detected on the day of rash onset. The IgM and IgG classes rise rapidly; IgG persists, but IgM begins to wane

Table 31.1 Common transient and permanent manifestations in infants with congenital rubella syndrome

Transient manifestations	Permanent manifestations
Hepatosplenomegaly, hepatitis	Hearing impairment (deafness)
Thrombocytopenia with purpura/petechiae Dermal erythropoiesis (blueberry muffin syndrome)	Congenital heart defects (e.g., patent ductus arteriosus, pulmonary arterial stenosis)
Long bone radiolucencies Intrauterine growth retardation	Eye defects (cataracts, pigmentary retinopathy, congenital glaucoma, microphthalmos)
Meningoencephalitis Interstitial pneumonitis	Central nervous system involvement (e.g., microcephaly, mental and motor delay, autism)

(see Sect. 7). However, in a small percentage of persons, IgM persists for long period of time [30]. This persistence can be confused with acute infection. To help differentiate IgM associated with acute infection, testing for seroconversion of IgG or avidity testing is recommended.

9 Control

9.1 Vaccine Development

As noted earlier, the development of vaccines was spurred by the 1962–1965 epidemics in Europe and the United States. Shortly after the isolation of rubella virus, investigators attempted to develop an inactivated virus vaccine, but their attempts were unsuccessful. Either the vaccines were not antigenic or if antibodies were produced, it was questionable if the preparation was contaminated with live virus [87].

With the issues of the inactivated vaccine, several groups were interested in developing a live-attenuated vaccine in the 1960s. Parkman and colleagues were the first to successfully attenuate RV with 77 passages in African green monkey kidney cell cultures and to give the attenuated strain HPV77 [88]. Between 1969 and 1970, three vaccines were licensed in the United States, including HPV-77.DK12 (dog kidney), HPV-77.DE5 (duck embryo), and Cendehill (rabbit kidney) [46]. The Cendehill vaccine was licensed in Britain in 1969, and shortly thereafter, the RA 27/3 vaccine (human diploid cells) was licensed in Europe. In Japan, the initial vaccines licensed were the Takahashi (rabbit kidney) and Matsuura (Japanese quail-embryo fibroblasts) vaccines. Three additional vaccines were licensed in Japan: Matsuba (rabbit kidney), DCRB 19 (rabbit kidney), and TO-336 (rabbit kidney) [89].

By 1979, all three of the vaccines licensed in the United States were replaced by RA27/3. RA27/3 vaccine generally induces higher antibody titers and produces an immune response more closely paralleling natural infection than the other vaccines. HPV-77.DK12 was withdrawn due to the higher incidence of side effects as compared to other vaccines.

After the development and licensure of the initial rubella vaccines globally, additional vaccines were licensed in various geographic locations. In 1980, a rubella vaccine (BRD-2) was developed in the People's Republic of China using a local RV strain from a child, isolated in human diploid cells. In a trial comparing the BRD-2 vaccine and RA 27/3 vaccine, the seroconversion rate and mild side effects were similar [90]. In Japan, currently, five different rubella vaccines are in use, including the TO-366 vaccine [91]. Even though additional vaccines have been licensed and developed, RA27/3 continues to be the most widely used vaccine strain globally.

Rubella-containing vaccine is available as either a single antigen or combined with measles (MR), measles and mumps (MMR), and measles, mumps, and varicella (MMRV).

9.2 Response

9.2.1 Clinical Reactions

Vaccines can develop mild rubella, including rash, lymphadenopathy, fever, sore throat, and headache. However, the incidence of each of these side effects varies directly with age, being almost absent in infants and increasing with age. Fortunately, the minor side effects are seldom severe enough to cause days to be lost from school or work [92–94].

In a double-blind study of vaccination with MMR in twins, there was a 1 % incidence of arthropathy and little evidence of other reactions [95]. In 1991, the Institute of Medicine of the National Academy of Sciences published a committee report on four possible adverse effects of rubella vaccine: acute arthritis, chronic arthritis, neuropathies, and thrombocytopenia [96]. The committee concluded that RA27/3 causes acute arthritis. With regard to chronic arthritis, the committee stated doubtfully, "The evidence is consistent with a causal relation between the currently used rubella vaccine strain (RA27/3) and chronic arthritis in adult women, although the evidence is limited in scope and confined to reports from one institution." Since that time, large vaccination campaigns conducted in millions of Latin Americans, including women of childbearing age, have not been accompanied by additional reports of significant chronic arthropathy [64].

In 2011, IOM was asked to review adverse events associated with several vaccines. In their report, the IOM concluded that the evidence is inadequate to accept or reject the causal relationship between MMR vaccine and chronic arthralgia or arthritis in women [97].

9.2.2 Shedding of Virus

Because of the risk of spreading of vaccine virus to susceptible persons including pregnant women, considerable effort has been made to detect the spread of vaccine virus to susceptible contacts. Early contact studies documented no evidence of spread to susceptible contacts. However, there was a rare asymptomatic seroconversion that could not be explained fully [98, 99].

Virus has been recovered from breast milk of women vaccinated postpartum. Transmission of the virus to the infant has been documented, but the infection is asymptomatic and transient.

9.2.3 Serological Response

Rubella vaccine is usually administered ≥12 months of age, since maternal antibodies have usually disappeared by that age. The seroconversion rate for children ≥12 months is >95 %. The age at first vaccination does not appear to be as critical for rubella as for measles vaccine. Passively transmitted maternal antibodies to rubella have been found in approximately 5 % of infants from 9 to 12 months and 2 %

from 12 to 15 months of age. Studies of rubella-containing vaccine administered at 9–12 months of age has demonstrated a seroconversion rate of >90 %. Rubella vaccine is usually offered to children with measles vaccine (MR) or measles and mumps vaccines (MMR).

9.3 Rubella Vaccination Strategies: Their Impact on Rubella and Congenital Rubella

9.3.1 Epidemiological Approach

The goal of rubella vaccination programs is the prevention of the intrauterine infection that causes CRS. There initially were two basic approaches: the US (indirect protection) (see Sect. 5.1) and the UK (direct protection). However, with over 30 years of experience with introducing rubella vaccine into countries, the strategies have evolved.

In 2000, the WHO convened a meeting to review the worldwide status of CRS and its prevention [100, 101]. Since the previous international meeting on CRS and rubella in 1984, some of the changes included availability of more data on the CRS disease burden in developing countries, an increase in the number of countries with national rubella immunization programs, and advances in laboratory diagnosis. In 1996, only 83 countries/territories used rubella vaccine in their national immunization programs. Since the 2000 meeting, additional countries have introduced rubella-containing vaccine, two WHO regions (Regions of the Americas and Europe) have established rubella elimination goals by 2010 and 2015, respectively, and one WHO region (Western Pacific) has established an accelerated rubella control and CRS prevention goal by 2015. As of 2010, this number had increased to 130 countries.

In 2011, the WHO rubella vaccine recommendations were updated [64]. The WHO recommends that countries that have not introduced rubella vaccination take the opportunity offered by accelerated measles control and elimination to introduce rubella vaccine. The measles vaccine strategy platform provides the opportunity to use combined vaccine and an integrated measles-rubella surveillance system. The preferred strategy for introduction of rubella vaccination is to begin with MR/MMR vaccine in a campaign targeting a wide range of ages together with immediate introduction of MR/MMR vaccine into the routine program. In 2011, GAVI (formerly the Global Alliance for Vaccines and Immunization) opened a window for introduction of rubella-containing vaccine into GAVI eligible countries (for more on GAVI, see Chap. 1. Of the remaining 63 countries that had not introduced rubella vaccine in 2011, 51 (81 %) are GAVI eligible. GAVI funding will support MR vaccine for catchup campaigns targeting children 9 months to 14 years 11 months and introduction grant. It is estimated that 30 countries will have introduced rubella vaccine by the end of 2015, and all GAVI eligible countries will have introduced it by 2018. With GAVI support, the goal of rubella eradication may be within reach.

9.3.2 Vaccination in Pregnancy

Although there is now abundant evidence for the safety of RA 27/3 for the fetus, pregnancy remains a contraindication to rubella vaccination, and women are advised to take precautions against pregnancy for 1 month (28 days) after vaccination. Prior to the efforts to eliminate rubella from Latin America, there was limited data on vaccination of unknowingly pregnant women [102]. To eliminate rubella and CRS in the region of the Americas, countries conducted campaigns in adult. As part of the campaigns in several countries, women who were vaccinated and subsequently learned that they were pregnant at the time of vaccination were followed up. On the basis of serological evaluation, 2,894 (10 %) women were classified as susceptible at the time of vaccination; of their pregnancies, 1980 (90 %) resulted in a live birth. Sera from 70 (3.5 %) of these infants were rubella IgM antibody positive, but none of the infants had features of CRS as a result of rubella vaccination. The maximum theoretical risk for CRS following rubella vaccination of susceptible pregnant women was 0.2 %. In all the available literature on vaccination of pregnant women, approximately 3,000 susceptible women with live births have been followed up and none of the infants had features of CRS.

9.3.3 Persistence of Vaccine-Induced Immunity

Studies on the long- term persistence of antibodies after rubella immunization of susceptible persons have documented that immunity probably persists for life in the majority of vaccines. Although antibody titers fall over time, sometimes to very low levels, immunological memory persists, and a secondary immune response will occur on exposure to rubella.

Follow-up studies have shown that 95–100 % RA27/3 vaccines are seropositive 10–21 years after immunization [103]. The high seroconversion rate, the persistence of antibodies, and an amnestic response when revaccinated do not support the need for a second dose of rubella vaccine. However, based on the indications for a second dose of measles- and mumps-containing vaccine, a second dose of MMR is now offered in most industrialized countries, and this helps to boost low rubella antibody concentrations.

9.3.4 Reinfection

Reinfection is usually subclinical and is more likely to occur in persons with vaccine-induced immunity than in those whose immunity is naturally acquired. It is not due to antigenic variants of rubella virus [104]. Reinfection is defined as a significant rise in antibody concentration in a person with

preexisting antibodies. In a clinical situation, preexisting antibodies can be confirmed by testing an earlier stored serum, but if no such serum is available, evidence of preexisting antibody may be accepted if there are at least two previous laboratory reports of antibodies ≥10 iu/ml obtained by reliable techniques (not HI) or a single result of antibodies ≥10 iu/ml obtained after documented rubella vaccination [105].

The concern for reinfection is a pregnant woman as it might lead to fetal infection. Several challenge tests have been conducted with some attempting to document viremia. In one of these studies [106], the viremia was detected in persons with low levels of antibody titers. The risk of congenital infection following reinfection in the first 12 weeks of pregnancy has been estimated in the United Kingdom to be about 8 %, while the risk of congenital rubella defects is probably no more than 5 %, which is considerably less than the >80 % risk of primary rubella during the same period of pregnancy [107]. Thus, it is important to be able to use laboratory tests to distinguish reinfection from primary rubella in pregnancy.

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Part III

Viruses Causing Acute and Chronic Syndromes and/or Malignancy

Hepatitis Viruses: Hepatitis B and Hepatitis D

32

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1 Hepatitis B Virus

The hepatitis B virus (HBV) virus is a small, partly double-stranded enveloped DNA virus and is the etiological agent of human hepatitis B infection. HBV is the world's most common cause of chronic viral hepatitis and hepatocellular carcinoma (HCC). The latter condition is covered extensively in Chap. 34. It is estimated that there are up to 400 million people chronically infected with HBV around the globe, most of whom are unaware of their infection status [1–3]. The virus is >50 times more transmissible than HIV, but an effective vaccine is available and is having an impact on the global burden of disease caused by hepatitis B [1]. The viral infection and its control has been the focus of intensive scientific research since its discovery.

1.1 Historical Background

Hepatitis as a syndrome has been recognized since ancient times, but the distinction of two forms – one usually transmitted via the fecal-oral route and one more often parenterally transmitted – began to emerge in the early- to mid-twentieth century [4, 5]. Although it could be shown that the parenterally transmitted agent had properties consistent with a virus with a long incubation period [6], identification of the agent itself eluded researchers until the mid-1960s when Blumberg, Alter, London, and colleagues discovered a serum protein, named the "Australia" antigen, present in the blood of some Australian aborigines, individuals with Down syndrome, and oncology patients [7, 8]. The initial evidence suggested that the presence of this protein might be a marker of risk for development of leukemia. Through an exhaustive series of epidemiologic studies, it became clear that the Australia antigen might instead be associated with an infectious agent. This hypothesis took precedence when it was demonstrated in a clinical study that a subject previously negative for the antigen became positive, coincident with biochemical evidence of liver inflammation [4, 9].

Further studies led to the visualization of the virus particle (called the Dane particle) by electron microscopy [10, 11] and demonstration of its transmissibility in animal studies and via blood transfusion [4, 9]. Using banked blood from transfusion recipients, Prince [12] showed that the circulating antigen was first detected during the incubation period for posttransfusion hepatitis, was likely a component of the virus particle, and was persistently detected for long periods after apparent recovery in some patients, consistent with what was then referred to as serum hepatitis, i.e., a hepatitis with a long incubation period and an asymptomatic carrier state [12]. Studies by Krugman et al. in institutionalized developmentally disabled patients had separately identified sera that were capable of transmitting serum hepatitis with clinical characteristics similar to those seen by Prince [5].

Since "infectious hepatitis" transmitted via the fecal-oral route was known as hepatitis A, the serum hepatitis virus was called the hepatitis B virus (HBV). The Australia antigen was shown to be the surface protein of the viral particle, now called hepatitis B surface antigen or HBsAg. The test for HBsAg quickly became an important tool for prevention of posttransfusion hepatitis by its use in screening blood donations [9].

Two major discoveries quickly followed confirmation of the association of HBsAg with serum hepatitis. The first of

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these was the observation that noninfectious HBsAg particles could be used to produce an effective vaccine against HBV [13]. Shortly after this came the confirmation that chronic HBV infection is a major cause of hepatocellular carcinoma worldwide [14, 15].

1.2 Methodology Involved in Epidemiologic Analysis

1.2.1 Source of Mortality Data

Mortality from acute HBV infection is uncommon although as many as 1 % of the primary infections lead to decompensated liver disease and failure and death. However, most HBV-related deaths are from hepatocellular carcinoma (HCC) or liver cirrhosis (LC). The World Health Organization (WHO) estimates that 600,000 individuals worldwide die each year as a result of HBV infection [16]. In the United States, the number of deaths is approximately 3,000 per year [17]. In vital statistics data, HBV-related deaths may be undercounted because HCC and LC deaths are often not attributed to specific viral etiologies on death certificates [18, 19]. Among 1,788 deaths in the United States where HBV was listed as a cause of death, the demographic groups with the highest standardized mortality rates were found in males (approximately 3-fold higher than females) and non-White/non-Black racial groups (approximately 5-fold higher than Whites). Age- specific mortality rates peaked in the 55–64-year-old age group [18]. In areas of the world where the prevalence of chronic HBV infection is high, its impact on mortality is reflected directly in HCC and LC death rates.

1.2.2 Sources of Morbidity Data

Acute HBV infection is a reportable disease in many parts of the world. In populations where the infection is endemic, however, the majority of infections occur at younger ages and are likely to be asymptomatic and to result in chronic infection, which is not generally included in reportable disease statistics. In recent years, the United States has seen a dramatic decline in reported acute HBV cases, falling from 8,036 cases nationwide in 2000 to 3,350 in 2010. In 2010, the age group with the highest incidence rate for reported cases was the 30–39-year-olds. Among those with risk factor reported, sexual behaviors (sexual partner with known or suspected HBV infection, multiple sexual partners, and/or men who have sex with men) were found more frequently than injection drug use and medical or occupational exposure [18]. Chronic HBV has been a reportable disease since 2007, but only a few states routinely report cases. Because of the difficulties in determining chronicity, enhanced surveillance programs provide the most reliable data [20]. In 2010, the CDC received reports of 10,515 chronic cases from eight reporting sites. The peak prevalence was found in the 25–54-year age group and in Asian/Pacific Islanders [18].

1.2.3 Serologic Surveys

General population serosurveys have examined the prevalence of HBV markers in many populations worldwide [21–23]. These data have been used to define the general epidemiologic patterns of chronic HBV infection seen worldwide (see Sect. 1.4). In a 2012 systematic review, Ott et al. [24] used 396 published articles with data from general populations to estimate global HBsAg prevalence at 3.7 %. Surprisingly, age-specific prevalence was highest in children in West Africa (~8.5 %). East Asian age-specific prevalences exceeded those of West Africa only in adults age 45 and older.

Carefully constructed large-scale national serosurveys in China, conducted in 1992 and repeated in 2005, have been used to estimate the impact of disease control measures, particularly immunization, on age-specific prevalence of HBV markers. In these two studies, the prevalence of HBsAg in children under 5 years old declined from 9.7 % in 1992 to 1.0 % in 2005, but no change was seen in prevalence in adults age >20 years. All age groups showed a reduced prevalence of anti-HBc, however, suggesting that overall exposure to HBV has been reduced in the population through immunization of infants and other public health measures [25]. Within countries such as the United States, general surveys such as the National Health and Nutrition Examination Surveys (NHANES), good indicators of the health status of the majority of the population, have underestimated prevalence of HBV and other diseases that affect the minority or disadvantaged groups not fully included in the sampling approach. The CDC-adjusted estimate of chronic HBV seroprevalence in the United States was 1.2 million persons in 2012 [17]. The true prevalence may be as high as 2–2.2 million, the difference being largely due to undercounting of foreign-born persons [26, 27].

1.2.4 Laboratory Diagnosis

A number of laboratory tests are available for the detection of current and past HBV infection and immunity. The markers and their interpretation in combination are summarized in Table 32.1. Most markers are detected by standard enzyme immunoassay (EIA) or radioimmunoassay (RIA) and reported as either positive or negative. Some markers may also be quantitated by these methods.

HBsAg is the viral surface (envelope) glycoprotein coat. While HBsAg is not itself infectious, its detection in the circulation is an indication of current infection and the potential for infectiousness to others. It is present in large amounts in the circulation of most infected persons, both as full viral particles and as large numbers of empty particles, round or filamentous in shape, which contain no viral DNA [28, 29].

Table 32.1 Patterns of serologic markers of HBV infection and their usual significance [1, 31]

	HBsAg	Anti-HBs	Anti-HBc	IgM anti-HBc	HBeAg	Anti-HBe	HBV DNA
	Viral glycoprotein coat	Antibody to HBsAg	Total antibody to viral core	IgM class antibody to viral core	e antigen, associated with viral core	Antibody to HBeAg	Viral DNA
Susceptible	–	–	+/–	–	–	–	–
Natural immunity (past infection)	–	+	+	–	NA	NA	–
Vaccine-induced immunity	–	+ (≥10 mIU/mol considered adequate)	–	–	NA	NA	–
Early acute infection	+	–	+	+	+	–	+
Chronic infection	+ Presence for ≥6 months indicates chronic infection	–	–	–	+/–	+/–	+/–
Resolved or resolving infection	–	+	+	–	–	–	–
"Occult" infection	–	–	+	–			+/–

NA not applicable

These subviral particles may be present at 100-fold or more excess in relation to viral particles [29, 30].

HBsAg is detectable in serum beginning 1–2 months after infection, sometimes much earlier, and prior to the onset of symptoms. Persistence for 6 months or longer is an indication that chronic infection has been established. In acute infection, HBsAg levels begin to subside after the onset of symptoms and disappear by 1–5 months after infection, as symptoms resolve [31, 32]. In chronic infection, HBsAg remains detectable for extended periods, often for the lifetime of the infected individual. In general, circulating antigen load decreases after the first few months of chronic infection but remains easily detectable by standard assays [28, 32]. Quantitation of circulating antigen load is emerging as a clinical marker of disease stage or treatment response in chronically infected individuals [30].

The so-called occult HBV, i.e., apparently HBsAg-negative HBV infection (defined by HBV DNA in the liver or blood), has been described in some populations. In some cases HBsAg is present but in a variant form not recognized by standard commercial assays. In other cases, HBsAg is present but suppressed to very low levels by host immunologic state, viral mutation, and/or coinfections with other viruses (e.g., HIV, HCV). In some cases, serologic markers such as anti-HBc and anti-HBs may be absent as well [33]. Clinically, occult infections are significant because blood and body fluids from patients may still be infectious and some occult HBV patients are at high risk of HCC and chronic liver disease [34, 35].

Antibody to HBsAg (anti-HBs) becomes detectable in the serum of acutely infected individuals after the decline of HBsAg [32]. It indicates the development of protective immunity in natural infections and in response to HBV vaccination. It can be quantitated by routine clinical assays, and a level of ≥10 mIU/ml is an indicator of protective immunity [31]. While rare, the co-occurrence of HBsAg and anti-HBs positivity have been reported in some chronic infections [36].

Antibody to hepatitis B core protein (anti-HBc) is non-neutralizing, is detectable in all HBV infections, and persists indefinitely. It is not detected in persons who are immune to HBV through vaccination [31]. It arises first as IgM class anti-HBc, after the appearance of HBsAg in both acute and chronic infection [28, 32]. A high level of IgM anti-HBc is an indication of recent infection, and a predominantly IgM response may be detectable for a short time even after resolution of HBsAg in acute infection. Thus, high-titer IgM anti-HBc in the absence of HBsAg is accepted as the laboratory component of the case definition for reportable acute HBV infection [31, 37]. Nevertheless, IgM anti-HBc may also occasionally be detected in chronic HBV infection as well, often in the course of seroconversion from HBeAg to anti-HBeAg positivity [38].

Hepatitis B e antigen (*HBeAg*) is a soluble protein secreted from HBV-infected hepatocytes and encoded in the open reading frame (ORF) for the C protein, with an alternate initiation site. Its precise function is not fully understood. Its presence is not necessary for viral replication, but clearance of circulating HBeAg usually coincides with a reduction in viral load [28]. HBeAg is present during acute infection but clears usually prior to the clearance of HBsAg [39]. In chronic infection, it persists for a variable period, influenced by host characteristics and viral genotype. In some chronically infected persons, HBeAg may reappear and disappear repeatedly over the course of infection [40].

The appearance of *antibody to HBeAg (anti-HBe)* accompanies or follows clearance of HBeAg in both acute and chronic infections. In chronic infection, this is usually an indicator of transition to less active disease, evidenced by

lower liver transaminases and low to undetectable viral load. In 10–30 % of anti-HBe seroconversions, however, active hepatitis will continue, with elevated liver transaminases and detectable viral load. In another 10–30 % who initially have less active disease after the development of anti-HBe, more active disease returns at a later time [41].

HBV DNA can be detected in circulation and in liver tissues. Commercial assays using hybridization or amplification methods for quantitation of circulating viral load are now widely available on several commercial platforms. Many assays are able to detect viral loads as low as 6 IU/ml (30 viral copies/ml) [42]. HBV DNA is first detected in blood very early in both acute and chronic infection, the earliest of the virus-specific markers. In acute infection, it drops to very low levels by the time of HBsAg clearance, but using very sensitive methods, HBV DNA can still be detected at times in the circulation of subjects who otherwise appear to have cleared infection [28, 43]. In chronic infection, viral load is high initially but may drop to low or undetectable levels later [28]. Very high viral loads (>200,000 IU/ml) occur when the host immune system does not recognize the virus as foreign. During active immune response, viral loads diminish and may drop to at or below the limits of detection when disease moves into the inactive phase [41]. Viral isolation or viral culture are not used in the routine detection or monitoring of HBV infection.

1.3 Biological Characteristics of the Organism

HBV is classified as a Hepadnavirus, a family of DNA viruses that also includes mammalian (woodchuck, ground squirrel) and avian (duck) viruses that are similar in structure and in their hepatotropism. Viral replication occurs in hepatocytes through a reverse transcription step that is unique to this family of viruses [44, 45]. Infections of cells other than hepatocytes, if they occur, do not appear to cause significant disease [29]. Humans appear to be the only natural host for HBV, but infections of nonhuman primates have been produced in the laboratory [31].

The circular, partially double-stranded viral genome is small, ~3,200 nt, and encodes four distinct proteins in overlapping reading frames. These are the polymerase (P), the envelope (S), the nucleocapsid or core (C), and the X protein. Within the S ORF, three different surface proteins are encoded by different initiation codons – the S, pre-S_1, and pre-S_2. Similarly, the C region encodes the core (HBcAg) and e antigens (HBeAg) [28, 44]. Upon entry into the host cell, viral genomes contained in core particles are repaired to form covalently closed circular DNA (cccDNA), which in turn acts as the transcription template for viral mRNA and genomic viral RNA. Genomic copies are packaged in the cytoplasm, after which the viral polymerase reverse transcribes the template into the DNA in the partially double-stranded form found in viral particles in the circulation. In addition to these particles, smaller subviral particles composed of envelope proteins but no genetic material are secreted from infected cells in great excess [28]. In a chronic infection, as many as 10 [11] viral particles per day may be released into circulation, with a half-life of approximately 1 day [46].

Serotypic variants of envelope proteins, designated as adr, adw, ayr, and ayw, have long been noted in HBV infection. While these do not appear to strongly associated with differences in clinical disease, they vary considerably by geography and have been used epidemiologically to study transmission and potential vaccine escape mutants [47, 48].

Genotypic variants of HBV have been classified into ten different types (A–J) with >8 % diversity at the nucleotide level, each with a characteristic geographic distribution [44, 49, 50]. Genotypes A–H are considered to be the major ones worldwide and have been the most extensively studied. Subgenotypes (diversity of 4–8 %) have been described for most of the major genotypes. HBV genotypes differ in the frequency with which clinically significant mutations in the precore (PC), basal core promoter (BCP), and pre-S1 regions are found [41, 47, 49]. The clinical significance of different HBV genotype and mutation combinations is an area of ongoing research. This is briefly reviewed below (Sect. 3.19).

HBV remains intact under many environmental conditions, but under most circumstances it can be handled safely at Biosafety Level 2. It is resistant to freezing, ether, and acid. When dried on surfaces at room temperature, it can remain intact for at least 1 month. Boiling for at least 1 min or heating to 60 °C for at least 1 h will inactivate the virus as will standard autoclaving or dry heat methods. Hypochlorite, glutaraldehyde, formaldehyde, alcohol, and some other commonly used disinfectants can inactivate HBV with sufficient exposure time. HBsAg is resistant to UV irradiation [1, 45, 51].

1.4 Descriptive Epidemiology

The World Health Organization (WHO) has described three epidemiologic patterns of HBV infection based on the prevalence of serologic markers (Fig. 32.1). In the lowest-risk regions (North America, Australia, Northern/Western/Central Europe), the prevalence of HBsAg is <1 % in the general population and 4–6 % for anti-HBs. The intermediate-risk regions (Eastern Europe, Central/South America, Mediterranean, Southwest Asia) have HBsAg in up to 7 % and anti-HBs inasmuch as 55 %. In the highest-risk regions (Southeast Asia, China, sub-Saharan Africa), the HBsAg prevalence may be as high as 20 % and anti-HBs positivity is nearly universal, ~95 % [52].

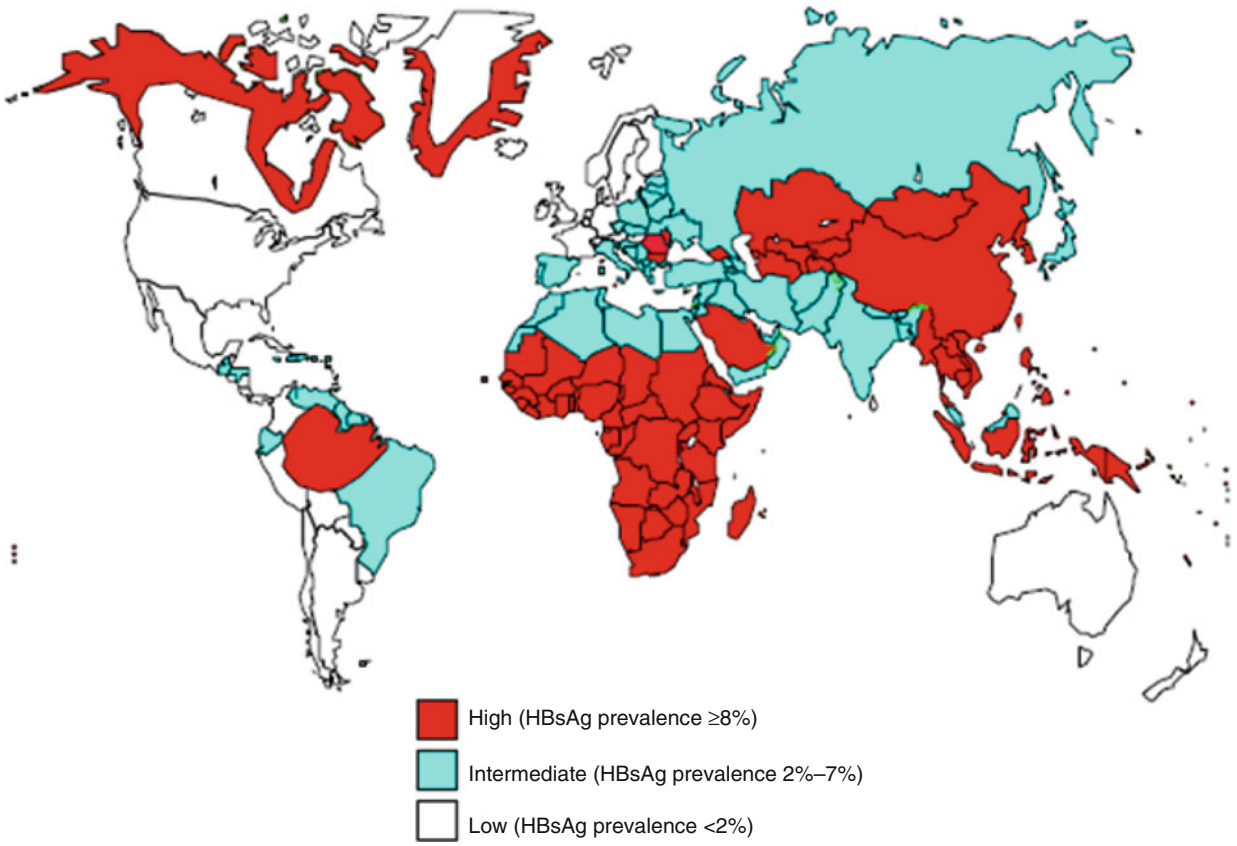

Fig. 32.1 Worldwide areas of high, intermediate, and low prevalence of HBsAg (From WHO [53])

1.4.1 Prevalence and Incidence

The prevalence of chronic HBV infection is the best indicator of the geographic burden of disease (Fig. 32.1). In areas of high prevalence, i.e., ≥8 % HBsAg positive, 70–90%of the population may become infected with HBV prior to 40 years of age. In these areas, infection in the perinatal period or early childhood is common and is responsible for the bulk of chronic infections. Acute hepatitis B is rarely seen since most people are exposed early in life. High rates of LC and HCC in adults in these populations reflect the impact of these early life infections. The high prevalence areas include about 45 % of the world's population [1, 31, 53].

In areas of intermediate prevalence (2–7 % HBsAg positive), acute hepatitis B is more common since age at infection is shifted to older groups. Lifetime risk of infection may be as high as 20–60 %, including both perinatal/early childhood infections and parenteral and sexual exposures later in life. The intermediate areas comprise about 43 % of the world's population.

In the low prevalence regions (<2 % HBsAg positive), most new infections occur in adults through parenteral, sexual, and occupational routes. Lifetime infection risk is <20 %. Immigrants from higher-risk areas of the world may comprise a significant proportion of chronically infected individuals in the population. The low prevalence areas represent about 12 % of the world's population [1, 31, 54].

1.4.2 Epidemic Behavior

Widespread epidemics of hepatitis B infection have not been reported frequently. Small outbreaks may go unrecognized if links between apparently sporadic cases are not identified. In recent years, detected outbreaks in the United States have been most frequently associated with nosocomial exposures in nonhospital settings. Improper infection control procedures in the use of blood glucose monitoring equipment have been involved in multiple outbreaks in long-term care facilities. Hemodialysis and other outpatient medical settings have been implicated as well [55, 56]. Similarly, in the United Kingdom, outbreaks are most frequently associated with nosocomial exposures [57] but have also been documented in groups with shared risk behaviors such as injection drug use and heterosexual intercourse [58]. In the past, nosocomial infections caused by infected health-care workers' contacts with patients have been reported but have decreased in incidence with the widespread use of HBV vaccination and universal precautions against blood-borne infections [54].

1.4.3 Age

Age-specific risks of infection with HBV differ by populations, as described above in *Prevalence and Incidence*. Age at infection is a strong determinant of chronicity in HBV infection. Infants infected perinatally have a risk of developing chronic infection of about 90 %. When infection occurs after the perinatal period through about 5 years of age, the risk of chronic infection is reduced to 25–30 %. In older children and adults, about 5–10 % of infections become chronic [59, 60]. Most chronic infections are asymptomatic for long periods following infection. In acute infections, the development of symptoms is also age dependent, with young children showing symptoms in only 5–15 % of infections but older children and adults in 33–50 % [60, 61].

1.4.4 Sex

HBV infection rates are higher in males in many populations due to two factors. The male predominance in acute infection may be due to higher numbers of males in risk groups for parenteral exposure such as injection drug users. The prevalence of chronic infection is also higher in males in most populations worldwide, even where prevalence of markers of past HBV infection does not differ by gender. This may be due to a predisposition in males to develop chronic infections or to a higher prevalence of other predisposing factors among men, e.g., immunosuppression [52, 61, 62].

1.4.5 Race

Race may be considered a risk factor for HBV infection insofar as it reflects origins in populations of high endemicity, i.e., Asians, Pacific Islanders, Alaska Natives, and Africans.

1.4.6 Occupation

Persons employed in any setting where there is risk of contact with blood or body fluids are at increased risk of HBV infection. Health-care and public safety workers [63, 64], embalmers [65], staff in residential facilities for the developmentally disabled [63], and body piercers and tattoo artists [66] are among those for whom measures to prevent bloodborne infections, including HBV immunization, have been recommended.

1.5 Mechanisms and Routes of Transmission

Humans are the only natural reservoir of HBV infection. Transmission occurs through parenteral routes, percutaneously or permucosally. Body fluids and tissues of infected persons that have been shown to contain HBV and are therefore potential sources of infection include blood and blood products; body secretions that contain blood, semen, vaginal secretions; and cerebrospinal, peritoneal, pericardial, synovial, and amniotic fluids [67]. Saliva of infected persons contains small amounts of virus, and transmission through bites is possible, while other contacts with saliva (e.g., through kissing) have not been shown to transmit infection. Breast milk from infected mothers has not been shown to be a source of HBV infection but caution is recommended if nipples are cracked or bleeding [68, 69]. Natural transmission of HBV by urine, feces, tears, sweat, or respiratory droplets has not been reported [31, 70].

1.5.1 Direct Parenteral Exposure

Without prophylaxis, mother-to-child transmission occurs most often when mothers are HBeAg positive and/or have high circulating viral load during pregnancy. Babies born to these mothers have rates of infection as high as 70 % [71, 72]. In mothers who are HBsAg positive without HBeAg, perinatal transmission risk is lower, about 10 % [31]. Most vertical transmission occurs in the perinatal period, but a small proportion of cases show evidence of intrauterine transmission [73, 74].

Other parenteral exposures contribute to HBV infection as well. In medical settings, transmission from patient to patient has been frequently documented where infection control practices or blood donation screening methods are inadequate [59, 75]. Unsafe injections in medical settings were estimated to be responsible for 21 million new HBV infections worldwide in 2000, nearly one third of all new infections [76]. Injection-associated risk can be attributed to the reuse of needles and other devices used for immunizations, finger-stick devices, and acupuncture needles [61]. Hemodialysis settings are particularly problematic because of the high potential for environmental contamination [77, 78]. Transmission from infected health-care providers has been reported, particularly in the past when universal precautions and immunization were less frequently used. In recent years in the developed world, such transmission has been reported only rarely, and risk appears to be found largely in individuals with high viral loads who performed invasive procedures [79].

Injection drug users are at risk for HBV infection through sharing of injection equipment, particularly in communities where there is prevalent infection and low rates of immunization [31]. Receipt of tattoos in prison or other settings where sterile equipment is not available has also been shown to be associated with HBV transmission, while tattoos performed with sterile equipment are not associated with transmission [80–82].

1.5.2 Inapparent Parenteral Exposure

While HBV infections occur in settings without obvious direct parenteral exposures, it is thought that inoculation

occurs through openings due to skin conditions, injuries, or insect bites rather than through intact skin or mucosal surfaces [59, 83]. The ability of HBV to remain infectious for long periods in the environment contributes to infections that occur through inapparent parenteral means. Horizontal transmission may occur within families, particularly among children due to their high viral loads [60, 70, 84] and from infected mothers to their children even after the perinatal period [54]. Prolonged sharing of living quarters and communal use of personal items such as razors and toothbrushes is associated with infection in households where at least one person is already infected [67, 85]. Risk of infection continues through early childhood in children of infected mothers [54].

Unprotected sexual contact is a common means of transmission of HBV infection, in both heterosexuals and men who have sex with men (MSM). In age-adjusted HBV marker prevalence data among US adults, increasing numbers of lifetime sexual partners, earlier age at first intercourse, and MSM were associated with higher prevalence of HBV infection [86]. Nonimmune sexual partners of HBV-infected persons have a high rate of incident infection [87]. History of other sexually transmitted infections and anal intercourse have also been identified as risk factors.

1.6 Pathogenesis and Immunity

Pathogenesis in HBV infection is primarily but not exclusively confined to the liver. Extrahepatic manifestations such as polyarteritis nodosa, glomerulonephritis, joint inflammation, and skin rashes can occur in both acute and chronic infections [88, 89] as a result of deposition of immune complexes [90]. While there is some evidence that viral replication is possible in cells other than hepatocytes, there is no consistent evidence that this contributes to pathogenesis [29]. Moreover, HBV replication is usually not directly cytotoxic to hepatocytes. During incubation, there is a long period during which viral replication is ongoing, but there are no symptoms, no biochemical evidence of liver inflammation, and no detectable immune response [28, 90]. It is when immunologic response to infection is detected that there is evidence of liver damage. This is consistent with the idea that it is the immunologic response rather than the virus itself that is largely responsible for hepatocellular damage [28, 29]. Possible exceptions to this may be in fulminant hepatitis B where some viral variants associated with this outcome have in vitro characteristics that may be cytotoxic in vivo [91] and in fibrosing cholestatic hepatitis, a rare outcome after liver transplant in HBV-infected patients [92]. Otherwise, the observed sequence of appearance of serologic markers indirectly supports the lack of direct cytopathic effect of HBV infection.

Shortly after exposure, there is high-titer viremia, followed by the detection of HBsAg, usually at 4–10 weeks after infection, and then HBeAg. Humoral immune response is first detected as anti-HBc, several weeks after the appearance of viral antigens, by which time most of the hepatocytes have been infected [28, 29, 92]. Infected individuals with immature or suppressed immune response are more likely to develop immune tolerance to infection and become chronically infected without evidence of significant liver damage [28, 92].

In acute transient infections, specific T lymphocyte responses to many HBV antigens are detectable only after several weeks of infection during which viral particles and antigens are present circulating at very high levels [28, 90]. Once this response is initiated, circulating viral load begins to drop, and infection is rapidly cleared from nearly all hepatocytes [28]. The exact means by which clearance is accomplished is not fully understood but appears to require the rapid replacement of infected hepatocytes killed by CTLs along with releases of cytokines that reduce viral replication in the remaining infected cells without killing them [28, 29, 93]. Circulating HBsAg and HBeAg diminish as well and generally clear by 4–6 months after infection [32]. While transaminase levels may rise to very high levels during this period leading up to clearance, it appears to occur without massive destruction of infected hepatocytes [28, 29]. Even after apparent recovery, patients who have had acute HBV infections may have detectable HBV DNA in circulation for long periods of time [28, 92].

In infections that become chronic, the specific cellular immune responses to viral antigens, especially cytotoxic T lymphocyte responses, are much weaker and narrower than in acute infections [90]. Humoral response, however, is similar to that seen in acute infection except in that anti-HBs is rarely detected [28]. An immunosuppressive role for HBeAg has been postulated as partial explanation for the failure to clear infection [29]. Because chronic infection is a more likely outcome in infections of very young or immunosuppressed individuals, the functional capacity of host immune response clearly has a role as well. Over time, however, chronically infected individuals may exhibit many changes in circulating markers of viral infection, liver damage, and immune response. These are dependent on both host and viral characteristics.

1.7 Patterns of Host Response

HBV infection produces a variety of host responses ranging in severity from self-limited asymptomatic infection to lifetime chronic infection. In acute symptomatic infection, signs and symptoms are nonspecific and similar to other forms of acute hepatitis, including malaise, nausea, vomiting, rash,

joint pains, and abdominal pain. Acute hepatitis may occur with our without jaundice [67].

Chronic infections often do not begin with obvious symptoms, and their manifestations differ between individuals and over the same individual's lifespan. Three defined phases of chronic infection – immune tolerant, immune active, and inactive hepatitis – are defined by the combination of HBeAg/anti-HBe, viral load, and evidence of liver inflammation.

Chronic infections occurring later in life usually begin in the immune-active phase with HBeAg present [41, 94]. Immune tolerant is the initial phase for individuals infected perinatally, and it may persist for decades with positive HBeAg and high viral load but little or no evidence of liver damage [95]. Many of these individuals eventually progress to the immune-active phase in adulthood though the triggering events for this transition are poorly understood [95].

These later acquired infections usually begin in or quickly transition to the immune-active phase with some evidence of immune response to infection. In this phase, viral loads are lower than in the immune-tolerant phase but still readily detectable, and HBeAg is present. Immune-mediated liver damage results in elevations of ALT and histologic evidence of liver inflammation as well as fibrosis in some. The risk of the most serious morbidity and liver-related mortality increases with the duration of the phase. For some, however, there is successful transition to less active disease, indicated by HBeAg to anti-HBe conversion, reduction of viral load, and normalization of liver enzymes. Prior to this transition, there may be a transient hepatitis flare. After HBeAg clearance, most individuals enter the inactive hepatitis phase, characterized by lower viral load, normalization of ALT, and reduction of histologic evidence of liver damage [2, 41].

After seroconversion to the anti-HBe-positive state, a subset of individuals will continue to have moderate to high viral loads and evidence of liver inflammation. This is referred to as anti-HBe-positive active hepatitis and is associated with ongoing hepatocellular damage [96, 97]. Others who convert to anti-HBe positive and enter the inactive phase may experience one or more reversions to HBeAg-positive immune-active phase, during which viral load rises and liver inflammation increases [96, 98], and the risk of cirrhosis is high compared to those who sustain transition to inactive hepatitis [97].

1.7.1 Serologic Patterns of Infection

The incubation period of acute HBV infection is usually between 60 and 90 days from the time of infection to the onset of jaundice or ALT elevation [32, 63]. The first detectable serologic marker is HBV DNA, followed by HBsAg, both of which appear before the onset of symptoms. The length of the incubation period is related to both the amount and route of inoculation as well as host factors [67]. Serologic markers and the patterns associated with different clinical manifestations of infection are summarized in Table 32.1. Table 32.2 summarizes the serologic patterns seen in the different phases of chronic infection [2, 41].

1.7.2 Clinical Outcomes of Infection

In acute, self-limited infections, about 70 % are asymptomatic. Among the remaining 30 %, symptoms range from mild to severe and can persist for some months [32, 39]. Recovery from infection is accompanied by the appearance of anti-HBs, an indicator of immunity to reinfection [63]. Some 1 % of acute infections develop into fulminant hepatitis with massive hepatic necrosis [67]. Risk of fulminant hepatitis is increased by coinfection with other hepatotropic viruses, e.g., HCV or HDV [2].

Chronic infections often begin with no obvious symptoms, especially in infants and young children. Some decades may pass before signs and symptoms of liver inflammation occur as the host transitions into the immune-active phase. Chronic infections do sometimes resolve spontaneously, at a rate of clearance of HBsAg 0.5–1 % per year, usually during the immune-inactive phase [41]. It is estimated that 10–30 % of chronically infected individuals will eventually develop LC or HCC [15, 39, 99]. In addition to disease activity, age, gender, family history, and lifestyle habits (e.g., alcohol consumption) can affect risk [100–102]. Conversion and reversion between HBeAg positive/negative or high-titer HBV DNA positive/negative are also associated with higher risk of HCC [40, 103].

There is considerable evidence for variation in the natural history of disease for different HBV genotypes and subgenotypes, but the full range of comparisons is not possible because of the geographic specificity and rarity of some variants. Moreover, some observed differences in natural history between genotypes/subgenotypes may not be direct effects of genetic variation. For example, a variant that appears to be more likely to cause chronic infection may do so indirectly through prolonging the HBeAg+ period in chronic infection which would in turn lead to higher rates of perinatal transmission and, consequently, younger age of infection, which itself is associated with increased risk of chronic infection. There are also known differences between genotypes and subgenotypes in the prevalence of viral mutations that are independently associated with disease progression [49, 104, 105], and these cannot always be taken into account in natural history studies due to limitations of sample size or laboratory resources.

Despite this caveat, within populations some clear genotype/subgenotype differences in natural history have become apparent. The best studied is the comparison outcomes in genotype B vs. genotype C in Asian populations where both are found. In Taiwan, adults with genotype B chronic infection had higher age-specific prevalence of HBeAg and higher

Table 32.2 Characteristics of the phases of chronic HBV infection

	Immune tolerant	Immune active	Inactive hepatitis
HBeAg	Present	May be present or absent	Absent, conversion to anti-HBe+
Viral load	High (>200,000 IU)	>20,000 IU/ml (HBeAg+ form) >2,000 IU/ml (HBeAg− form)	Low or undetectable (<2,000 IU/ml)
Usual ALT level	Normal	Elevated	Normal
Liver biopsy findings	Normal or minimal inflammation/fibrosis	Inflammation, often with fibrosis	Minimal inflammation, resolving fibrosis
HBsAg	Present throughout	Present throughout	Present but may resolve
Duration	Often many years	Variable	Variable
Risk of cirrhosis/HCC while in phase	Very low	Increases with duration	Low but increases with reversion to immune-active phase

Adapted from [2, 41]

incidence of spontaneous clearance of HBeAg compared to genotype C patients [49, 106]. Infection with genotype C in Taiwan is also associated with higher risk of HCC in HBeAg− individuals [102].

Studies in Alaska have been particularly revealing because several HBV genotypes are represented in Native Alaskan populations, facilitating within-population comparisons. In a large longitudinal study with a median follow-up time of 20 years, Livingston et al. identified subjects with genotypes A, B, C, D, and F at baseline, and age-adjusted HBeAg+ status was associated more strongly with genotypes C, D, and F than with A and B. For those HBeAg+ at baseline, genotype C subjects were much less likely than those with other genotypes to have seroconverted to HBeAg− by the end of follow-up. Genotype C patients also had a significantly older age at HBeAg− seroconversion than all other genotypes (47.8 years vs. 16.1–19.4 years) [107].

Within genotype A, there are three defined subgenotypes, A1–A3. Of these, A1 is the most common genotype in sub-Saharan Africa. In a case–control study in South Africa, Kew et al. [108] found that genotype A was associated with a 4.5-fold risk of HCC compared to all other genotypes. Moreover, all of the genotype A cases who were subgenotyped were subgenotype A1. Genotype A cases also occurred in significantly younger individuals than the cases of other genotypes; this observation is consistent with the known pattern of HCC in this part of Africa, where the risk in younger, HBeAg− men appears to be higher than in other parts of the world [105].

The significance of HBV genetic variation is an evolving area of research. Of particular importance are studies that include both genotyping and viral mutant detection and represent the full range of variability seen in HBV worldwide. The functional significance of many genotype and subgenotype differences has yet to be characterized. There are indications in some clinical studies that viral genetic variants may play a role in treatment response to antiviral therapies, especially interferons [109, 110]. The potential for HBV variants to inform future drug discovery remains to be fully explored. Equally important is further study of the approximately 60 % of chronically infected individuals who never develop serious liver disease [32, 39, 41]. Protective factors are not well understood.

1.8 Control and Prevention

1.8.1 Immunization

The development of the first HBV vaccine was undertaken by Blumberg and Millman in 1969, based upon the observation that some infected persons appeared to develop protective antibodies upon recovery and that antibodies to HBsAg were usually absent in those with apparent chronic infections [111]. Empty HBsAg particles, i.e., particles that do not contain viral DNA, are abundant in the circulation of chronically infected individuals. The first vaccine was produced by the separation and purification of these particles from donor sera. The purified particles were demonstrated to produce a protective immune response in HBV-naïve individuals [13, 111]. A vaccine was formulated and in randomized, placebo-controlled trials was shown to be highly effective in preventing clinical hepatitis or asymptomatic antigenemia [4, 112].

Safe and effective vaccines against HBV infection have been available since 1981. Their use as a routine vaccination has been shown to reduce incidence of acute and chronic infection. In Taiwan, the impact of universal infant HBV immunization, begun in 1984, has been reflected in reductions in HCC incidence in the vaccinated age cohorts [113]. Worldwide, however, many populations at risk for HBV do not have good access to routine immunization. In 2011, 180 countries had included HBV vaccine in routine childhood immunization programs. The average three dose series completion rate in these countries is 75% [114]. Using estimates of prevalence of HBsAg and HBeAg among women of childbearing age, Goldstein et al. [115] modeled the potential impact of different HBV immunization strategies on HBV-related mortality globally and by region in the year 2000 birth cohort. Potential global reduction in HBV-related

mortality was estimated for 90 % vaccine coverage at between 68 and 84 %, depending on whether the vaccine was given at birth.

Early HBV vaccines were derived from the empty HBsAg particles in the blood of infected donors. Current vaccines are made from yeast-derived recombinant subunits of HBsAg and contain no viral DNA. The vaccine does not contain thimerosal. It may be delivered as a single-antigen vaccine or in combination with other antigens. It is given intramuscularly, and three doses are usually the recommendation though these may vary with different vaccine manufacturers and age/risk groups.

When given as directed in healthy persons, the vaccine is both highly immunogenic (stimulates the production of antibodies) and has a high protective efficacy (reduces infection rates in vaccinees). In infants, 80–95 % develop protective antibody titers after two doses, and 98–100 % after three doses. Protective antibody titers are seen in up to 95 % healthy vaccinated children after three doses. In adults, immunogenicity of standard vaccine regimens decline with age, so that by age 60 only 75 % of those receiving three doses have protective titers. For this reason, older adults and individuals of any age with chronic conditions causing immunosuppression (e.g., hemodialysis) may require higher and/or increased numbers of doses [31]. In those who do produce protective antibody, the vaccine is 80–100 % effective in preventing HBV infection or clinical hepatitis. Antibody titers decline with age, but in most cases an anamnestic response is elicited when individuals are exposed to HBsAg, demonstrating that immune memory remains intact [116, 117]. Booster doses are not recommended for most populations [61, 118].

1.8.2 Prevention of Perinatal Transmission

Transmission of HBV infection from mother to child during the perinatal period is a major cause of chronic HBV infections worldwide, particularly in populations with high seroprevalence of HBsAg with HBeAg and/or high viral loads. Several approaches to prevention of perinatal transmission have been proposed in different settings, their use being dependent on a population's resources and rate of perinatal vs. other forms of HBV transmission [53]. When the infection status of the mother is known, the most effective intervention to prevent perinatal infections is the administration of the first dose of hepatitis B vaccine ("birth dose") and one dose of hepatitis B immune globulin (HBIG) within 24 h of birth (preferably within 12 h), followed by completion of the standard 3-dose regimen for Hep B vaccine. This strategy has been shown to be 85–95 % effective in preventing perinatal HBV transmission in infants of infected HBeAg-positive mothers [119–121].

Implementation of this strategy, however, depends on the availability of accurate prenatal HBsAg screening for pregnant women [122] and doses of both HBV vaccine and HBIG in birthing centers. Routine delivery of a birth dose of HBV vaccine is recommended in all infants in the United States, regardless of known maternal HBV infection status, to provide early protection in case of erroneous or unavailable maternal HBsAg status at birth [121]. This is the approach recommended by the WHO as well in countries where perinatal HBV infection risk is high [53]. In regions where many births occur outside of medical facilities, HBV vaccine doses can be made available to community health workers and birth attendants. Vaccine administration as soon as possible after birth is recommended for maximum protection. In these settings it is usually not possible to provide HBIG as well, due to cost of purchase and maintaining appropriate storage conditions [53].

1.8.3 Treatment

There are seven widely approved antiviral therapies for chronic HBV infection, two interferons and five nucleos(t)ide analogs, designed to inhibit activity of the viral DNA polymerase [123]. These treatments are effective in reduction of viral load and/or HBeAg seroconversion in some patients, and expert groups worldwide have produced detailed guidelines for the selection of appropriate patients for their use [124–131]. The relatively short-term endpoints achievable with antiviral therapies, however, do not necessarily translate into long-term reductions of morbidity and mortality from HCC and liver cirrhosis [2]. Antiviral treatment may reduce but does not eliminate the risk of HCC among chronically infected individuals who achieve long-term control of viral replication and improvement of liver histology [132, 133, 134]. Not all treated patients reach these endpoints, however, and those who do often require costly long-term therapy to achieve it, bringing with it risk of antiviral resistance mutations and medication side effects [133]. Many chronically infected individuals who might benefit from antiviral treatment lack access to it because they are unaware of their infection status, are not receiving appropriate medical monitoring, or cannot afford the medications [3, 135]. Moreover, the majority of chronically infected individuals do not fall within the current antiviral treatment guidelines since available medications have not been shown to be effective in some classes of patients, notably those in the immune-tolerant or immune-active phases [136].

1.9 Unresolved Problems

Since the discovery of HBV, great strides have been made in reducing its impact on human health. Nevertheless, hundreds of millions of people worldwide are chronically infected with HBV. Millions more will become infected every year. In addition to immunization, public health mea-

sures for prevention of perinatal HBV transmission and prevention of exposure to blood-borne agents have the potential to reduce or eliminate new HBV infections if implemented appropriately and consistently. For those already chronically infected, current antiviral treatments have the potential to substantially reduce the burden of disease and mortality, but they are not widely available in endemic populations due to cost and medical care access limitations [137]. Even in the developed world, many chronically infected individuals do not know their infection status or do not receive appropriate medical management for it [3]. Cost reduction for antiviral agents and better education of medical professionals on their appropriate use will require new resources and strategies within the already burdened health-care systems.

Continued basic research in HBV virology, immunology, and therapeutics are needed. Despite intensive research, key elements in the natural history of infection such as how the virus gains entry into hepatocytes and how infections are cleared are still unknown [29, 90, 138]. Currently available antivirals are not effective in many groups of chronically infected individuals. Novel approaches and new targets for therapeutics are needed [136, 138, 139]. The staging of liver disease, assessment of treatment response, and early detection of cirrhosis and HCC in the clinical setting would be more accessible and safer for patients with the use of noninvasive methods in the place of liver biopsy, and further work in this area is needed to identify better approaches [139, 140].

Worldwide eradication or elimination of HBV may be an achievable goal [141]. To achieve it, however, will require not only basic science and clinical advances but public health innovation and actions to bring the means of HBV control to all populations.

2 Hepatitis Delta Virus (HDV)

2.1 Historical Background

First reported by Rizzetto et al. [142] in 1977, the hepatitis delta virus (HDV) was initially identified as a novel antibody-antigen system found in the blood and liver of persons infected with HBV and associated with more severe liver damage. The delta antigen was detected in the nuclei of hepatocytes. Transmission studies in chimpanzees demonstrated that the so-called "delta agent" was distinct from HBV but dependent on the presence of HBV infection [143–145]. Further studies characterized the agent as a single-stranded circular RNA virus, defective in that it cannot establish or maintain infection without the copresence of HBV. HDV does not share sequence homology with HBV but is similar to some known plant viroids [146–148].

2.2 Methodology Involved in Epidemiologic Analysis

2.2.1 Morbidity and Mortality Data

It is estimated that about 15 million people worldwide are infected with HDV, mostly in areas of high HBV prevalence or in immigrants from those areas [149]. Population-level data on HDV epidemiology is not widely available since it is not usually part of reportable disease surveillance systems. Exceptions to this are found in areas of higher prevalence of HDV infection, including Italy, Greece, and Australia [150–153]. Even in areas where the disease is reportable, HDV prevalence and disease burden may be underestimated since screening and diagnosis are undertaken only in those known to be infected with HBV. In the United States, some states include HDV in reportable disease statistics but consider these to be underestimates of prevalence or incidence [154]. In the United Kingdom, Cross et al. [155] noted that many laboratories do not routinely test HBV-infected patients for HDV markers. This is likely the case in many populations, and it would lead to underestimation of disease burden [156]. The impact of HDV is also hard to ascertain from vital statistics mortality data since liver disease and HCC deaths are often not attributed to specific viral etiologies in death certificates. Furthermore, in vital statistics data, HBV-related deaths may be undercounted because HCC and LC deaths are often not attributed to specific viral etiologies on death certificates [19].

2.2.2 Surveys

Because of the unique relationship with HBV infections, HDV prevalence surveys worldwide have focused in populations at risk for HBV infection. Geographic areas of HDV endemicity have been identified in the Mediterranean, Middle East, and parts of Asia and Africa [156–158]. Both within and outside of those geographic areas, high prevalence of HDV infection has been reported among injection drug users, people with many sex partners, and other groups at high risk for blood-borne infections in some populations [159–162]. Prevalence of HDV infection worldwide had been declining in some areas where HBV immunization coverage is good, e.g., many parts of Southern and Western Europe [159]. However, the decline of prevalence in Europe observed through the 1990s appears to have halted more recently [156], possibly due to immigration from areas of higher prevalence [158, 163]. Prevalence may be increasing in some parts of the world not originally considered endemic areas for HDV because of immigration from known areas of endemicity [159]. There is some evidence that more effective treatment for HIV infection has indirectly led to either stable or increasing prevalence of HDV infection among these high-risk groups in Europe because of increased survival [160].

2.2.3 Laboratory Diagnosis

Laboratory criteria for diagnosis of HDV infection require the detection of antibody to HDV (anti-HDV), HDV RNA, and/or HDV antigen (HDAg) in blood or liver tissue. Commercial availability of these tests is variable. For epidemiologic studies of the prevalence of HDV exposure, total anti-HDV is the best single marker; however, this approach may underestimate prevalence of recent infections (<30 days) [164] and past infections [157]. Anti-HDV is not an indicator of protective immunity against HDV infection.

Clinically, the distinction between HDV/HBV coinfection (i.e., infections occurring simultaneously) and superinfection (HDV infection occurring in established chronic HBV infection) is considered important. Upon initial infection in either of these, HDV RNA and HDAg are at least transiently present in the liver and blood. Detection of HDAg in blood is not always feasible, however, because of the presence of neutralizing antibodies [165–167]. Unless there is documentation of existing HBV infection, it can be difficult to distinguish co- and superinfections at presentation. Coinfections usually have a longer incubation period – 3–7 weeks from exposure to ALT elevation and onset of symptoms – depending on the size of the inoculum. Superinfection incubation periods are shorter – as little as one week [159, 168].

Coinfection: When HBV/HDV infections occur simultaneously, HBV markers (HBsAg, HBeAg, HBV DNA) are detectable in the blood before any HDV markers appear. HDV RNA and HDAg appear transiently, and HDAg is seen in blood only in about 25 % of patients [165]. A more useful marker in coinfection is IgM anti-HDV (IgM class antibody to HDV), which appears during the acute phase in >90 % of coinfections [167]. Resolution of acute coinfection is accompanied by a rise in IgG anti-HDV (IgG class antibody to HDV). Persistence of IgM anti-HDV beyond the acute phase indicates the development of chronic infection. In resolved coinfection, all HDV markers including IgG anti-HDV may eventually clear. As a result, there is no consistent marker of past infection [157].

Superinfection: When an individual already infected with HBV is superinfected with HDV, HDV RNA, and/or HDAg, detection in serum coincides with a decline in HBsAg, sometimes to undetectable level [166]. This may lead to difficulties in diagnosis in the early phase of disease since HDV testing is often not considered unless the presence of HBV infection is evident. Appearance of HDV DNA is followed by detection of anti-HDV. The persistence of IgM anti-HDV is more frequently found in superinfection rather than in coinfection and is an indicator of chronicity.

2.3 Biological Characteristics

HDV is a defective single-stranded RNA virus that requires HBV to establish infection. Specifically, HDV uses the protein coat of HBV (HBsAg) to encapsulate its RNA genome. HDV cannot survive or replicate without the presence of HBV. With a genome of 1.7 kb, HDV is the smallest known animal virus and the only known animal virus to have a circular RNA genome. Because of these unique qualities, HDV is classified as its own separate genus, *Deltavirus*, of which it is the only known member [165]. Initially, HDV has classified into three major genotypes worldwide, each with both geographic and demographic differences in prevalence [169]. Recent work has further broken down the genotypes into on into eight clades: HDV-1 is found worldwide; HDV-2 (HDV-IIa) is localized to Japan, Taiwan, and Yakutia, Russia; HDV-3 to the Amazon basin; and HDV-4 (HDV-IIb) also to Taiwan and Japan; and HDV-5, HDV-6, HDV-7, and HDV-8 are found in Africa [170]. Coinfections with more than one HDV genotype have been reported [171]. The clinical significance of HDV genotypes and genetic variants is not fully understood. Humans are the only known natural host for HDV infection though experimental infections of chimpanzees and woodchucks infected with HBV or WHV (woodchuck hepatitis virus) are possible [67, 171].

2.4 Descriptive Epidemiology

2.4.1 Prevalence and Incidence

It is estimated that 5 % of those infected with HBV are also infected with HDV, leading to a worldwide prevalence of approximately 15 million individuals. Good epidemiologic data on HDV prevalence are not available for much of the world, and highly affected subpopulations may not be reflected in national-level data. In 2001, the WHO designated the areas of highest prevalence as the Mediterranean Basin, Middle East, Central Asia, West Africa, Amazon Basin, and specific islands of the South Pacific. In contrast, most areas of East Asia have lower prevalences. In North America, Australia, and Western Europe outside of the Mediterranean Basin, prevalence is very low [168]. Even in areas of the world where it is suspected that HDV is endemic, such as sub-Saharan Africa, few or no data on its prevalence or geographic distributions are available [172].

The global picture of HDV endemicity does not necessarily coincide with that of HBV infection [149]. This may be due to assortative contact patterns within populations of HBV-infected persons. For example, HDV is rare among HBV-infected chronic liver disease patients in Hong Kong except in those who are injection drug users, where HDV prevalences of >90 % have been reported [173]. Among HBV-infected persons across the Asia Pacific region, the highest HDV infection rates have been reported among those with behavioral risk factors (e.g., sexual activity, injection drug use) and in some medical settings (e.g., hemodialysis) [174]. Since the discovery of HDV in the late 1970s, some

populations (e.g., in the Amazon basin and Southern Italy) have seen a clear reduction in HDV prevalence because of HBV immunization and measures to prevent transmission of other blood-borne diseases [156]. In other populations where HDV had previously been rare, there have been reports of increasing prevalence and HDV-associated acute hepatitis outbreaks due to migration or increases in injection drug use [149, 161, 175, 176].

2.4.2 Specific Risk Groups

Individuals infected with hepatitis B are at risk for acquiring an HDV superinfection. Those who are susceptible for HBV infection, especially those who are in high-risk groups for hepatitis B infection, are at risk for developing HBV/HDV coinfection.

Ethnicity/Race

Those individuals born in parts of the world where HDV is prevalent are at higher risk for HDV infection. There is no evidence for a biological difference in risk due to race or ethnicity.

Behavioral

Behavioral risk factors for HDV infection are similar to those for other blood-borne infections, i.e., injection drug use, high-risk sexual activities, close contact with persons at high risk, and unsafe tattooing and piercing [161, 173, 175, 177, 178].

Other

HBV-infected individuals who receive unscreened blood or blood products are at increased risk of HDV infection, particularly in higher prevalence areas. Other exposures in medical settings may carry risk [178]. HBV-infected hemodialysis patients in developing countries, for example, may be at high risk for superinfection [179].

2.5 Mechanisms and Routes of Transmission

HDV is transmitted similarly to HBV, through direct contact with blood and infected body fluids. There is a risk of HDV transmission through sexual contact, though it is lower than HBV [159, 171]. HDV is rarely transmitted vertically from mother to infant [168]. Horizontal intrafamilial transmission may occur through inapparent percutaneous or permucosal means but not through casual contact [180].

2.6 Pathogenesis and Immunity

In the clinical setting, HDV infection is associated with more severe liver disease compared to infection with HBV alone. Nevertheless, some infected individuals are asymptomatic and have little to no histologic evidence of liver disease [95, 149, 157, 181]. The pathogenesis of HDV infection is not well understood. Damage to infected hepatocytes is increased with the level of HDV replication, but whether this is a direct cytopathic or indirect immune-mediated effect is not clear [169]. HDV replication requires the presence of HDAg, but HDAg appears not to be cytopathic [182, 183]. As with HBV infection, adaptive immune response in HDV infection is thought to play a role in pathogenesis, but this is not fully understood [159].

2.7 Patterns of Host Response

Initially, HDV infection presents with signs and symptoms of acute hepatitis, whether in co- or superinfection with HBV, with variable severity but generally more severe than what is seen in HBV monoinfection [159]. This initial phase may be more severe than in other viral hepatitides, with 2–20 % leading to fulminant hepatitis [169]. In most (80–95 %) of HBV/HDV coinfections, the course is self-limited and results in clearance of both viruses [159, 169]. Only 2–5 % become chronic. In HBV/HDV superinfections, as many as 20 % may resolve spontaneously [169], but 70–80 % become chronic infections with more rapid progression of liver disease than is seen in HBV monoinfection [149, 159]. Chronic HDV infection leads to cirrhosis in up to 80 % of cases, and it is associated with greater risk of developing chronic liver disease and dying from liver disease than HBV infection alone [168]. The association of HDV infection with the development of HCC is not clear. A review by the International Agency for Research on Cancer (IARC) in 1994 found the evidence for HDV's carcinogenicity inadequate [184]. Nevertheless, several cohort studies since that time have shown a risk of HCC in HBV/HDV infections of 2–6-fold over HBV infection alone [185, 186]. Whether this is a direct carcinogenic effect of HDV or an effect mediated through the higher risk of cirrhosis in HDV infection remains to be seen.

2.8 Control and Prevention

There are no specific measures for prevention of HDV. HBV immunization and general measures to prevent blood-borne infections are the most commonly used and effective means of prevention. Individuals who are already chronically infected with HBV may reduce the risk of HDV superinfection by undergoing antiviral treatment for HBV as well as avoiding behaviors that increase the risk of parenteral infections. In those where HDV/HBV infection is already established, treatment with interferon-alpha has shown some benefit [149].

2.9 Unresolved Problems

Despite many years of study, the epidemiology of HDV infection is not fully understood in some populations due to the lack of population-based data. In addition to viral genetic variability, host characteristics and environmental factors need to be better described. The role of other virus infections such as HCV and HIV may be key to the rapidity of liver disease development in some populations, but has not been widely studied outside of clinic populations. The aims could be accomplished by better availability of surveillance data worldwide.

HDV infection, particularly when it presents as fulminant hepatitis, is a significant clinical challenge. Further studies of natural history and aspects of HDV pathogenesis are needed, with the hope that these may lead to better therapies. Until new therapies or preventive strategies are discovered, more focus is needed on eliminating HBV infection, to continue to decrease the reservoir of susceptibles for future HDV infections. More active campaigns are also needed to educate people on safe practices to prevent HDV infection in those already infected with HBV.

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1 Introduction

Hepatitis C virus (HCV) is a bloodborne human pathogen with a current global prevalence rate of approximately 2–3 %, representing approximately 130–180 million infected individuals [1–5]. Infection with HCV may cause acute hepatitis, but the majority of persons with acute infection are asymptomatic. Persons chronically infected with HCV are at risk of chronic hepatitis and of developing serious hepatic complications such as cirrhosis, hepatocellular carcinoma (HCC), or liver failure. In the developed world, HCV is the leading cause of cirrhosis and primary liver cancers [6, 7] and the major cause of end-stage liver disease leading to liver transplantation [8, 9]. A recent report commissioned by the Institute of Medicine (IOM) of the National Academies estimates that up to 75 % of HCV-infected persons have not been diagnosed [10].

2 Historical Background

The recognition of what was eventually identified as HCV began in the 1970s with the first descriptions of posttransfusion hepatitis not attributable to hepatitis A virus (HAV) or hepatitis B virus (HBV). This disease entity was termed at that time "non-A, non-B" (NANB) hepatitis [8, 11, 12]. Hepatitis C virus was discovered in 1988 and was shown to be the primary etiologic agent of parenterally transmitted NANB hepatitis [8, 11–14]. Leading up to the discovery of HCV, both cohort and case-control studies of persons with NANB hepatitis demonstrated specific risk factors associated with acquiring the disease, including transfusion of blood and blood products, occupational exposure to blood, sex with known infected partners, injection drug use, and sex with multiple partners [15–21]. Together, these studies led to the conclusions that the primary etiological agent of NANB was predominantly both transfusion related and community acquired.

3 Methods for Epidemiologic Analysis

3.1 Sources of Morbidity and Mortality Data

Morbidity and mortality data on HCV infection are acquired from a variety of sources including death certificates, hospital admissions, surveys, research studies, and required reporting of cases. In the USA, HCV is a separate reportable disease, and health-care providers, hospitals, and laboratories are required to send reports of cases of HCV infection to state and local health departments that include them within their jurisdiction.

Among HCV-infected persons, there are a rapidly increasing number of deaths, which in the USA now surpass deaths among HIV-infected persons. US multiple-cause mortality data from 1999 to 2007 of approximately 21.8 million death certificates demonstrated an increase in the mortality rate from HCV infection over that time period [22]. A statistically significant average annual age-adjusted mortality rate increase of 0.18 death per 100,000 persons per year related to HCV was observed ($P = 0.002$). In addition, the relatively young ages at death (45–64 years) of most HCV-infected persons portend a large and ever-increasing health-care burden in the years to come. In the USA alone, estimates forecast a peak in the number of incident cases of end-stage liver disease due to HCV of approximately 38,600 in the year 2030, 3.9 times the predicted annual incidence in 2010 [23].

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The annual number of liver transplants and deaths are forecast to peak about 2–3 years later at approximately 3,200 transplants and 36,100 deaths. Of the approximately 2.9 million pre-cirrhotic patients infected with chronic hepatitis C in 2005, it is estimated that without treatment, 24,900 or 0.9 % will have died from hepatitis C by 2010; 379,600 or 13.1 % by 2030; and 1,071,229 or 36.8 % by 2060. Globally, estimates indicate that up to four million persons are newly infected each year, 170 million people are chronically infected and at risk of developing cirrhosis and liver cancer, and 350,000 deaths occur annually due to all HCV-related causes [24, 25].

3.2 Surveys

The National Health and Nutrition Examination Survey (NHANES) refers to a series of assessments that have periodically collected data on the health and nutritional status of a large sample of adults and children in the USA. The information includes HCV prevalence, based on interview, physical examination, and laboratory testing, allowing clinicians to target at-risk groups with educational services and therapeutic options. The latest NHANES survey from 1999 to 2002 included 15,079 total participants and is the largest epidemiological study ever conducted on HCV prevalence in the USA. According to NHANES, 1.6 % or 4.1 million persons in the USA, most of whom were born between 1945 and 1964 were anti-HCV seropositive [3]. The survey, however, sampled from a noninstitutionalized civilian population and did not include certain high-risk persons, namely, the incarcerated, homeless, nursing home residents, hospitalized patients, those in active military service, and immigrants. The survey also missed some groups with an expected high prevalence of HCV infection (e.g., health-care workers and persons on long-term hemodialysis) because of their underrepresentation in the sample studied. As a result, data from NHANES may have underestimated the true prevalence of HCV in the USA by at least one million infected persons [26, 27].

Globally, the prevalence of HCV varies geographically. Recent developments in modeling allow the seroprevalence of anti-HCV to be used to estimate the global burden of disease for HCV infections. Specifically, an international collaborative, The Global Burden of Diseases, Injuries, and Risk Factors 2010 (GBD2010) Study, is an effort to estimate the global burden of HCV infection. The GBD Study defined 21 regions such that detailed data in one country can plausibly be extrapolated to other countries in the region in order to create burden estimates [25, 28]. Based on a systematic review and meta-analysis of primary national data sources and articles published for peer review between 1980 and 2007, the following global estimates of HCV have been made: Central and East Asia and North Africa/Middle East are estimated to have a high prevalence of HCV infection(>3.5 %), and South and Southeast Asia; sub-Saharan Africa; Andean, Central, and Southern Latin America; Caribbean; Oceania; Australasia; and Central, Eastern, and Western Europe have moderate prevalence (1.5–3.5 %), whereas Asia Pacific, Tropical Latin America, and North America have low prevalence (<1.5 %).

3.3 Laboratory Diagnostics

There are two different types of assays that are used in the diagnosis and evaluation of HCV infection— serologic and molecular assays. Serologic assays detect antibody to HCV (anti-HCV) and can be used to screen for and diagnose prior or current HCV infection. Serological assays include widely used enzyme immunoassays (EIAs) with specificities greater than 99 % and therefore remain the best screening tests for the diagnosis of hepatitis C. False-negative anti-HCV testing may occur, albeit rarely, in the setting of severe immunosuppression such as HIV infection, solid organ transplantation, agammaglobulinemia, or in patients on hemodialysis [29–32]. False-positive anti-HCV with EIA tests may also occur, particularly in populations with a low prevalence of HCV infection. Signal to cut-off ratios (>4.0 associated with true HCV exposure or infection) with EIAs can be used to help guide whether further confirmatory testing with recombinant immunoblot assay (RIBA) can be helpful. The RIBA test assesses the serological reaction of a patient to multiple HCV antigens and is a confirmatory test to the anti-HCV EIA test. A positive RIBA in conjunction with a positive anti-HCV antibody test indicates a true past or present infection with HCV. A negative RIBA in conjunction with a positive anti-HCV antibody test indicates a false-positive anti-HCV antibody test, and the patient can be reassured without further testing [33]. In persons with positive HCV screening tests, molecular assays to detect viral nucleic acid, and therefore active viral infection, are recommended and can be either qualitative or quantitative. Real-time polymerase chain reaction (PCR)-based assays and transcription-mediated amplification (TMA) assays have largely replaced qualitative molecular assays and are widely used for monitoring during response-guided therapy. These assays have sensitivities of 10–50 IU/mL and have excellent specificity (98–99 %). Serologic and molecular assays do not assess disease severity or prognosis. Additionally, the most current consensus proposal distinguishes six genotypes based on phylogenetic cluster analysis of complete genomes. Genotype testing is performed in the evaluation of HCV infection in order to maximize the chance of successful treatment outcome for each individual patient as treatment type, duration, dose, and effectiveness are influenced by genotype. While phylogenetic analysis with

direct sequencing of an HCV genomic region is considered the gold standard for identifying different HCV genotypes, this method is expensive and time consuming. For this reason, commercial genotyping kits were developed for routine determination of HCV genotypes.

4 Biological Characteristics

A member of the family Flaviviridae, HCV is classified within a separate genus, Hepacivirus, due to differences from other members of the family in the organization of the structural proteins that make up the amino terminal third of its polyprotein. The virus has an enveloped, positive-sense, single-strand RNA genome of approximately 9.6 kb in length with extraordinary genetic diversity [34]. The RNA contains a single large open reading frame that encodes for a 327 kD polyprotein of approximately 3,000 amino acids that is flanked by nontranslated segments [35]. The polyprotein is processed by host cell peptidases and viral proteases that cleave it into structural and nonstructural proteins needed for viral replication [36]. The 5′-end includes structural proteins (core, E1, and E2) that encode nucleocapsid and envelope proteins, and the 3′-end includes nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) required for replication (see Fig. 33.1).

The nonstructural proteins have distinct enzymatic functions including production of proteases (NS3, NS4A), helicases (NS3), and polymerases (NS5B). The HCV genome contains both highly conserved and highly variable regions. Six major genotypes of HCV have been described, and there are subtypes within each genotype. The genotypes are differentiated by sequences in the relatively conserved core, E1 and NS5B regions [36]. Pairwise differences in the nucleotide sequences of the six HCV genotypes are on the order of 31–33 % and the geographic distribution of specific genotypes varies [34]. In the USA, genotype 1 is the most common (75 %). In Europe, genotypes 1 and 3 are most common, while in Egypt genotype 4 predominates. Additional variants, known as quasispecies, are present in infected individuals and are a result of the high error rate of the viral RNA polymerase during replication.

5 Descriptive Epidemiology

5.1 Morbidity and Mortality

Global morbidity and mortality from HCV infection are a tremendous public health burden. Available estimates indicate that worldwide, there were 54,000 deaths and 955,000 disability adjusted life years associated with HCV infection [25]. The major burden from HCV infection comes from sequelae from chronic infection. Estimates indicate that three to four million persons are newly infected each year, 170 million people are chronically infected and at risk of developing liver disease including cirrhosis and liver cancer, and 350,000 deaths occur each year due to all HCV-related causes [24, 25].

5.2 Prevalence and Incidence

The available data suggest that the global prevalence of HCV infection has increased from 2.3 % (95 % uncertainty interval [UI]: 2.1–2.5 %) to 2.8 % (95 % UI: 2.6–3.1 %) and >122 million to >185 million between 1990 and 2005 [4, 24]. These estimates are based on systematic reviews of published prevalence data, including volunteer blood donor studies, and therefore they may underestimate the true population prevalence [37]. Although HCV infection is endemic worldwide, there is a large degree of geographic variability in its distribution (see Fig. 33.2).

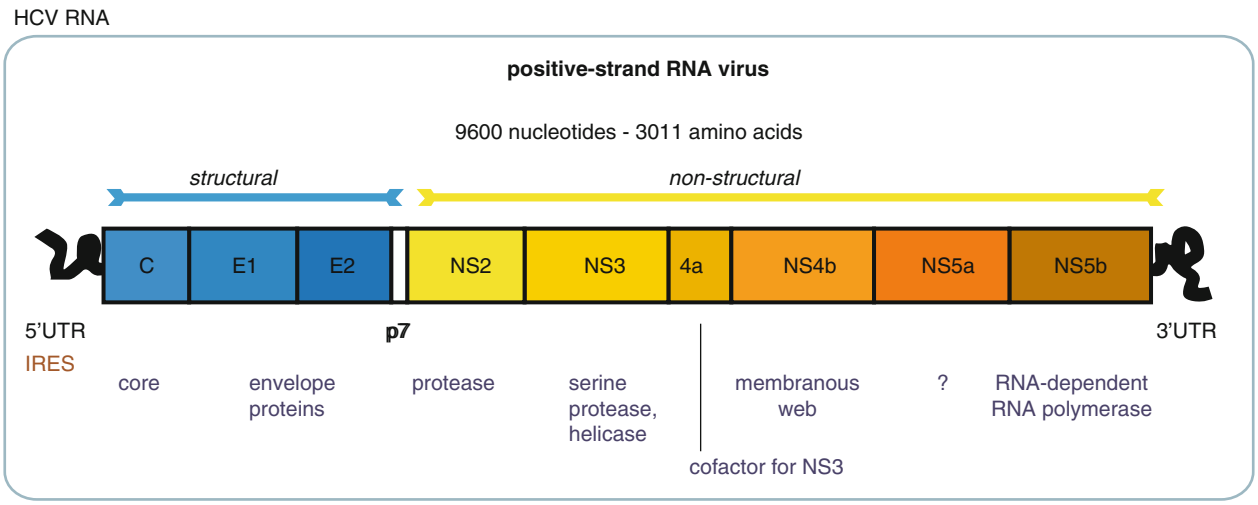

Fig. 33.1 HCV RNA (Data from Heim [262])

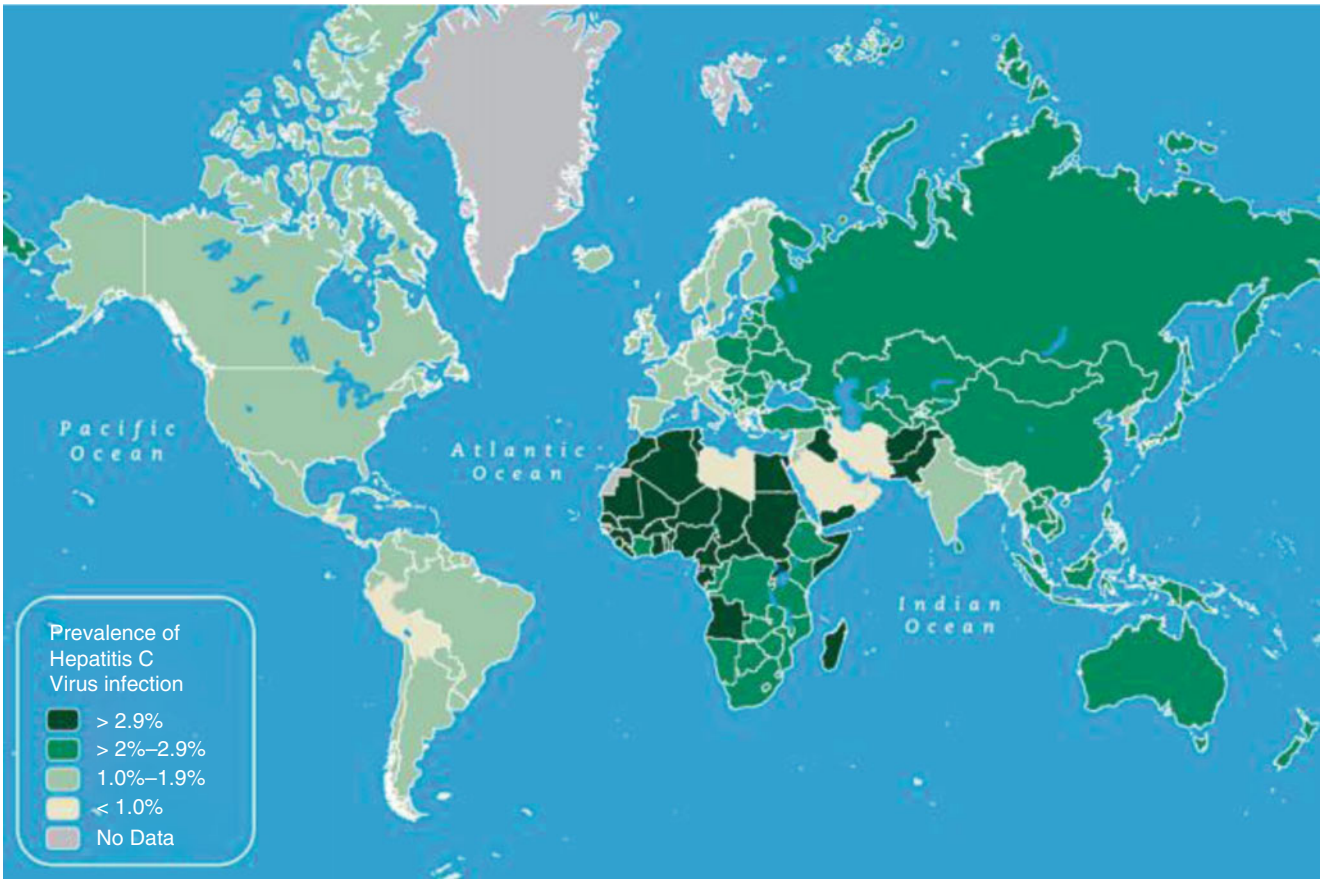

Fig. 33.2 Geographic prevalence of hepatitis C virus infection (Data from the CDC accessed online September 19, 2012 from http://www.cdc.gov/immigrantrefugeehealth/guidelines/domestic/viral-hepatitis-figure5.html)

The prevalence of anti-HCV antibody is quite variable throughout the general population, with the highest rates among persons with repeated percutaneous exposures through injection drug use. Additional risk groups include hemophiliacs, patients on hemodialysis, patients transfused with unscreened blood and blood products, inmates of long-term correctional facilities, and persons with occupational exposure [38–45]. Data regarding the incidence of HCV infection are difficult to obtain, since most acute infections (60–70 %) are asymptomatic and there is no widely available test to distinguish acute from chronic infection [46, 47]. Surveillance data from the CDC demonstrate a decrease in the annual number of incident cases (see Fig. 33.3). These data are derived by adjusting rates from the Sentinel Counties Study of Viral Hepatitis (1982–2006) and the Emerging Infection Program (2007) for underreporting and asymptomatic infection.

5.3 Epidemic Behavior

Outbreaks of hepatitis C are limited in scope because of the predominantly bloodborne route of transmission. Historically, most early outbreaks of HCV infection were reported in the setting of hemodialysis and blood or plasma donation [48–52]. However, many outbreaks have occurred in other health-care settings as a result of poor infection control practices; failure to use aseptic technique during preparation or delivery of therapeutic injections has commonly led to cross-contamination from reused needles and syringes, multidose saline vials, infusion bags, heparin solutions, and pain treatments [53–64].

Additionally, outbreaks of acute HCV infection have been reported in IDUs and HIV-infected MSM [65, 66].

5.4 Geographic Distribution

Data from the WHO and CDC concur in their indication that the prevalence of HCV infection varies in different geographic regions [67]. Data from GBD2010 estimates higher prevalences of HCV in East and Central Asia (3.7–3.8 %), Eastern Europe (2.9 %), North African (3.6 %), and central and west sub-Saharan Africa (2.3–2.8 %), while prevalence is lower in North America (1.3 %) and Latin America (1.2–2.0 %) [5, 68]. Additionally, there are variances with respect to age and peak prevalence [24]. The highest prevalence of HCV is

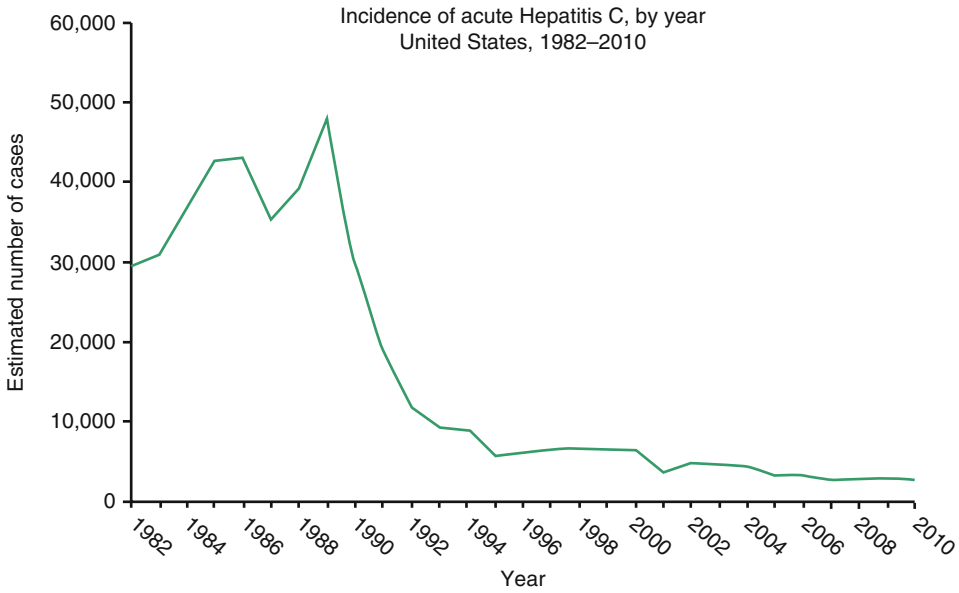

Fig. 33.3 Decrease in the number of acute HCV cases, by year in the USA (Data from the CDC. Accessed online September 19, 2012 from http://www.cdc.gov/hepatitis/Statistics/index.htm)

15 % in Egypt, while the lowest reported prevalences are <1.0 % in the UK and Scandinavia [24, 67].

5.5 Temporal

Countries with similar overall prevalence of HCV infection have different age-specific prevalence patterns; three main patterns have been described [67]. The first pattern, found in the USA and Australia, is characterized by a low age-specific prevalence among persons less than 20 years and greater than 50 years with the majority of HCV infection occurring in persons 30–49 years old. This pattern suggests that most HCV transmission occurred in the last 20–40 years and primarily in young adults [3, 69, 70]. The second pattern, found in a number of countries including Turkey, Spain, Italy, Japan, and China, is characterized by low age-specific prevalence in children and young adults with the majority of infections occurring in persons over 50 years old [71–74]. This pattern suggests a cohort effect where most HCV transmission occurred 30–50 years ago. The third pattern, found in Egypt, is characterized by high prevalence of infection among persons in all age groups with HCV infection increasing steadily with age [75, 76]. This pattern suggests increased risk in the distant past followed by ongoing high risk of transmission.

5.6 Age

Acute hepatitis C infection may occur in all age groups but appears to occur mostly among young adults. According to US data from NHANES, prevalence of anti-HCV increased with age from 1.0 % in those 20–29 years of age to a peak of 4.3 % in those 40–49 years of age [3]. Two-thirds of HCV-positive cases in this survey were born between 1945 and 1964. Among young injection drug users, the annual incidence of HCV infection ranges from 10 to 36 % [77–81]. The overall age distribution of disease is likely related to patterns of exposure (injection drug use in young and middle-aged adults; transfusion in older adults) and possibly to age-specific variation in clinical expression of disease.

This is in contrast to the age-specific prevalences of HCV infection which increase steadily with age in many countries as outlined above [72–74, 82–84]. This suggests a cohort effect in which the risk for HCV infection was higher in the past. Where the emergence of HCV infection occurred in the distant past, the burden of HCV-related chronic disease might already have reached its highest magnitude. However, changes in disease transmission patterns that result in younger persons acquiring infection could lead to future increases in chronic disease as this cohort ages. In Egypt, where there has been an ongoing high risk for decades, the high magnitude of the current burden of HCV-related chronic disease is predicted to continue [85].

5.7 Sex

Data from the USA demonstrates that HCV infection is more common in men (2.1 %) than women (1.1 %). It is not well understood why this difference exists, although differences by gender in risk factors such as injection drug use likely contributes [3]. Males with HCV infection are more likely (2.5 times) to develop cirrhosis and hepatocellular carcinoma when compared with females, suggesting that estrogen may have protective effects against fibrosis progression [86–88].

5.8 Race

Hepatitis C occurs worldwide in all racial/ethnic groups studied. In the USA, data from the most recent NHANES demonstrated a higher overall prevalence among non-Hispanic black persons (3.0 %) compared with non-Hispanic white persons (1.5 %) and was almost entirely attributable to differences among older participants [3]. According to Armstrong and colleagues, this finding suggests that younger non-Hispanic black persons may not be subject to the disproportionately high burden of disease that was seen in the previous generation. However, the authors do note that the small number of younger anti-HCV-positive participants limits definitive conclusions [3]. Although sparse data make comparisons difficult, observed racial differences in prevalence or mortality rates are likely due to the differences in exposures and risk factors in different locations.

5.9 Occupation

The route of acquisition of HCV is similar to that of hepatitis B virus (HBV) in the developed world, with exposure to blood playing the principal but not exclusive role in transmission. HCV is not transmitted as efficiently as HBV through occupational exposures to blood and is largely confined to health-care workers who have sustained contaminated needlestick injuries. The average incidence of anti-HCV seroconversion after accidental percutaneous exposure from an HCV-positive source is 1.8 % (range: 0–7 %) [89–91]. Transmission has been associated with hollow-bore needles, deep injuries, HIV coinfection, and a high titer of HCV in the source patient's blood [89–93]. Study of dentists has demonstrated an increased risk of HCV infection compared to controls (1.75 % vs. 0.14 % OR 12.9, 95 % CI 1.7–573), particularly among oral surgeons, as has also been shown for HBV [94].

6 Mechanisms and Routes of Transmission

HCV entry into hepatocytes is assumed to be a multistep process that requires sequential interactions between cellular factors and viral proteins. Replication depends on viral and host proteins and occurs in association with intracellular membranes [95]. Historically, HCV research has been hampered by a lack of adequate in vitro and in vivo model systems. In vivo study of HCV infection is restricted to humans and chimpanzees due to a lack of small animal models. Another major limitation in the study of the HCV life cycle is the inability to culture wild-type strains of HCV efficiently in cell culture. Much has been learned regarding RNA replication from replicons, each of which contains an adapted HCV genome encoding a selectable marker [96]. However, replicons do not reproduce other aspects of the life cycle such as virion production and infection [96, 97]. Two additional advances came in 2003 and 2005 with the development of retroviral pseudoparticles bearing unmodified HCV glycoproteins (HCV gp) and the discovery that a genotype 2a isolate (JFH1) from a patient with acute fulminant hepatitis could undergo the complete virus life cycle in cell culture, respectively [98].

Direct percutaneous exposure to blood is the most efficient mode of HCV transmission. Higher rates of transmission occur with large and repeated percutaneous exposures such as injection drug use and with transfusions or transplantation from infectious donors (see Fig. 33.4) [99]. Lower rates of transmission occur with single, small dose percutaneous exposures such as accidental needlesticks [91, 99]or by mucosal exposures to blood- or serum-derived fluids (e.g., birth to an infected mother, sex with an infected partner) [99–101].

6.1 Iatrogenic Exposure

Transfusion-associated HCV infection was a major worldwide risk before HCV testing became available in 1989. Prior to screening of the blood supply, transmission of HCV occurred in 5–18 % of people receiving a transfusion [102–104]. In some groups who were transfused large amounts of blood or pooled blood-derived products prior to screening, such as hemophiliacs, prevalence of HCV infection ranged from 59 to 99 % [105, 106]. The risk of acquiring HCV via blood transfusion has been virtually eliminated in countries that have implemented routine HCV testing of donors (less than one in a million per unit transfused) [107]. However, some countries have not prioritized blood transfusion safety and/or lack the resources to implement donor screening, and in these countries blood transfusion remains an important source of infection [108].

Worldwide, there is a substantial preventable burden of HCV due to iatrogenic transmission. There is a high prevalence of transfusions, reuse of needles and syringes, needlestick injuries among health-care workers, and unnecessary medication injections [109, 110]. It has been estimated that contaminated health-care injections cause approximately two million of the HCV infections acquired annually and may account for up to 40 % of all HCV infections worldwide [111]. In addition to unsafe injection practices, in some countries, poor or nonexistent infection control in health and dental care facilities may be a source of HCV transmission [112]. In Egypt, where the prevalence of HCV is the highest in the world, the reuse of glass syringes during the parenteral therapy campaigns to control endemic schistosomiasis is a widely suspected to be the source of iatrogenic transmission [113]. However, there may have been considerable other concurrent iatrogenic exposure at the time [114]. More recent evidence

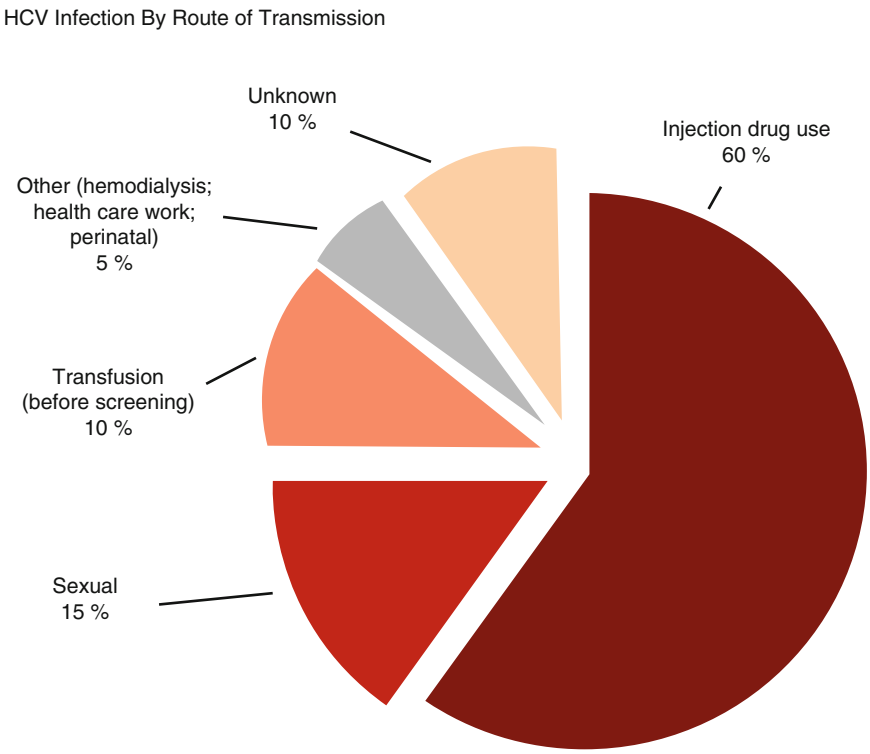

Fig. 33.4 HCV infection by route of transmission (Data from the CDC. Accessed online September 19, 2012 from: http://commons.wikimedia.org/wiki/File:Hepatitis_C_infection_by_source_(CDC)_-_en.svg)

in Egypt supports a continuation of iatrogenic exposures including unsafe medical and dental practices that is contributing to ongoing HCV transmission [112, 115].

Iatrogenic transmission of HCV is not limited to resource poor countries. Outbreaks of HCV transmission have occurred in resource-rich countries as well [55, 56, 59, 61–63]. Most of these outbreaks were reported in the setting of hemodialysis and poor infection control leading to cross-contamination from reused needles and syringes, multidose saline vials, infusion bags, heparin solutions, and pain treatments.

6.2 Injection Drug Use

Injection drug users (IDUs) have the highest overall prevalence of HCV infection compared with any other risk group. Worldwide, it is estimated that 16 million people injected drugs in 2007 [116]. Data from one systematic review suggests that ten million IDUs globally are HCV seropositive [117]. Anti-HCV prevalence is noted to be as high as 60–80 % in IDUs. Injection drug use has been the leading mode of transmission during the past four decades in the USA and Australia and now accounts for most newly acquired infections in many countries in Europe. Eastern Europe, East Asia, and Southeast Asia have the largest populations of IDUs infected with both HBV and HCV.

The WHO recommends that IDUs be a key target group for prevention and treatment of HCV (WHO Resolution A63/15 2011). Indirect drug sharing and preparation practices including back loading and using cotton (filters), cookers (drug solution containers, such as spoons), and rinse water have all been associated with HCV transmission [81, 118–120].

6.3 Sexual Transmission

Sexual practices and exposures predictive of anti-HCV positivity include the number of recent and lifetime partners, high-risk sexual practices, other STDs (HSV-2 and Trichomonas), and HIV infection particularly in MSM [71, 121–128]. The magnitude of risk for transmission of HCV infection by sexual activity has been controversial. Sexual transmission of virus occurs when infected body secretions or infected blood are exchanged across mucosal surfaces [99]. The presence of virus in body secretions or blood is necessary but may not be sufficient for transmission to occur [101]. Other factors that may influence transmission include the titer of virus in body fluids, the integrity of the mucosal surfaces, and the presence of other genital infections. Studies to detect HCV RNA in semen, vaginal secretions, cervical smears, and saliva have yielded mixed results [121, 122, 125, 129, 130]. Studies have demonstrated that when HCV RNA is detected in these secretions, it is present in lower concentrations than in blood. Low levels of virus in genital secretions may be one reason that HCV is sexually transmitted less efficiently than HBV or HIV. Also, the absence of appropriate

target cells in the intact genital tract may require the presence of abnormal mucosa for transmission. A review of the literature demonstrated that the risk for sexual acquisition of HCV appeared to be related in large part to HIV coinfection [131]. There have been at least two cross-sectional studies demonstrating increased risk of acquiring HCV infection among heterosexual HIV-infected persons [132, 133].

The clearest and least equivocal sexual risk behavior that leads to HCV transmission is unprotected sex among HIV-positive men who have sex with men (MSM) [131]. The risk of HCV transmission by sexual activity differs by the type of sexual relationship. Persons in long-term monogamous partnerships are at lower risk of HCV acquisition (up to 0.6 % per year) than persons with multiple partners or those at risk for sexually transmitted infections (up to 1.8 % per year) [101]. Data from the CDC Acute Hepatitis Sentinel County Surveillance program demonstrated that 18 % of individuals with acute HCV infection reported no other risk factor except sexual contact with an anti-HCV-positive person in the preceding 6-month period or multiple sexual partners.

HIV coinfection appears to increase the rate of HCV transmission by sexual contact although the precise mechanism is unknown. Cross-sectional studies from the USA have demonstrated a two- to fourfold higher risk of heterosexual HCV infection in HIV-infected than in HIV-uninfected subjects [132, 134]. Epidemics of sexually transmitted HCV infection among HIV-infected MSM have emerged in Northern Europe, the USA, and Australia in the last decade [65, 128, 135–138]. Risk factors for transmission in HIV-infected MSM include multiple partners, fisting, use of sex toys, and presence of genital ulcerative disease [139].

6.4 Mother-to-Child Transmission

Prior to the testing of blood for HCV in 1992, blood transfusion was the predominant mode of acquisition for HCV infection in children. Since then, mother-to-child transmission has become the leading source of HCV infection in children where blood screening is routine. The rate of perinatal transmission of HCV is 4–7 % per pregnancy and requires detectable HCV RNA in maternal serum at delivery [67]. The exact timing of transmission of HCV from the mother to the child is unknown. Both in utero and intrapartum infections are possible. The outcome of perinatal transmission of HCV in twin pregnancies is often discordant, with transmission of HCV more likely to affect the second twin and with neonatal HCV RNA undetectable at delivery; these observations suggest that intrapartum transmission may be more frequent than in utero transmission [140]. Risk factors for vertical transmission include higher maternal serum HCV RNA levels (above ten [6] copies per mL), prolonged labor after membrane rupture, internal fetal monitoring, and coinfection with HIV [100, 141–150]. There is no apparent increased risk of vertical transmission of HCV with the mode of delivery except in women who are also infected with human immunodeficiency virus (HIV). The increased risk of perinatal HCV transmission in mothers coinfected with HIV may be related to higher HCV viral loads in coinfected persons as a result of HIV-mediated immunosuppression [145]. Other factors may increase perinatal transmission in HIV-coinfected mothers. One such factor is facilitation by HIV infection of HCV entry into blood cells with subsequent replication; another is concomitant use of injection drugs by coinfected mothers [151–153]. The reason for increased risk of vertical transmission in coinfected mothers who are IDUs is not known. It has been postulated that greater maternal peripheral blood mononuclear cell (PBMC) infection confers an increased risk for vertical transmission. Internal fetal monitoring and prolonged rupture of membranes should be avoided if possible in all HCV-infected pregnant women. Patients should be advised that breastfeeding is permissible since there is no evidence that breastfeeding increases risk of HCV transmission.

6.5 Hepatitis C Virus Transmission to Health-Care Workers

HCV is not transmitted efficiently through occupational exposures to blood. Nosocomial transmission is largely confined to health-care workers who have sustained contaminated needlestick injuries. The average incidence of anti-HCV seroconversion after accidental percutaneous exposure from an HCV-positive source is 1.8 % (range: 0–7 %) [89–91]. This risk is intermediate between that resulting from similar exposures to HIV (~0.3 %) and in a susceptible person from exposure to HBV (6–30 %) [154]. Transmission has been associated with hollow-bore needles, deep injuries, HIV coinfection, and a high level of HCV viremia in the source patient [92, 93]. Transmission rarely occurs from mucous membrane or non-intact skin exposures to blood, and no transmission to health-care workers has been documented from intact skin exposures to blood [155]. The prevalence of HCV infection among health-care workers is no greater than in the general population, averaging 1–2 % [67]. Even more rarely, HCV-infected health-care workers have transmitted HCV to patients through needlestick injuries or other percutaneous exposure, with an extremely low risk—averaging about 0.5 %, even for those episodes involving surgeons.

6.6 Hepatitis C Virus Transmission in Other Settings

Percutaneous exposure to blood can occur in a variety of other practices, during which transmission of HCV may occur. These exposures include but are not limited to tattooing, body

piercing, intranasal drug use, ritual scarification, circumcision, acupuncture, and cupping. There are currently insufficient data to determine the extent to which these risks factors may contribute to overall HCV transmission.

7 Pathogenesis and Immunity

The majority of people with acute HCV infection progress to chronic infection (85 %) [156–159]. The key features of acute and chronic hepatitis C virus infection are reviewed in this section.

7.1 Acute Infection

The natural history of HCV infection generally begins with a subclinical and unrecognized acute phase following infection. Symptomatic acute hepatitis occurs within 2 weeks to 6 months of infection in only a minority of cases (10–15 %) and is clinically indistinguishable from acute hepatitis of other causes—with a combination of fever, fatigue, loss of appetite, nausea and vomiting, dark urine, jaundice, and liver tenderness, accompanied by elevated levels of serum bilirubin and alanine and aspartate aminotransferases (ALT and AST, respectively) [160]. The majority of cases of HCV infection progress to chronic infection, but in up to 15 % of people, infection may resolve spontaneously. Evidence suggests that patients with symptoms in the setting of acute HCV infection tend to have a higher rate of spontaneous clearance [161–163]. Other factors that might contribute to spontaneous clearance include infection in infants and young women, selected host polymorphisms near the gene encoding IL28b, infection with HCV genotype 3, having a low peak viral load, Caucasian race, and rapid decline in viral load within the first four weeks of diagnosis [161–166]. Persons with alcohol consumption and HIV coinfection are less likely to spontaneously clear acute HCV infection [159, 163, 167].

Early detection of progression to chronic HCV infection can be difficult, as it almost always occurs in the absence of symptoms. The differentiation of acute from chronic HCV infection depends largely on the clinical presentation including presence of symptoms, recognition of a time-limited exposure, and documentation of ALT elevation and its duration. After exposure, HCV RNA is usually detectable before antibody appears in serum. HCV RNA can be identified as early as 7–21 days following exposure, whereas anti-HCV is generally not detectable before 8–12 weeks [30, 156, 168, 169]. When symptoms of acute HCV infection occur, they may include malaise, fatigue, myalgias, nausea, and right upper quadrant pain. Fulminant hepatic failure is a rare presentation of acute HCV infection unless other underlying chronic liver disease is present [170, 171].

7.2 Chronic

Individuals with chronic hepatitis C (CHC) are at increased risk of liver-related morbidity and mortality. The majority of persons with CHC are asymptomatic. The most common symptom of CHC infection is fatigue [172, 173]. Other symptoms may include myalgias, nausea, anorexia, arthralgias, and difficulty concentrating. Progressive hepatic fibrosis leading to cirrhosis is the major complication of chronic HCV infection and accounts for almost all HCV-related morbidity and mortality [9]. In patients who develop cirrhosis, symptoms include worsening fatigue and anorexia, fluid overload, difficulty concentrating or confusion due to hepatic encephalopathy, and gastrointestinal bleeding. Physical examination findings can include signs of chronic or end-stage liver disease including palmar erythema, spider angiomas, ascites, and asterixis. Laboratory changes include abnormal liver tests including transaminase elevations.

8 Patterns of Host Response

Persons with CHC are at risk for progression to cirrhosis, liver failure, and/or hepatocellular carcinoma (HCC). However, the disease course in persons with CHC is generally protracted and variable (see Fig. 33.5) [174]. The risk of developing cirrhosis is 5–25 % over 25–30 years [175, 176]. There are a number of host, viral, and environmental factors that are associated with disease progression.

Host factors associated with more rapid fibrosis progression include older age (above 40 years old), longer duration of infection, male gender, obesity, hepatic steatosis, insulin resistance, and hepatic iron overload [67, 86, 176–186]. Age appears to be the predominant risk factor for more accelerated fibrosis progression [86, 177]. Data from Poynard and colleagues indicate that median time to cirrhosis was 44 years in patients infected at under 20 years of age, 30 years in patients infected between 31 and 40 years of age, and 12 years in patients infected over the age of 50 years. The concept that the aging liver is more susceptible to fibrosis is also supported by experience with liver transplantation, where older donor age is a strong risk factor for rapid fibrosis in the graft [187, 188]. A number of mechanisms have been proposed to explain this: increased vulnerability to oxidative stress, a reduction in hepatic blood flow, and mitochondrial capacity [189–191]. Additionally, there are genetic factors that influence the natural history of HCV and treatment response. Both the innate and adaptive immune responses are important for clearance of an acute HCV infection. After an HCV infection, the innate immune response is initially important for controlling viral replication with the adaptive immune response peaking at 8 weeks after infection [192]. Ultimately, a coordinated effort between the innate and adaptive immune

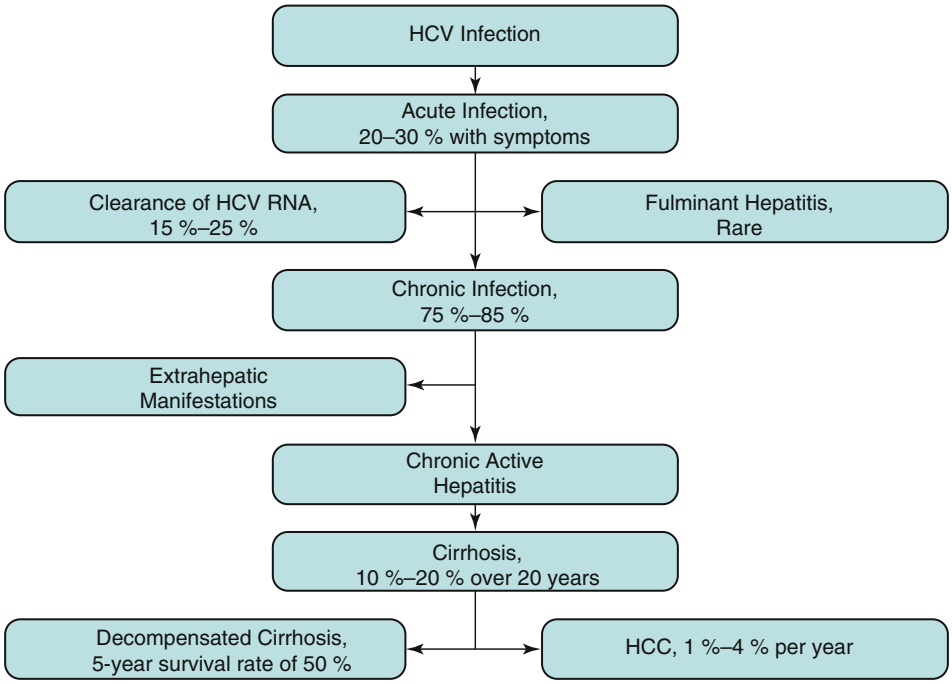

Fig. 33.5 Natural history of HCV infection (Reprinted with permission from International Journal of Medical Sciences. Chen and Morgan [174]. Available from http://www.medsci.org/v03p0047.htm)

responses is necessary to eliminate HCV from the liver [192]. Genetic variation of the host immune system may affect fibrosis progression rates by way genetic factors that may influence activators of hepatic stellate cells, the family of proteolytic enzymes known as matrix metalloproteases (MMPs), and the tissue inhibitors (TIMPs) of MMPs.

Coinfection with HIV and/or HBV is also associated with progression of CHC [193–198]. While viral interactions with interference between HBV and HCV in patients with HBV-HCV dual infection with inhibition of the replication of one of the viruses have been described in several studies [199–205], this phenomenon is incompletely understood, and the mechanism by which this occurs is yet to be established [206, 207]. Dual infection with HBV and HCV is characterized by one virus dominating over the other and can result in more accelerated progression to cirrhosis [193, 194]. Approximately 15–30 % of HIV-infected persons are coinfected with HCV [208]. With HIV coinfection there is an increased rate of end-stage liver disease, hepatocellular carcinoma, and death, in addition to the increased progression to cirrhosis [195–198, 209–211]. Accelerated fibrosis progression is also seen with high alcohol intake (daily consumption >50 g of alcohol) and marijuana use [212–215].

While the incidence of HCV infections is decreasing in the USA and other developed nations, due to the prolonged course of CHC, the number of infected people with complications related to end-stage liver disease is still increasing. The prevalence of hepatitis C-related cirrhosis and its complications, including hepatic decompensation and HCC, is expected to continue to increase through the next decade [216]. Patients with HCV-related cirrhosis are at increased risk of hepatic decompensation with signs/symptoms of liver failure and/or portal hypertension including ascites, portosystemic encephalopathy, and portal hypertension-related gastrointestinal bleeding [217, 218]. Patients with HCV-related cirrhosis are at an approximately 3 % per year risk for developing hepatocellular carcinoma [219–221].

8.1 Extrahepatic Manifestations

Chronic HCV infection can have serious consequences for organ systems other than the liver. HCV infection may affect the central nervous system, endocrine system, lymphatic system, eyes, kidneys, blood vessels, skin, joints, and peripheral nerves [222, 223]. Approximately 38 % of patients with chronic HCV infection will manifest at least one symptomatic extrahepatic manifestation during the illness [224]. Most extrahepatic manifestations of chronic HCV infection are immunologically mediated, and chronic infection seems to be necessary for their development. The most prominent manifestations include mixed cryoglobulinemia (MC) vasculitis, lymphoproliferative disorders, renal disease, insulin resistance, type 2 diabetes, sicca syndrome, and rheumatoid arthritis-like polyarthritis. Presence of these conditions can have a substantial impact on patient management as they can affect morbidity and mortality (for a detailed review, see [223]).

9 Control and Prevention

9.1 Public Health Approaches

Prevention of HCV-related liver disease should remain a key focus of clinical and public health interventions [10]. Major steps to prevent new HCV infections have been taken in several countries including routine screening of blood donors, implementation and enforcement of standard precautions, eliminating reuse of injection equipment, and facilitating safer drug injecting behaviors with adoption of harm reduction programs. Interventions using combined strategies of education, behavioral interventions, substance-use treatment, syringe access, and syringe disinfection can reduce the risk of HCV seroconversion by 75 % [225]. Therefore, efforts to prevent infection, reduce harms, and treat HCV-infected IDUs are essential. These steps should be extended worldwide. As part of secondary prevention, HCV-infected individuals should be counseled to minimize their risk of transmitting HCV and referred for medical evaluation and consideration of antiviral therapy. Of course, the latter approaches rely on the identification of persons with chronic HCV infection. In the USA, the CDC estimates that although 27 % of the population was born during the interval 1945–1965, they account for approximately three-fourths of all HCV infections and 73 % of HCV-associated mortality, and they are at greatest risk for hepatocellular carcinoma and other HCV-related liver disease. As a result, CDC has recently revised prior recommendations to include onetime testing for persons born during 1945–1965 without prior ascertainment of HCV risk [226]. This recommendation for onetime screening in persons born during the 1945–1965 has now been endorsed by the US Preventive Services Task Force [227].

To address occupational risk of HCV infection, individual institutions should establish policies and procedures for HCV testing of persons after percutaneous or mucosal exposures to blood, and they should ensure that all personnel are familiar with these policies and procedures. Health-care professionals who provide care to persons occupationally exposed to HCV should be knowledgeable regarding the risk for HCV infection and appropriate counseling, testing, and medical follow-up.

Finally, while there is currently no approved vaccine for HCV, attempts toward developing a vaccine are currently underway. Despite difficulties associated with extreme variability and mutability of hepatitis C virus (HCV), several vaccines that prevent initial infection or viral persistence, or that clear viremia in individuals with chronic HCV infections, are currently in development [228]. The likely goal for a prophylactic vaccine against HCV is to present persistence of the virus, rather than to prevent primary infection. The is based on the fact that a small proportion of people infected with acute HCV spontaneously clear it and this appears to be prominently mediated by cellular immunity [229]. Current HCV vaccine approaches include peptide, plasmid DNA, recombinant proteins, and vector-based vaccines. Virally vectored vaccines are the most promising in terms of T-cell induction—in particular adenoviral and modified vaccinia Ankara vectors. An understanding of the combination of functions possessed by the T-cell population is needed to effectively assess HCV vaccine efficacy. Parameters of interest when assessing a vaccine-induced HCV-specific T-cell population include breadth, cytokine production, cytolytic capacity, magnitude, phenotype, and proliferative capacity. A major challenge in HCV vaccinology will be the assessment of vaccine efficacy in well-characterized at-risk populations. Early phase vaccine trials are currently underway.

9.2 Treatment

The first successful therapies for HCV infection became available with the use of interferon to treat NANB hepatitis in 1986 [230]. Sustained virological response (SVR) is analogous to a cure and the goal of treatment. An SVR is defined by the absence of detectable HCV RNA in a patient's blood 6 months after the conclusion of antiviral therapy. Viral genotype testing is performed in the evaluation of HCV infection in order to maximize the chance of successful treatment outcome for each individual patient as treatment type, duration, dose, and effectiveness are influenced by genotype. Historically, persons infected with HCV genotypes 1 and 4 have required longer durations of treatment and have had lower SVR rates. Since the early treatments, significant and rapid progress has been made in establishing effective therapy for HCV infection. There are now an increasing number of potent options for treatment.

The standard of care for HCV treatment for the last decade until 2011 has been a combination of pegylated interferon and ribavirin [30]. Pegylated interferon alfa is a potent inhibitor of HCV replication that acts by inducing interferon-stimulated host genes that have antiviral functions, and given its diverse actions, it is not associated with viral resistance [95]. Ribavirin acting synergistically with pegylated interferon alfa is an integral component of the treatment for HCV, although the mechanism of action is not well known. Treatment for HCV infection varies according on genotype.

The first major advancement in HCV treatment in over a decade, occurred in 2011 with introduction of the first generation direct-acting antiviral agents (protease inhibitors) in combination with pegylated interferon and ribavirin [231–235]. Challenges with these new regimens included additional side effects, increased pill burden, increased drug-drug interactions, and antiviral resistance [95]. Additionally, use of these agents in real world practice had lower SVR results than reported in registration trials [263].

At the end of 2013, another major advancement of HCV therapy was realized with approval of second generation direct-acting antiviral agents, Sofosbuvir and Simeprevir [236]. Sofosubuvir, a nucleotide analogue NS5B polymerase inhibitor, is a component of the first all-oral, interferon-free regimen approved for treating chronic hepatitis C. Interferon-free therapy for treatment of hepatitis C reduces the side effects associated with use of interferon [237, 238, 239, 240, 241]. Many additional agents with different mechanisms of actions, improved safety profiles and cure rates greater than 95 % in registration trials are currently in various stages of clinical development [242, 243]. The short-term prospects for continued improvements in treatments with other direct acting antivirals in conjunction with pegylated interferon and ribavirin are in the pipeline and are quite promising, and these advances will likely lead to increased rates of cure in the coming years [244–246]. Additionally, all oral interferon-free regimens are now on the horizon, which will lessen treatment side effects and likely increase patient adherence and tolerability [240, 241]. However, it is important that both patients and providers be aware that there is no protective immunity and reinfection is possible with ongoing risk behavior and exposure.

Successful treatment of HCV infection eliminates transmissibility by eradication of viral infection (i.e., cure). It is reasonable to assume that transmissibility is likely reduced by reduction in HCV viral load even if cure is not accomplished.

9.2.1 Perinatal HCV Infection

Immune globulin and antiviral agents are not recommended for postexposure prophylaxis of infants born to HCV-positive women. Children born to HCV-positive women should be tested for HCV infection. Early diagnosis of HCV infection can be done by testing for HCV RNA at age 1–2 months and should be repeated after 3 months within the first year [100, 153, 247, 248]. Umbilical cord blood should not be used for the diagnosis of perinatal HCV infection because cord blood can be contaminated by maternal blood. Testing of infants for anti-HCV should be performed no sooner than 15–18 months of age, when passively transferred maternal anti-HCV declines below detectable levels. If positive for either anti-HCV or HCV RNA, children should be evaluated for the presence or development of liver disease, and those children with persistently abnormal ALT levels should be referred to a specialist for medical management.

9.3 Postexposure Management

It has been estimated that in the year 2000, 16,000 HCV infections may have occurred worldwide among health-care workers due to their occupational exposure to percutaneous injuries [249]. Although no immediately prophylactic regimen exists for HCV, effective treatment is available. When a needlestick, percutaneous injury from other sharp instruments, or mucosal exposure to blood occurs, the human source of the exposure should be tested for antibody to HCV (anti-HCV), and all repeatedly reactive results by enzyme immunoassay should be confirmed by RIBA for anti-HCV. If the source is anti-HCV positive, the exposed person should be tested for anti-HCV and alanine aminotransferase level at baseline and follow-up (e.g., at 4–6 months) [250, 251]. For earlier diagnosis of HCV infection, testing for HCV RNA may be performed at 4–6 weeks. There are no recommendations for restriction of activities during the postexposure follow-up period. There is consistent evidence that treatment reduces the risk that acute hepatitis C will evolve to chronic infection [252, 253]. Currently, the American Association for the Study of Liver Diseases (AASLD) practice guidelines recommend treatment initiation in patients with acute HCV if serum HCV RNA is not eliminated spontaneously within 12 weeks of HCV transmission [30]. When HCV infection is identified early, the individual should be referred for medical management to a specialist knowledgeable in this area.

10 Unresolved Problems

Although effective diagnostic tools and treatments are available, HCV remains a major public health problem. In many countries, including the USA, HCV-associated disease is the leading indication for liver transplantation, and it is a leading cause of HCC in the USA [37, 89, 254]. HCC and cirrhosis have been increasing among persons infected with HCV [255, 256], and these outcomes are projected to increase substantially in the coming decade [23, 257]. HCC is the fastest growing cause of cancer-related mortality, and infection with HCV accounts for approximately 50 % of incident HCC [7].

Transmission of HCV infection continues despite current prevention strategies. Early diagnosis of persons with HCV infection has been difficult. Because 80 % of acute HCV infections are asymptomatic, and more than 60 % of persons with chronic HCV infection are asymptomatic [156, 163], early diagnosis has relied on screening for asymptomatic infection and disease. Most persons with HCV do not know they are infected, are not evaluated for treatment, and do not receive needed care (e.g., education, counseling, and medical monitoring) [258–260]. In the USA alone, 75 % of infected persons remain unaware of their infection [261]. Additional strategies for and resources to support prevention and treatment of HCV are still needed. Education of persons at risk, implementation of standard precautions and infection control, and screening of blood donors for HCV are partially or entirely lacking in many parts of the world.

Finally, not all persons with HCV infection progress to end-stage liver disease or cirrhosis. However, we currently have no good predictors of who will suffer disease progression and therefore have only limited ability to target therapy toward those individuals. The morbidity and mortality associated with this disease continue to rise rapidly along with the costs of care. While treatment regimens with newly licensed drugs and others in clinical trials are evolving at a rapid pace, the challenge will be to make these therapies available and affordable to all persons with infection.

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Suggested Reading

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Hepatitis Viruses: Hepatocellular Carcinoma

34

Ju Dong Yang, Roongruedee Chaiteerakij, and Lewis R. Roberts

1 Introduction

Liver cancer is the fifth most common cancer in men and the Eighth in women. The estimated number of new liver cancer cases worldwide has increased by 70 % between 1990 and 2008. It has been projected that the number will increase by 30 % to one million new cases in 2020. Viral hepatitis is the most common cause of hepatocellular carcinoma (HCC) in the world. Hepatitis B virus (HBV) is the most common etiology of HCC in most Asian and African countries and accounts for more than half of all HCCs in the world. Hepatitis C virus (HCV) infection is the leading cause of chronic liver disease and HCC in most Western countries including the USA. The prevalence of HCV infection has increased and the proportion of patients with liver cirrhosis or HCC from HCV has also increased in the USA over the past several decades. Carcinogenesis is a complex and multistep process involving a sequence of tumor initiation, promotion, and progression over several years. Common molecular mechanisms of HCC occurring in the cirrhotic liver include cell-cycle dysregulation, sustained angiogenesis, apoptosis resistance, and cellular immortalization through reactivation of telomerase reverse transcriptase. In HBV- and HCV-induced hepatocarcinogenesis, the interplay between HBV and HCV, the host immune response to infection, and chronic inflammation in the liver contribute to malignant transformation. HBV and HCV are involved in carcinogenesis directly through induction of genetic mutations and through oncogenic effects of viral proteins. Indirectly, both viruses share some common carcinogenesis pathways, in particular, chronic inflammation, oxidative stress, and tumor suppressor gene *p53* inactivation. The goal of HCC surveillance is to decrease HCC mortality through the early detection of HCC in asymptomatic patients. In real-world practice, the majority of at risk individuals are not under HCC surveillance and HCC is usually detected at a late stage when patients become symptomatic from advanced HCC. There is strong evidence that HCC surveillance increases the early detection of tumors and improves survival in patients with HCC. However, the efficacy of HCC surveillance is still controversial mainly because of the lack of robust level 1 evidence supporting the use of HCC surveillance. Noninvasive diagnosis of HCC in individuals with cirrhosis can be made by contrast-enhanced four phase CT (non-contrast, arterial, portal, and delayed phases) or dynamic contrast MRI. Arterial enhancement and portal venous or delayed phase washout are highly specific characteristic features of HCCs. Percutaneous liver biopsy is indicated when lesions (>1 cm) develop without background liver cirrhosis or dynamic imaging modalities show inconclusive results. The treatment of HCC depends on multiple factors, including the tumor extent, severity of liver dysfunction, performance status of the patient, socioeconomic support, and the availability of different types of treatment. Surgical resection, liver transplantation, and local ablative treatment are potentially curative treatment modalities for patients with early-stage HCC. Transarterial chemoembolization and radioembolization are locoregional treatment modalities applicable for patients with intermediate-stage

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HCC. Sorafenib is a targeted systemic treatment that is offered to patients with advanced stage HCC.

2 Historical Background of the Association Between Viral Hepatitis and Hepatocellular Carcinoma

A possible association between hepatitis B virus (HBV) infection and hepatocellular carcinoma (HCC) development was first reported in the 1950s [1]. Subsequently, the association between HBV infection and HCC was supported by several epidemiologic studies in the 1970s, mostly from Africa and Asia [2–6]. A causal relationship between HBV infection and HCC was confirmed by a Taiwanese prospective study showing that HBV-infected patients had a 98.4-fold increased risk for developing HCC compared to individuals without HBV infection [7]. There is also a lower but nevertheless increased risk of HCC associated with chronic HBV carrier status [8]. The relationship between hepatitis C virus (HCV) infection and HCC was first noted in the early 1980s [9, 10]. As HCV (then described as non-A, non-B chronic hepatitis) became endemic in Japan in the late 1940s and early 1950s, the majority of the early evidence of a causal link between HCV and HCC was from studies performed in Japan. Due to the lag time of 20–40 years between the establishment of chronic HCV infection and the development of HCC, a dramatic increase in HCC-related mortality in Japan occurred beginning in the 1970s and most of these HCCs developed in patients with non-A, non-B hepatitis [11]. Subsequent to the identification of the hepatitis C virus in 1990 and the development of specific assays for the infection, among those with chronic non-A, non-B hepatitis, antibody to HCV (anti-HCV) was detected in 94.4 % [10]. Epidemiologic studies from Spain and Italy also reported that 61–75 % of HCC patients were positive for anti-HCV [12–14]. The initial causative role of HCV infection in HCC development was supported by a Japanese study showing that HCV RNA was detected in HCC tissue and the surrounding noncancerous liver tissues of patients with HCC [15]. A meta-analysis of 32 case-control studies published between 1993 and 1997 concluded that chronic HBV-infected patients had 13.7-fold increased risk for HCC compared to individuals without chronic HBV infection, and HCV-infected patients had 11.5-fold increased risk for HCC compared to individuals without HCV infection [16]. This meta-analysis also reported that the risk for HCC increased to 165-fold for those infected with both HBV and HCV, indicating a synergistic interaction of HBV and HCV coinfections on the risk for HCC development [16]. Given the evidence for the association between HBV and HCV infections and HCC risk, both HBV and HCV were classified as human carcinogens by the International Agency for Research on Cancer (IARC) in 1994 [17]. A different meta-analysis found that HBV and HCV infections increase HCC risk by 13.5-fold and 12.2-fold, respectively, while HBV and HCV co-infections additively increase the HCC risk by 51.1-fold [18].

3 Incidence of HCC

Worldwide, liver cancer is the fifth most common cancer in men and the eighth in women [19]. The global incidence of HCC has been rising, from 437,408 cases in 1990 to 782,000 in 2012. HCC cases are projected to increase further to 997,955 new cases by 2020 and 1,251,520 new cases by 2030 [19]. The trends in HCC incidence reflect the temporal and geographical changes in prevalence of risk factors for HCC. The rising incidence is primarily occurring in countries with low and intermediate HCC incidence. In the USA, the annual HCC incidence has been shown to have increased by threefold between 1975 and 2005 (1.5–4.9 cases per 100,000 persons) [20]. Indeed, a study from Olmsted County, Minnesota, has confirmed the increasing trend of HCC incidence, showing that the incidence of HCC increased from 3.5 to 6.9 per 100,000 person years between 1976 and 2008 [21]. In England and Wales, the HCC incidence increased by 46 % between 1971 and 2001 in males (1.8 to 2.7 cases per 100,000 persons) and increased by 8 % in females (0.8 to 0.9 cases per 100,000 persons) [22]. The rising trend in HCC in these countries is mainly attributable to HCV infection. It has been projected that the HCV-related HCC incidence in the USA will peak in 2015–2020 and then gradually decline [23].

In contrast to the rising trends of HCC incidence in low and intermediate prevalence areas, the opposite trends have been observed in high-prevalence countries. For example, the age-standardized HCC incidence in Japan declined steadily between 1995 and 2005 from 41.5 to 24.0 cases per 100,000 persons among men and from 10.8 to 7.3 cases per 100,000 persons among women [24]. These declines are attributed to the marked reduction in the incidence of HCV infection after implementation of a nationwide primary prevention program [24]. Similarly, a gradual decline in HCC incidence has also been observed in Taiwan where HBV is the major cause of HCC. Within 10 years after initiation of a nationwide vaccination program, the HCC incidence in children aged 6–14 years had decreased dramatically from 0.76 per 100,000 in the period from 1982 to 1986 to 0.36 per 100,000 in the period from 1990 to 1994 [25].

The overall incidence of HCC rises sharply after age of 40 and increases with age in both genders [19]. The incidence peaks at age 70 and then declines [20]. The age distribution of HCC varies worldwide. Several factors, including gender, age at HBV or HCV infection, and coexistence of other risk factors, account for the global variations in the peak age of

onset. The peak age of onset in Africa, another region where HBV is a major cause of HCC, is generally younger than in other parts of the world, suggesting that viral, genetic, environmental, or other risk factors may modify the risk for HCC development. For example, the peak age of onset in Mali is 40 years [26].

4 Geographical Variation of HBV and HCV-Induced HCC

Although HCC develops in all regions around the world, substantial global variation of HCC incidence is observed (Fig. 34.1). Over 85 % of HCC cases occur in East and Southeast Asia and in Middle and Western Africa, whereas the incidence is much lower in Europe (with the exception of South Europe), South America, North America, Australia, and New Zealand [19]. The variation in geographical distribution of HCC is explained by the prevalence of specific risk factors, in particular, chronic HBV and HCV infections. A strong correlation between HCC incidence and prevalence of chronic HBV and HCV infections has been reported (Fig. 34.1). It was estimated that 75–80 % of HCC cases worldwide are associated with either chronic HBV (50–55 %) or HCV (10–25 %) infection, while the global prevalences of HBV and HCV are about 6 and 3 %, respectively [27]. Most countries in Asia, except for Japan, Pakistan, and Mongolia, have a higher proportion of HBV-related HCC than HCV-related HCC. HBsAg seropositivity among HCC patients was 27–64 % in India, 53 % in Taiwan, 52–66 % in Turkey, 58–60 % in Thailand, 59–67 % in China, 62–82 % in Korea, and 61–93 % in Vietnam, whereas anti-HCV positivity was approximately 2 % in Vietnam, 5–15 % in Korea, 3–10 % in China, 4–53 % in India, 19–29 % in Turkey, 28 % in Taiwan, and 5–10 % in Thailand among HCC patients in these countries [28]. By contrast, only 18 % of HCC cases in Japan and approximately 30 % of HCC cases in Pakistan and Mongolia were positive for HBsAg. Japan had the highest proportion of HCC cases positive for anti-HCV (68 %) and anti-HCV positivity of HCC patients was 45 % in Pakistan and 40 % in Mongolia. Of the Asian countries, HBV and HCV coinfection was most commonly found in Mongolia (25 %) [28]. Similar to Asian countries, most countries in Africa have a much higher prevalence of HBV-related HCC cases than HCV-related cases. HBsAg positivity in HCC cases was approximately 65 % in Mozambique, 60 % in the Gambia, 45 % in South Africa, and 42 % in other African countries. The only exception is Egypt where the proportion of HCC cases with HBsAg+ was only 10 %. Egypt had the highest proportion of HCC cases with anti-HCV+

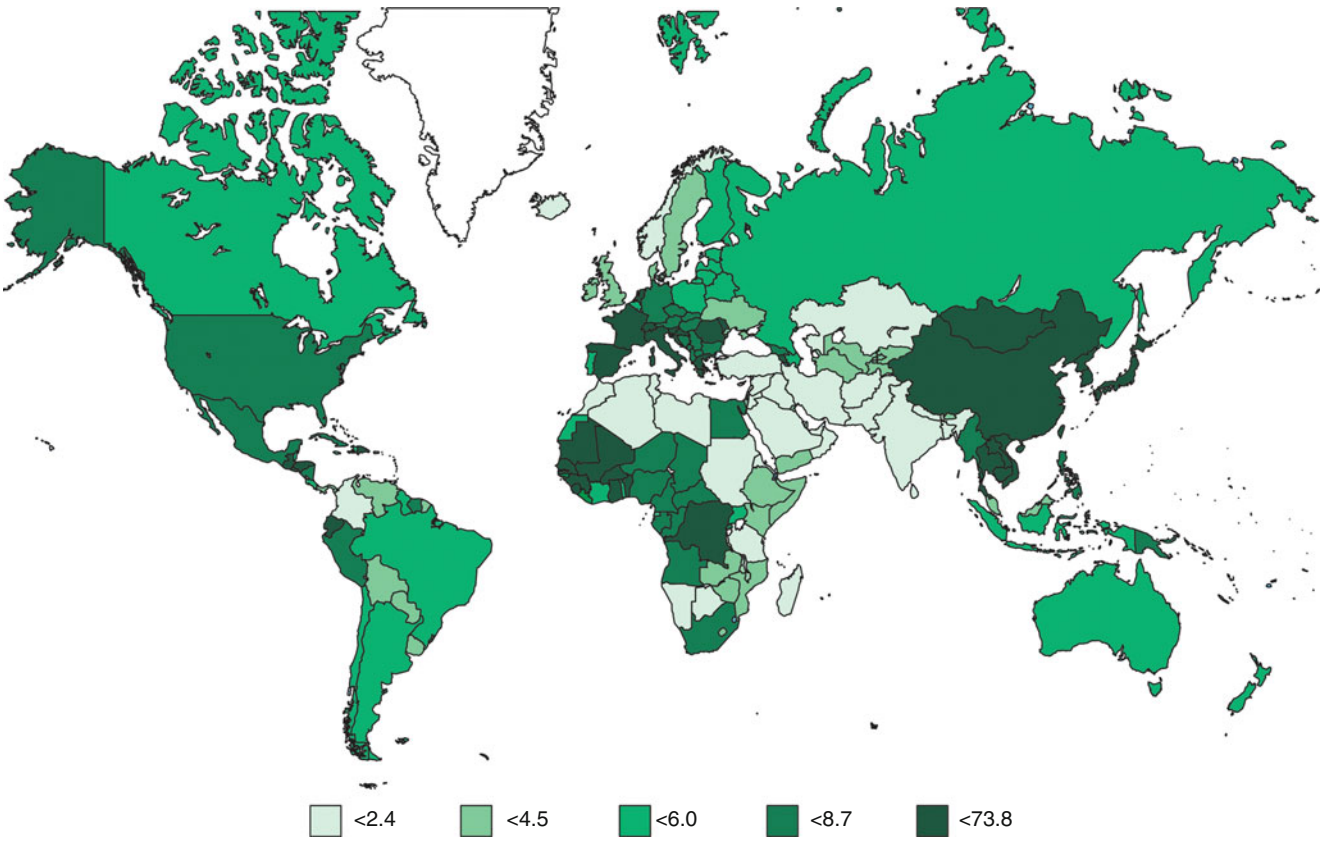

Fig. 34.1 (**a**–**c**) Worldwide incidence of HCC and prevalence of HBV and HCV

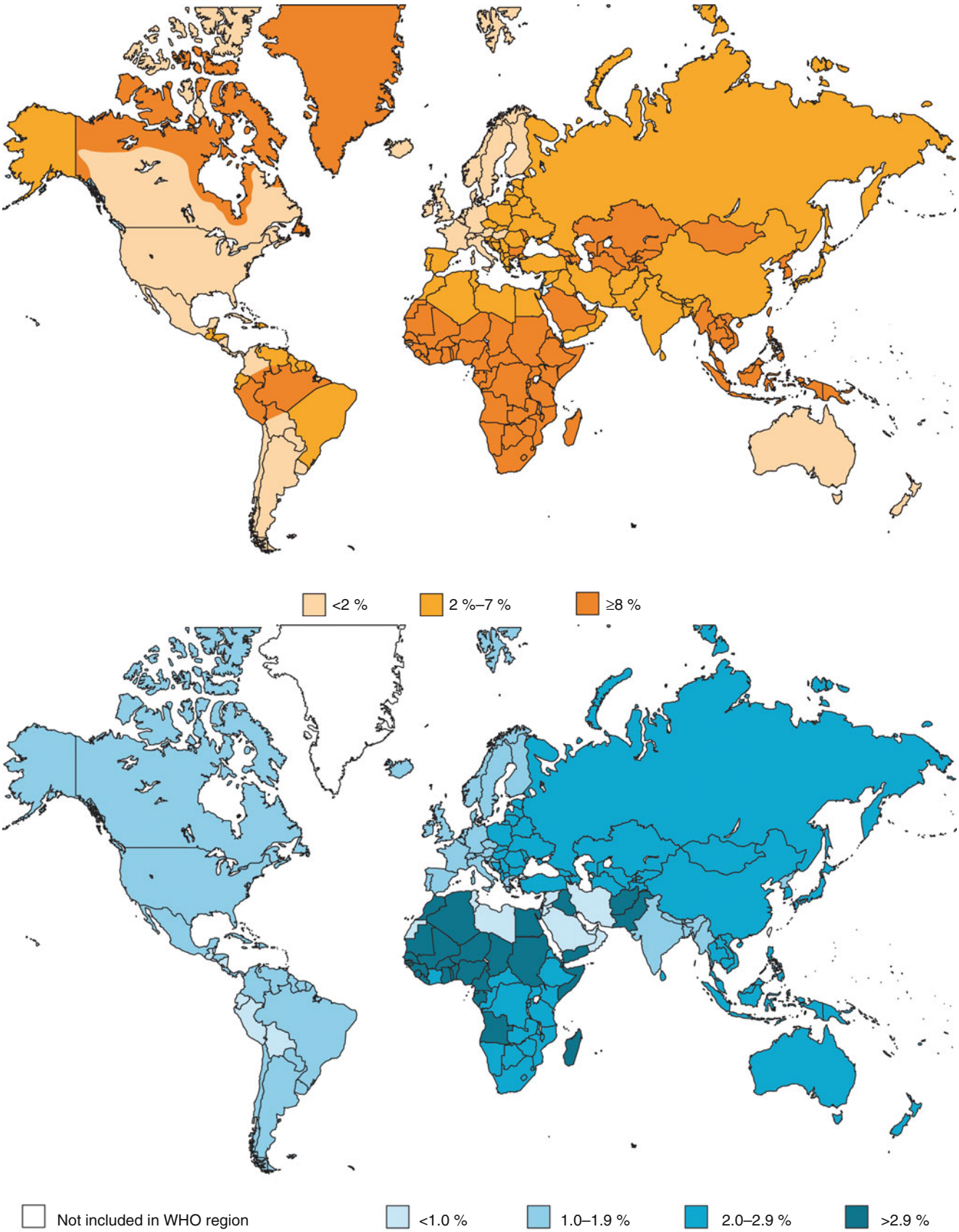

Fig. 34.1 (continued)

(69 %), whereas all other African countries reported a proportion of 5–25 % of HCC cases with anti-HCV+ [28]. The initial spread of HCV infection in Japan was thought to be due to mass treatment campaigns for endemic *Schistosoma japonicum* beginning in the 1920s [29]. Similarly, the high prevalence of HCV-associated HCC in Egypt is thought to be related to the iatrogenic transmission of HCV in that population during mass treatment campaigns for endemic *Schistosoma mansoni* [30].

Contrary to Asian and African countries, in most European countries except for Greece, the proportion of HCC patients with positive anti-HCV is greater than those with positive HBsAg. Anti-HCV positivity in HCC patients was 48 % in Spain, 43 % in Italy, 36 % in Austria, and 25 % in Belgium and the *UK*, while the proportion with positive HBsAg+ was approximately 10–20 % in these countries. In Greece, the proportion of HCC patients positive for HBsAg and anti-HCV was 56 and 16 %, respectively [28]. In Germany, the proportion of anti-HCV-positive cases (25 %) was close to that of HBsAg-positive cases (22 %). It is important to note that the proportions of HCC cases negative for both HBsAg and anti-HCV among European countries were relatively higher compared to the proportions in Asian countries (35 % in India and 5–25 % in all other Asian countries) and in Africa (less than 30 %). Strikingly, in Sweden 82 % of HCC cases were seronegative for HBsAg and anti-HCV, in contrast to the proportion that was seronegative for both viruses in other European countries (32–52 %) [28]. The high proportion of HCC cases seronegative for both viruses reflects the fact that risk factors other than chronic HBV or HCV infection, particularly alcohol use and increasingly fatty liver disease, are of substantial importance in contributing to HCC development in Europe. The proportion of HCC cases with positive HBsAg or anti-HCV varies greatly among countries in North, Middle, and South America. In the USA, HCV accounts for 30–50 % and HBV accounts for 5–15 % of HCCs. In Brazil, 40 % of HCC cases were seronegative for both HBV and HCV viruses and 37 and 18 % of HCC cases were positive for HBsAg and anti-HCV, respectively. In Peru and Mexico, 44 % of cases had positive HBsAg, 20 % of cases had positive anti-HCV, and 30 % of cases were seronegative for both HBV and HCV viruses [28].

5 Mortality

In 2012, an estimated 746,000 worldwide deaths were due to liver cancer [19]. Liver cancer was the second leading cause of cancer-related death in males, the sixth cause of cancer death in females, and the second most common cause of death from cancer overall [19]. Liver cancer is considered to be the second most lethal cancer, after pancreatic cancer, which had the highest mortality to incidence ratio of 0.96 [19]. The mortality to incidence ratio of HCC was 0.98 in 1990 and remained high at 0.95 in 2012 (Fig. 34.2) [31]. It has been predicted that the number of liver cancer deaths will increase to 929,368 in the year 2020 and to 1,175,804 in 2030 [19]. The 5-year survival rate for liver cancer worldwide is estimated at 15 % [32]. There are substantial geographical, regional, and local variations in HCC survival, in large part related to access to surveillance and therapy. Although the number of liver cancer deaths has been increasing due to the increasing incidence of HCC, the overall survival in patients with HCC appears to have improved over time. A study from Olmsted County, Minnesota, showed that overall survival in patients with HCC has improved over the past three decades, with an increasing proportion of patients receiving curative treatment over time and improvement in survival from a median survival time of 3 month in 1976 to 9 months in 2008 ($P = 0.01$) [21].

6 Etiology of HCC

6.1 Hepatitis B and Hepatitis C Viruses

HBV and HCV infections are the most common causes of HCC in the world. Alcohol is the next most common etiology of HCC. Due to the rising epidemic of obesity and metabolic syndrome, NAFLD (nonalcoholic fatty liver disease) is now recognized as one of the four major etiologies of HCC [33].

Variations in incidence of HCC in different racial and ethnic populations are generally related to the underlying prevalence of the major risk factors in these populations. In the USA, Blacks and Hispanics have a 2.5 and 2.0 times higher incidence compared to Whites, respectively [34]. In addition to the different risks of HCC, the clinical features and presentation also differ among different races. Caucasian carriers of HBsAg tend to develop HCC at an older age in a background of underlying liver cirrhosis, while Asians and Africans tend to develop HCC at a relatively early age with less cirrhotic change of the liver [23]. This difference may be explained by the difference in age at which HBV infection is acquired in the different populations as well as genetic variations of host and HBV genotype between the different races. It is well known that persistent HBV replication is associated with increased risk of HCC [35, 36]. HBV genotype C is the most prevalent genotype in Asians and known to be associated with the presence of hepatitis B virus e antigen (HBeAg), a decreased rate of spontaneous HBeAg clearance, higher rates of HBeAg reversion after HBeAg clearance, and an increased risk of HCC independent of HBV DNA levels [37–40]. Several publications from Asian countries have identified male sex, older age, elevated serum alanine aminotransferase (ALT), positive HBeAg status, elevated serum

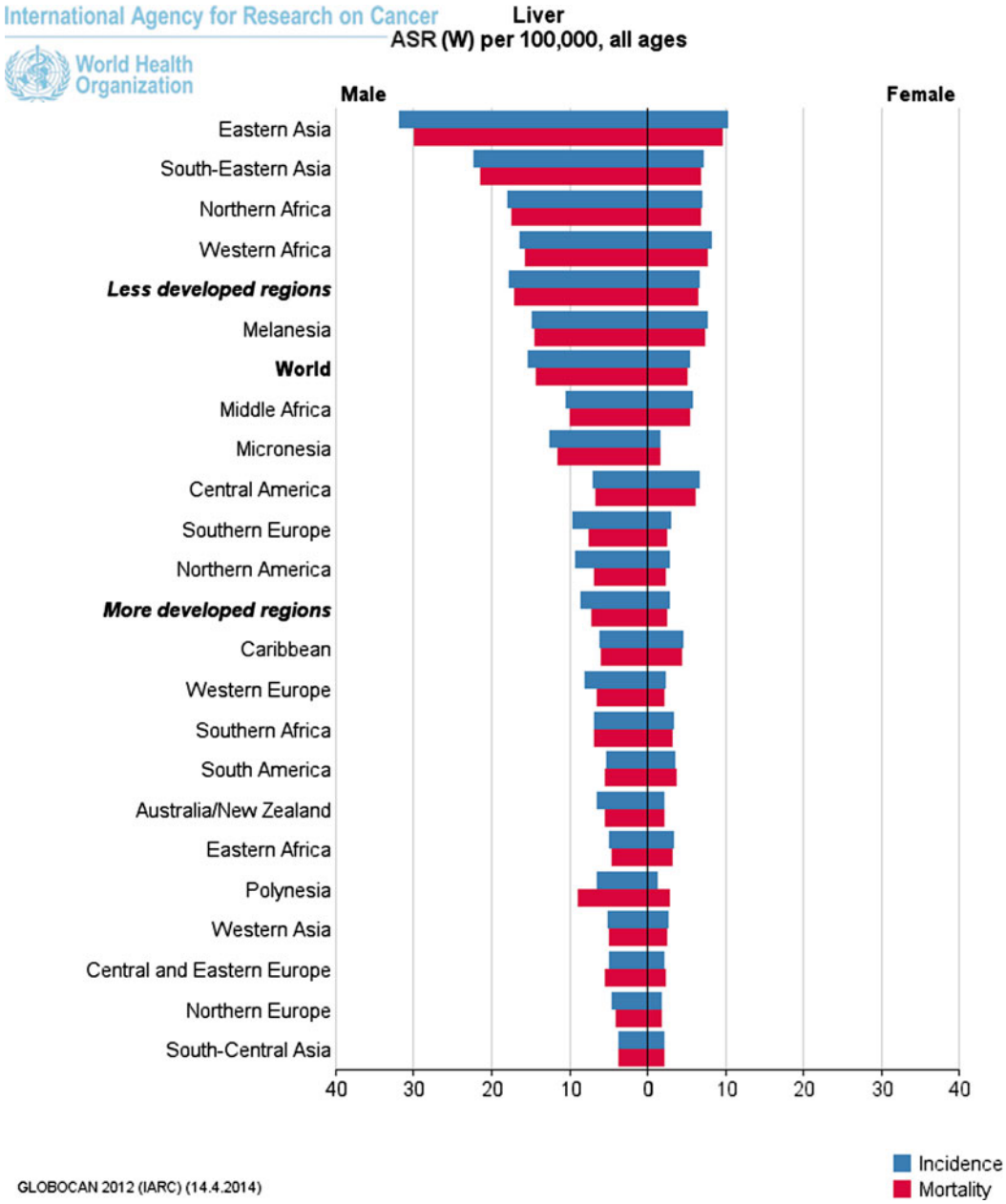

Fig. 34.2 Worldwide incidence and mortality of HCC in males and females

HBV DNA level, and HBV genotype C as independent predictors of HCC development [41, 42]. The risk for HCC can also be affected by long-term changes in serum levels of HBV DNA or ALT. A study from Taiwan showed that persistently high HBV DNA levels (1,000,000–10,000,000 copies/mL) are associated with a substantially increased risk of HCC, with a hazard ratio (HR) of 16.8 (CI, 7.3–38.4) compared to controls. The ALT level was also significantly associated with HCC risk [43].

Hepatitis C is the leading cause of chronic liver disease and HCC in most Western countries including the USA [21, 44]. The prevalence of HCV and the proportion of patients with liver cirrhosis or HCC induced by HCV in the USA have also increased over the past several decades [45]. A population-based study in Olmsted County, Minnesota, showed that HCV is the main driver of the increasing incidence of HCC, accounting for 45 % of HCCs between 2001 and 2008 [21]. Individuals with positive anti-HCV have a 28-fold increased risk of HCC compared to individuals with negative anti-HCV [46]. In Taiwan, a population-based study showed cumulative lifetime (age 30–75 years) incidences of HCC for men and women with positive anti-HCV were 23.73 and 16.71 %, compared to lifetime incidences for HBsAg-positive individuals of 27.38 and 7.99 % for men and women, respectively. HCV and HBV coinfection increases the risk of HCC; the cumulative lifetime

incidences of HCC in dually infected men and women were 38.35 and 27.40 %, respectively [47].

HCV transmission by blood transfusion was common in the USA and other highly developed countries prior to the introduction of screening; the risk of HCV transmission from a blood transfusion has been negligible in the USA since the implementation of donor blood screening for HCV in 1990 [48, 49]. More recently, the most common route of transmission has been by percutaneous exposure to blood via illegal intravenous or intranasal drug use and via high-risk sexual behaviors [50, 51]. Transmission of HCV among injection drug users (IDUs) remains problematic, as suggested by a report that the prevalence among them in England and Wales rose from 47 % in 1998 to 53 % in 2006 [51]. Iatrogenic transmission is not uncommon as a route of HCV transmission, even in more developed parts of the world. In a study from Spain, hospital admission was the most common risk factor preceding the diagnosis of acute HCV infection, which usually progresses to chronic HCV infection [52]. Although mother to infant vertical transmission is rare, a high HCV titer in the mother is known to increase the frequency of HCV transmission [53, 54].

6.2 Alcoholic Liver Disease

Alcoholic liver disease is the second most common risk factor for HCC in the USA [55]. Although alcohol intake is known to increase the risk for HCC, the threshold dose and the minimal duration of use associated with risk of HCC are not well established [56]. The risk of developing HCC appears to be lower in patients with alcoholic cirrhosis compared to patients with cirrhosis from other etiologies; in a recent Danish nationwide cohort study, the 5-year cumulative HCC risk for the alcoholic group was only 1.0 % (95 % CI, 0.8–1.3 %) [57]. It is known that females are more susceptible than males to liver injury from alcohol intake due to differences in alcohol metabolism [58]. One quarter of alcoholic patients in the USA have HCV infection [59]. Not surprisingly then, coexisting viral hepatitis and alcohol intake appears to increase the risk of HCC [56, 60]. A population-based study from Olmsted County, Minnesota, has shown that HCV patients with significant alcohol intake develop HCC at an earlier age compared to those without significant alcohol consumption [21]. Secular trends in per capita alcohol consumption in different countries or regions may therefore have significant impact on trends in HCC incidence.

6.3 Obesity-Related Nonalcoholic Steatohepatitis

Obesity-related nonalcoholic steatohepatitis (NASH), or nonalcoholic fatty liver disease (NAFLD), is emerging as an important new risk factor for HCC in many developed countries [61, 62]. Obesity has been associated with a significantly increased risk of mortality from HCC [63]. A study using the US Scientific Registry of Transplant Recipients for primary adult liver transplant recipients between 2001 and 2009 showed that NAFLD was the third most common indication for liver transplantation in the USA and is on a trajectory to become the most common indication. A US referral center study showed that NAFLD is the third most common cause of HCC [33]. Importantly, there is substantial and increasing evidence suggesting that NAFLD can lead to the development of HCC in the absence of established cirrhosis [64, 65]. The annual incidence rate of HCC in patients with NASH cirrhosis was reported to be 2.6 % [66]. Diabetes has been shown to be associated with a two- to threefold increased risk of HCC in multiple cohort and case-control studies [67]. A more recent large cohort study showed that well-controlled diabetes (HgbA1C <7 %) in patients is associated with a 44 % reduction in the risk of developing HCC compared to poorly controlled diabetes (HgbA1C >7 %) [68]. It is not clear however whether diabetes has a direct carcinogenic effect in the liver or whether it indirectly increases the risk of HCC by promoting hepatic inflammation and fibrosis. Metformin and statin drugs have been shown to be associated with a decreased risk of HCC, and metformin treatment was independently associated with an 80 % decrease in HCC occurrence in a cohort of patients with HCV-induced cirrhosis [69, 70]. A more recent meta-analysis which included approximately 105,495 patients with type 2 diabetes showed that metformin was associated with an estimated 62 % reduction in the risk of liver cancer among patients with type 2 diabetes ($P<0.001$) [71]. Fish oil also appears to be protective against the development of HCC. A Japanese population-based prospective cohort study of 90,296 subjects showed that consumption of n-3 polyunsaturated fatty acid (PUFA)-rich fish and individual n-3 PUFAs was associated with a 30–40 % risk reduction of HCC, in a dose-dependent manner. These inverse associations were similar irrespective of HCV or HBV status [72]. Statin use was associated with a 26 % decrease in risk of HCC in a matched case-control study nested within a cohort of VA patients with diabetes [73]. On the other hand, insulin treatment for diabetes has been shown to be associated with an increased risk of HCC [70, 74]. Based on the available evidence, no definitive statement can be made about the contribution lipid derangement (fatty liver) on the risk of HCC due to viral hepatitis.

6.4 Aflatoxin

Aflatoxin B is a mycotoxin which is a frequent food contaminant in sub-Saharan Africa and Eastern Asia and is a

well-known risk factor for HCC [75]. Aflatoxin B has a synergistic effect with HBV in HCC development by inducing p53 mutations (most commonly a G-to-T transversion in codon 249) and activating oncogenic pathways [76].

6.5 Other Cofactors

A positive family history of liver cancer increases the risk of HCC by two- to threefold independent of viral hepatitis [77]. Males are at two to four times greater risk for HCC than females. The male predilection varies around the world and is usually higher in high-incidence areas [19]. The sex discrepancy in HCC incidence is explained by two factors, namely, the difference in rates of exposure to known risk factors and sex hormone-related effects. Males are more likely to be exposed to hepatocarcinogens, in particular, viral hepatitis infection, alcohol, and smoking. Testosterone may also increase HCC risk by promoting hepatocyte proliferation and malignant transformation via induction of the androgen signaling pathway [78], whereas estrogenic hormones have a protective effect against HCC by suppressing the release of interleukin-6 from Kupffer cells in the liver [79]. Interleukin-6, a proinflammatory cytokine, inhibits apoptosis of HCC cells. In addition, male and female sex hormones have opposing effects on HBV replication, that is, testosterone increases HBsAg gene expression, whereas estrogen suppresses HBV replication [80–82].

7 Contribution of HBV and HCV Infection to Mechanisms of Hepatocarcinogenesis

Chronic liver inflammation, commonly induced by chronic viral hepatitis B or C infection, alcohol, or fatty liver disease, is a major driver of liver fibrogenesis and carcinogenesis. Hepatitis viruses, alcohol, fat, and other hepatotoxic agents induce acute liver cell injury and death [83]. Liver cell death induces inflammation and the production of cytokines and growth factors essential for regeneration of liver cells [84]. This leads to restoration of the normal cytology and architecture of the liver. Liver cell death as well as the cytokines produced in response to liver injury also produce reactive oxygen species which trigger tissue regeneration and repair. In the setting of chronic liver inflammation induced by chronic viral hepatitis B and/or C infection, repeated cycles of tissue injury, regeneration, and repair lead to telomere shortening, exhaustion of the capacity for liver regeneration, and eventually to hepatocellular senescence. Concurrently, the persistent liver inflammation induces stellate cell activation and liver fibrogenesis, contributing to architectural distortion of the liver tissue [85]. Within this context of exhausted liver regeneration, some hepatocytes escape from senescence through activation of the telomerase enzyme or alternative mechanisms, leading to cellular immortalization [86]. It appears that generation of a genotoxic inflammatory tumor microenvironment with a high concentration of reactive oxygen species leads to the accumulation of genetic alterations in immortalized hepatocytes, as well as to epigenetic alterations in DNA methylation, histone modification, and noncoding RNA expression that contribute to the development of the tumor phenotype [87]. The combination of oncogenic alterations, including oncogene-activating gene mutations, gene amplification, and increases in oncogene expression, as well as tumor suppressor inactivation by mutation, genetic loss, or epigenetic mechanisms, results in dysregulation of the cell cycle, the establishment of a proliferating mass of tumor cells, and consequent effects on angiogenesis, immune escape, and the tumor microenvironment that leads to overt malignancy [83, 88]. HBV and HCV contribute to HCC carcinogenesis through several mechanisms, in particular, the oncogenic effects of viral HBx and HCV core proteins [89]. The accumulation of pre-S1 and pre-S2 mutant HBsAg in the endoplasmic reticulum and the interaction between the HCV core protein and hepatocyte mitochondria trigger the generation of reactive oxygen species, a key pathogenic mechanism in hepatocarcinogenesis [90, 91]. HBx protein dysregulates the TSC1/mTOR signaling pathway through IKKβ activation resulting in hepatocyte proliferation [92]. HCV core protein promotes cell proliferation through activation of the Wnt/beta-catenin pathway, and NS5A nonstructural protein promotes hepatocyte proliferation through activation of the PI3K/Akt pathway [93, 94]. Concurrently, hepatocyte cell death is inhibited through the suppression of the p53-induced apoptosis pathway by NS5A [95]. HBV core and HCV E2 also interfere with TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis and tumor necrosis factor alpha (TNF-α)-mediated apoptosis [96, 97]. The HBx and HCV core protein can induce telomerase reverse transcriptase (TERT) and telomerase activity, leading to hepatocyte immortalization [98, 99]. Additionally, tumor angiogenesis is stimulated through HBx and HCV core protein-induced vascular endothelial growth factor (VEGF) activation [100, 101]. The HBx protein also enhances HCC invasion and metastasis by increasing the activity of matrix metalloproteinases, protease enzymes important for cancer cell migration and metastasis [101].

During the last few years, next-generation sequencing (NGS) technologies have been applied to elucidate additional details of the pathogenesis of HCC and have begun to provide a comprehensive genomic landscape of HCC. Many novel genetic alterations and pathways that potentially contribute to the pathogenesis of HBV- and HCV-induced HCC have been identified. Unlike noncancerous liver tissue in which HBV integrations take place in multiple

locations in the human genome, HBV integrations have been confirmed to occur commonly within or upstream of the telomerase reverse transcriptase gene *TERT* in HCC tissues [102]. A number of mutations in chromatin-remodeling family genes, in particular *ARID1A* and *ARID2*, have been identified in up to 50 % of HCC tumor tissues, suggesting that the chromatin-remodeling pathway could potentially be an important pathway in HBV- and HCV-related HCC [102, 103]. There is evidence that *ARID2* functions as tumor suppressor gene in HCC and that *ARID1A* may be a driver of HCC metastasis [103, 104]. *IRF2*, a gene involving in cell growth regulation, was found to function as a tumor suppressor by regulating the P53 pathway, one of the most important pathways in hepatocarcinogenesis [105]. A potential role of the oxidative stress pathway in HCC development has also been revealed. Mutations of the *NFE2L2* gene which encodes *NRF2*, a transcription factor regulating expression of antioxidant enzyme-coding genes, have been identified. *NFE2L2* has been shown to be significantly associated with mutations of *CTNNB1* in the Wnt/beta-catenin pathway, another major pathway in hepatocarcinogenesis [105]. Novel mutations in several genes regulating cell proliferation and cell survival, such as the cell adhesion molecule gene *VCAM1*, have also been indentified in HBV-related HCC [103]. The patterns of nucleotide substitutions of HBV-induced HCC and HCV-induced HCC are different, for example, C:G>A:T and T:A>A:T transversions are common patterns in HBV-related HCC whereas G:C>T:A transversion is a common pattern in HCV-related HCC [103, 105]. These results point to differences in the underlying etiologic mechanisms of HCC and will hopefully result in advances in individualized prevention and therapy of HCC.

8 Prevention

The most effective means of controlling HCC is primary or secondary prevention of chronic HBV and HCV infections, since chronic viral hepatitis and the resultant liver cirrhosis are the major causes of HCC. These control measures are covered in greater detail in Chaps. 32 and 33. As the majority of HBV and HCV infections are transmitted through direct blood exposure, avoidance of high-risk behaviors through public education and social awareness is a key approach to primary prevention of viral hepatitis-induced HCC. Iatrogenic transmission is a not uncommon route of hepatitis virus transmission. There are well-recognized risks of HBV and HCV transmission in dialysis units, endoscopy units, and other health-care settings. Therefore, it is critical to recognize and prevent transmission of hepatitis virus in health-care and para-health-care settings by implementing infection control measures such as screening blood products for the two viruses, use of disposable needles and procedure supplies, and thorough sterilization of surgical instruments. It is also important to avoid modifiable risk factors for HCC such as heavy alcohol use, diabetes mellitus, obesity, and smoking, particularly in patients with chronic viral hepatitis, as these factors can increase the risk of HCC in a synergistic manner [60, 106–108]. Global HBV vaccination has reduced the burden of HCC. HBV vaccination was introduced in 1984 and universal HBV vaccination had been implemented in 86 % of WHO member states by 2006 [23]. In countries with a high coverage of the population, HBV vaccination has resulted in dramatic decreases in the incidence of HBV infection and HCC development [109]. In contrast to HBV vaccination, there is currently no effective HCV vaccination available, due in part to difficulties related to the high mutation rate of HCV during viral replication.

Effective antiviral treatment has been shown to decrease the risk for HCC in patients with viral hepatitis. Lamivudine treatment decreases progression from cirrhosis to HCC in chronic HBV patients, and this preventive effect has also been seen in patients without liver cirrhosis [110–112]. Antiviral treatment for HCV which results in sustained virologic response decreases the risk of progression to HCC; in contrast, patients who do not achieve a sustained response continue to have a high risk of progression to HCC [113–115]. However, newer antiviral drugs appear to offer greater promise of a cure for HCV infection (Chap. 33). The role of antiviral treatment in patients with HCV cirrhosis is controversial [113–118]. The US HALT–C study examined the effect of interferon on the risk of HCC development. This trial included 1,048 patients with chronic hepatitis C and advanced fibrosis who did not have a sustained virologic response (SVR) to therapy. Participants were randomly assigned to groups given a half dose of peginterferon or no treatment (controls) for 3.5 years and followed up for a median of 6.1 (maximum, 8.7) years. After 7 years of follow-up, the cumulative incidences of HCC in treated and control patients with cirrhosis were 7.8 and 24.2 %, respectively (HR: 0.45); in treated and control patients with fibrosis but not cirrhosis (Ishak fibrosis stages 5 and 6), incidences were 8.3 and 6.8 %, respectively (HR, 1.44), suggesting that peginterferon treatment is effective in lowering the risk of HCC in patients with cirrhosis but not in patients without cirrhosis [119]. A similar European study showed a conflicting result. Patients with chronic hepatitis C with compensated liver cirrhosis who had failed to respond to interferon-alpha plus ribavirin were randomized to treatment with peginterferon alfa-2b or no treatment. Although the progression of liver cirrhosis was significantly slower for patients who received peginterferon alfa-2b compared with controls (HR, 1.56), there was no difference in the risk of HCC between patients received peginterferon alfa-2b and those receiving no treatment [120]. Evidence for a role of interferon treatment in preventing recurrence after liver

resection has been controversial. Although most previous studies suggest there is an effect of interferon in reducing the risk of HCC recurrence, a randomized controlled trial showed no difference in the risk of recurrence between groups who received 53 weeks of adjuvant IFNα-2b treatment and observation alone ($p=0.83$). In fact, adjuvant IFNα-2b treatment was associated with a significantly higher incidence of hematologic complications, including leucopenia and thrombocytopenia.

9 Surveillance for HCC

The goal of HCC surveillance is to decrease HCC mortality through the early detection of HCC in asymptomatic patients. In real-world practice, the majority of patients are not under HCC surveillance and HCCs tend to be detected at a late stage when patients become symptomatic from advanced HCC [33]. A population-based study using data from the Surveillance, Epidemiology, and End Results (SEER)—Medicare database—showed that only 17 % of cirrhotic patients are under regular HCC surveillance 3 years before diagnosis of HCC [121]. Similarly, of 13,000 patients with HCV-induced cirrhosis seen at 128 VA health-care facilities, only 12 % received routine HCC surveillance [122]. A more recent retrospective study with 156 cirrhotic patients identified from outpatient gastroenterology and primary care practices showed that patients with NASH cirrhosis were less likely to be under surveillance than patients with cirrhosis from other etiologies and were less likely to receive gastroenterology referral [123].

The prognosis of patients with HCC is dictated by the initial stage at diagnosis of HCC. Long-term overall survival or disease-free survival is possible only in patients who present with early-stage HCC which is amenable to potentially curative treatments, typically liver transplantation or surgical resection. There is substantial evidence that HCC surveillance increases the early detection of tumors and improves the survival of patients with HCC [124, 125]. Admittedly, this observation may in part be due to lead-time or length-time biases. However, the use of HCC surveillance is still controversial primarily because of the lack of robust level 1 evidence from randomized trials supporting the use of HCC surveillance. Thus far, two randomized controlled trials have investigated the efficacy of surveillance in reducing HCC mortality. Both studies were in cohorts of HBV patients from China. One randomized controlled trial was performed with 5,581 HBV patients [126]. This study used serum alpha-fetoprotein (AFP) as the primary tool for surveillance and showed that surveillance increased the detection of early-stage tumor but did not affect overall survival and liver cancer mortality. The second study of 19,000 HBV-positive individuals showed a positive effect of HCC surveillance on HCC mortality [127]. Although there was a high dropout rate of study participants, individuals under biannual surveillance with serum AFP and abdominal ultrasound (US) had a 37 % decreased risk of mortality from HCC than individuals not under surveillance. Unfortunately, no randomized controlled trials have investigated the efficacy of HCC surveillance in patients with etiologies other than HBV.

Table 34.1 Candidates for HCC surveillance

Cirrhosis	Non-cirrhotic HBV carrier
HBV cirrhosis	Asian men >40 years old
HCV cirrhosis	Asian women >50 years old
Alcoholic cirrhosis	Family history of HCC
Nonalcoholic steatohepatitis	Africans >20 years old
Primary biliary cirrhosis	
Other cause of cirrhosis	

Currently, based on studies showing an annual risk of HCC greater than 1.5 % per year, the American Association for the Study of Liver Diseases (AASLD) recommends that patients with cirrhosis, regardless of etiology, should be under HCC surveillance [128]. In addition, it is recommended that high-risk hepatitis B carriers (i.e., with annual HCC incidence >0.2 %) including Asian males aged over 40, Asian females aged over 50, Africans aged over 20, those with high viral load or elevated transaminases, and patients with a family history of HCC should enter HCC surveillance (Table 34.1) [128]. Surveillance should be performed every 6 months; this interval is based on how fast HCC tumors grow rather than the degree of HCC risk in each individual patient. In addition, it has been shown that a program of surveillance every 6 months can result in better clinical outcomes compared to surveillance every 12 months [129].

Abdominal US and serum AFP are the most commonly used tests for HCC surveillance in clinical practice. Although abdominal US is highly dependent upon operator experience and delineation of tumor nodules can be challenging in patients with obesity and nodular liver, abdominal US has been shown to have a sensitivity over 60 % and specificity over 90 % as a screening test for HCC [124]. These estimates may not be achieved in routine practice [130]. CT and MRI are not routinely used for HCC surveillance due to their high cost and the risks of repeated exposure to radiation (for CT) and contrast (for both modalities). These modalities are sometimes used as screening tests in patients listed for liver transplantation for whom the cost- and risk-benefit ratios may be in favor of their use. The use of serum AFP for HCC surveillance is controversial because of the poor sensitivity of AFP testing for early-stage disease; however, some proponents argue that AFP is still of value in patients for whom high-quality US evaluation cannot be obtained due to obese patient habitus or a lack of experienced ultrasonographers.

10 Diagnosis of HCC

The majority of HCCs can be diagnosed without biopsy of the tumor as HCC is a highly vascular tumor and has the specific radiologic finding of arterial enhancement and venous phase washout on contrast CT or MRI. The AASLD recommends a systematic approach for evaluating suspicious nodules found by surveillance US of patients at risk for HCC [128]. If the nodule is smaller than 1 cm, then follow-up with a repeat US every 3 months is recommended. If there is no change in the size of the lesion, US should be repeated every 3 months for up to 2 years without further investigation with CT or MRI. This is because most small nodules are noncancerous and small subcentimeter HCCs usually have not acquired the typical features of hypervascularity with venous washout, making a conclusive diagnosis by cross-sectional imaging difficult. If the nodule remains the same or regresses, it can be presumed that the nodule was not an HCC and regular 6 months surveillance can be reinstituted. If the nodule is larger than 1 cm on initial observation or becomes larger than 1 cm during follow-up, then further workup using contrast-enhanced four phase CT (non-contrast, arterial, portal, and delayed phase) or dynamic MRI is used for the confirmatory diagnosis of HCC (Fig. 34.3). If either MRI or CT shows the characteristic features of arterial enhancement and portal venous or delayed phase washout, then the diagnosis of HCC is confirmed (Fig. 34.4). If the initial cross-sectional imaging study – CT or MRI – shows inconclusive features, then the other test should be performed next to determine if it will reveal characteristic features of HCC. If the second imaging study shows characteristic features, then HCC is diagnosed. If not, percutaneous liver biopsy may be performed.

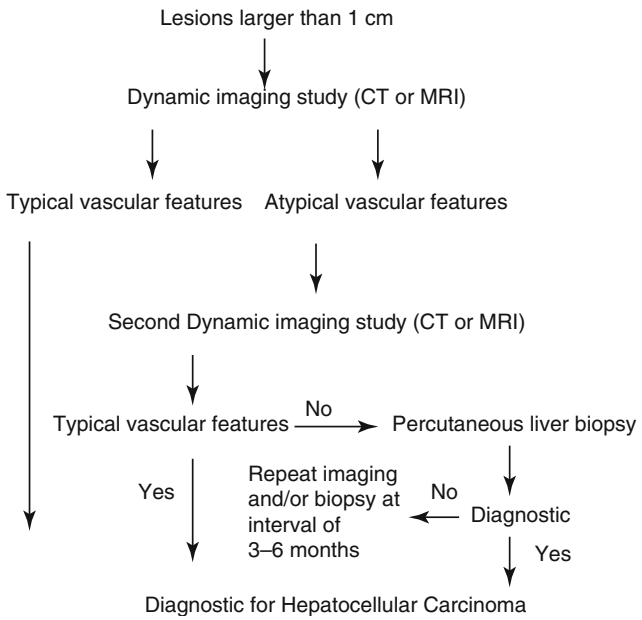

Fig. 34.3 Diagnostic algorithm of HCC

Percutaneous liver biopsy is indicated when lesions (>1 cm) develop without background liver cirrhosis or dynamic imaging modalities show inconclusive results. Since percutaneous needle biopsy is susceptible to sampling error with an up to 10 % false-negative rate for the biopsy of small HCCs, a negative result does not rule out HCC and close follow-up is required at 3- to 6-month intervals until the nodules disappear or acquire the typical radiologic features of HCC [131]. Percutaneous biopsy should be used with caution, particularly in patients who are eligible for curative treatment such as surgical resection or liver transplantation because of the 2–3 % risk of needle track seeding [132]. In the USA, since the United Network for Organ Sharing (UNOS) does not apply, Model for End-Stage Liver Disease (MELD) points are not assigned to patients with HCC until the masses reach at least 2 cm in size; in patients who are otherwise eligible for treatment with liver transplantation, many centers will follow >1 cm nodules with serial imaging until they reach 2 cm in size.

While the serum AFP is no longer used as a diagnostic criterion for HCC in the current AASLD practice guideline, levels of 400–500 or greater have 98 % or higher specificity for the diagnosis of HCC, similar to the specificity of the combination of arterial enhancement and venous washout by CT or MRI for the diagnosis of HCC; it may therefore have utility in selected settings.

11 Treatment of HCC

Treatment of HCC depends on multiple factors including extent of tumor, severity of liver dysfunction, performance status of the patient, socioeconomic support, and the local availability of different types of treatment. Surgical resection, liver transplant, and local ablative treatment are potentially curative treatment modalities that can be offered to patients with early-stage HCC (Fig. 34.5). Transarterial chemoembolization and radioembolization are locoregional treatment modalities that are used in patients with intermediate-stage HCC. Sorafenib is a targeted systemic multikinase inhibitor that shows limited effectiveness in patients with advanced stage HCC (Fig. 34.5).

11.1 Surgical Resection

Surgical resection can be curative for HCC in patients with well-preserved liver function and limited tumor extent. Candidates for surgical resection should be carefully

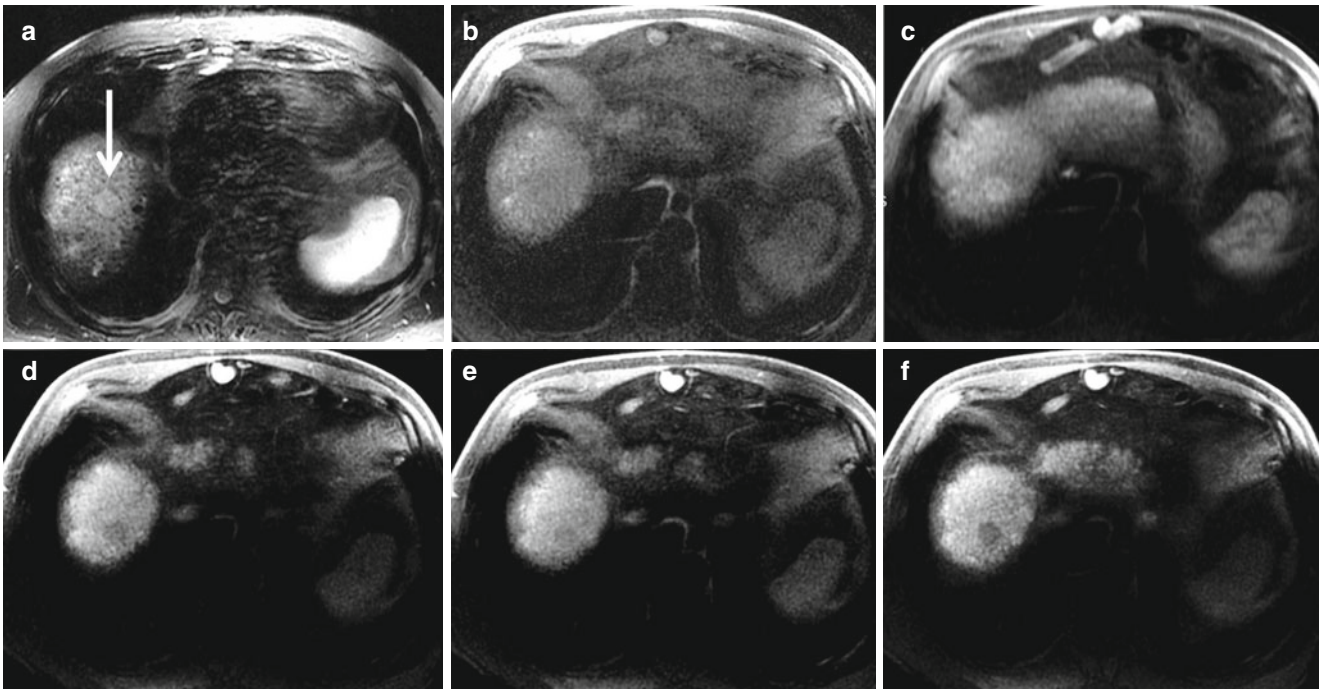

Fig. 34.4 Radiologic characteristics of HCC. HCC in a 67-year-old male with chronic hepatitis C. MRI study performed with intravenous Gd-EOB-DTPA (Eovist). HCC (*arrow*) is hyperintense on axial T2-weighted image (**a**), barely visible on pre-contrast T1-weighted image (**b**) shows hyperenhancement in arterial phase (**c**) and washout in portal venous (**d**) and delayed (**e**) phases. No uptake of contrast in the 20-min hepatocyte phase (**f**)

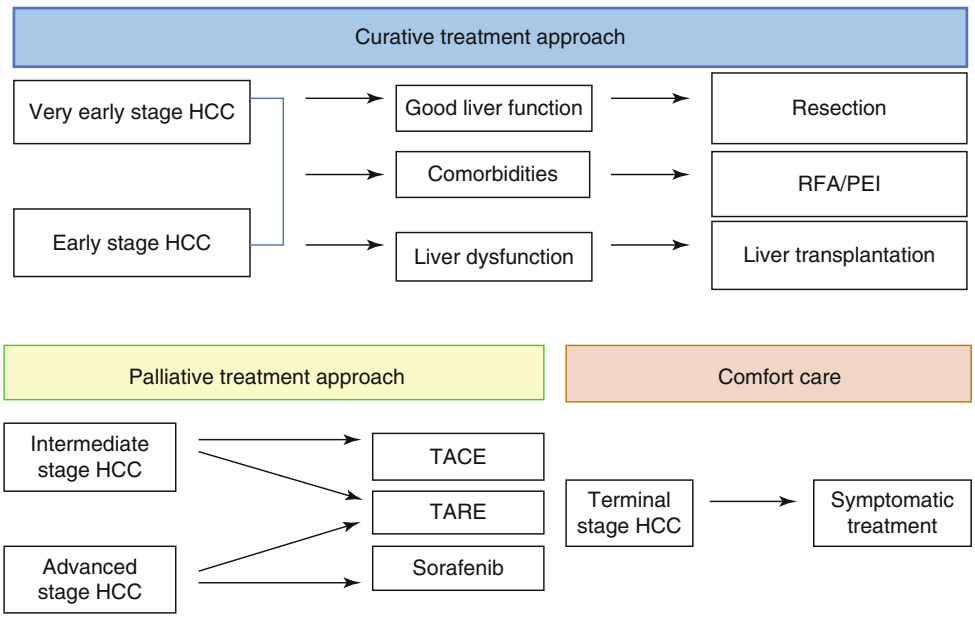

Fig. 34.5 Treatment recommendation according to a modified BCLC staging classification. *RFA* radiofrequency ablation, *PEI* percutaneous ethanol injection, *TACE* transarterial chemoembolization, *TARE* transarterial radioembolization

selected after thorough evaluation of underlying liver function and tumor extent as there is a risk of tumor recurrence and also of postoperative hepatic decompensation. In patients who develop HCC in the context of cirrhosis, the remaining liver continues to be at risk of development of cancer after surgical resection either because of the development of new HCCs in the cirrhotic liver or because of growth of micrometastasis from the previously resected HCC that was not visible in the initial imaging. The risk of recurrence after resection of HCC exceeds 70 % at 5 years [133]. Hepatic reserve is assessed by the indocyanine green retention test in some Asian countries and signs of clinically significant portal hypertension (thrombocytopenia, splenomegaly, varices) and bilirubin in Western countries [134].

A MELD score of 8 or less also reflects well-preserved liver function and is a strong predictor of both low perioperative mortality and long-term survival [135]. Dynamic multiphase CT or MRI is used for anatomic delineation of tumor extent. Surgical resection is typically not recommended for HCCs with associated vascular invasion or tumor metastasis, although it is performed in patients with macrovascular invasion in some Asian countries. Although large tumor size and number are associated with a high risk of recurrence after surgical resection, they should not preclude surgical resection in patients with resectable tumors and reasonable hepatic reserve [136]. A study using hospital discharge data from the Nationwide Inpatient Sample in the USA reported that the use of surgical resection has been decreasing over the past 15 years, whereas the use of liver transplant and radiofrequency ablation has increased in the USA [137].

11.2 Orthotopic Liver Transplantation

Orthotopic liver transplantation (OLT) is the most effective treatment for HCC. OLT completely removes the HCC and the rest of the benign liver and results in the best clinical outcomes. An increasing proportion of HCC patients in the USA receive OLT for HCC treatment [137]. The highly resource intensive nature of OLT and the requirement for long-term medical and social support are the main downsides of this treatment approach. There are significant racial disparities in likelihood of receiving liver transplant; African American and Pacific Islanders were about 40 % less likely to undergo OLT than Whites [138]. OLT is a preferred treatment modality in patients with early-stage HCC who are not good candidates for liver resection. A single center study in the USA showed that OLT is more commonly used in HCC patients with HCV, whereas surgical resection was more common in HCC patients with HBV. This difference was attributed to the smaller tumor extent and worse liver function in the HCV cohort versus the HBV cohort [139]. As the availability of organs is very limited, strict criteria exist for selection of candidates for OLT. The Milan criteria (a single tumor 5 cm or less in diameter or up to three lesions with the largest no more than 3 cm in diameter) are generally used to select candidates with HCC for OLT. Patients transplanted for HCC who meet these criteria achieve long-term survival that is similar to that of patients receiving OLT for non-HCC indications [140]. As some patients who are beyond Milan criteria can benefit from OLT, more liberal selection criteria have been proposed as expanded selection criteria for OLT, including the University of California San Francisco (UCSF) criteria [141, 142]. These criteria are yet to be universally implemented by UNOS. For HCCs that are initially beyond UNOS criteria, a downstaging strategy using local or locoregional therapy to reduce the tumor size and number to meet the Milan criteria, followed by subsequent transplantation, has shown encouraging results and is used in several institutions [143, 144]. A meta-analysis which included 720 patients who underwent transplantation following downstaging after initial presentation with disease outside the Milan criteria showed that absolute and disease-free survival rates in patients transplanted following downstaging are comparable to those in patients within the Milan criteria [145].

11.3 Living-Donor Liver Transplantation

Living-donor liver transplantation (LDLT) has been developed mostly in Asian countries and successfully replicated worldwide to address the lack of donor liver availability, despite significant morbidity and some mortality risk to donors [146]. LDLT may be an effective treatment modality in an environment of deceased donor liver shortage considering the current strict eligibility for listing and significant dropout rate from tumor progression or death while on the waiting list for OLT [147].

11.4 Percutaneous Ablation

Percutaneous ablation has been used more frequently in the USA over the past 15 years as it is less invasive than surgical treatment but results in similar clinical outcomes in selected patients. It is a potentially curative treatment for small tumors in patients who are not eligible for liver transplantation or resection due to comorbidities, liver dysfunction, or limited surgical resources. Percutaneous ethanol injection (PEI) has an excellent treatment effect for small tumors as complete necrosis is achieved in most HCCs smaller than 2 cm [148]. Currently, radiofrequency ablation (RFA) is now more frequently used than PEI. Randomized clinical trials have shown that complete tumor necrosis (96 % in RFA vs. 88 % in PEI), 3-year recurrence free survival (34–49 % in RFA vs. 12–43 % in PEI), and overall survival (62–78 % in RFA vs. 36–72 % in PEI) are significantly higher in patients with RFA than PEI [149]. Studies including two randomized control trials showed that RFA has similar efficacy to surgical resection in the treatment of early-stage HCCs with a lower complication rate and cost [150–153]. A study using a Markov model showed that RFA is as effective as surgical resection in treatment of small HCCs. A retrospective comparative study showed that RFA is superior to surgical resection in HCC less than 2 cm in terms of overall survival (72 % vs. 62 % for 5-year overall survival, $p<0.05$) and treatment complications (19 % vs. 51 %, $p<0.01$) [154].

11.5 Transarterial Chemoembolization

Transarterial chemoembolization (TACE) is the most commonly used treatment modality in patients with HCC [33]. TACE delivers a chemotherapeutic agent via the hepatic artery branches into the tumors while embolizing the tumor vasculature. TACE is primarily indicated in patients with unresectable HCC without portal vascular invasion or extrahepatic metastasis. Although patients with unresectable multifocal HCC have a poor long-term prognosis, TACE has been shown to improve overall survival in the intermediate time frame, when compared to supportive management (2-year overall survival of 31–63 % and 11–27 % in the TACE and control groups, respectively) in two randomized controlled trials [155, 156]. It was shown that patients who responded to the treatment have longer overall survival than patients who did not respond [157]. Although TACE was traditionally considered contraindicated in patients with portal vein invasion, one retrospective cohort study with 125 patients with HCC and main portal vein invasion showed that TACE improved the overall survival. The multivariate analysis showed that treatment with TACE with a hazard ratio of 0.26 ($P<.001$) and Child-Pugh class A status with a hazard ratio of 0.55 ($P=.004$) were independent predictive factors of a favorable outcome [158].

TACE is an alternative treatment option in patients with early-stage HCC when ablative treatment cannot be safely performed due to the tumor location. TACE is also frequently used for downsizing HCC tumors or as a bridging treatment prior to liver transplantation [143, 159]. Drug-eluting bead TACE is a newer technique that uses beads loaded with doxorubicin that are designed to gradually release the chemotherapeutic agent into the tumor, resulting in effective delivery of a high concentration of chemotherapeutic agent into the tumor while minimizing systemic release. This technique appears to be as effective as or superior to traditional TACE in terms of efficacy and safety [160]. As with TACE, patients with poor liver function or portal vein invasion should not be routinely treated with this treatment modality due to the high risk of acute liver decompensation [161]. One small randomized controlled trial showed that the combination of TACE and RFA was superior to RFA alone in patients with solitary HCC. The rates of local tumor progression at the end of the third year in the RFA and TACE-RFA groups were 39 and 6 %, respectively ($P=.012$) [162]. This result awaits further validation in larger groups of the patients. Combination TACE-RFA was also shown to be as effective as surgical resection in terms of overall survival in one retrospective study [163].

11.6 Transarterial Radioembolization

Transarterial radioembolization (TARE) with Y90-impregnated glass microspheres (TheraSphere®) or resin beads (SirSphere®) has been increasingly used for patients with unresectable multifocal HCC, including HCC with portal vein invasion. TARE appears to achieve equivalent survival outcomes with acceptable safety and improved tolerability as compared to TACE [164, 165]. A single center study in the USA analyzed 245 patients treated with TARE ($N=123$) and TACE ($N=122$) and showed that TARE resulted in a longer time to progression of HCC (13 vs. 8 months, $p<0.05$) with comparable overall survival (21 vs. 17 months, $p=0.23$) [166]. A number of multicenter cohort studies also support these findings [167].

11.7 Conventional Chemotherapeutics

Most HCCs are resistant to conventional chemotherapeutic agents. Moreover, HCC patients usually have poor tolerance to systemic chemotherapy due to hepatotoxicity and bone marrow suppression. Several molecular pathways have been implicated in liver carcinogenesis including receptor tyrosine kinases, Wnt/β-catenin, ubiquitin-proteasome, epigenetic promoter methylation and histone acetylation, PI3Kinase/AKT/MTOR, pro-angiogenic molecules, and telomerase [168]. Targeted therapies against these pathways are under active investigation. Sorafenib, an oral multikinase inhibitor that targets the Raf kinase, VEGFR, and PDGFR signaling pathways, is approved for the treatment of advanced HCC. In a phase 3 randomized placebo-controlled trial of 602 patients with advanced HCC, overall survival was 10.7 months in the sorafenib group compared with 7.9 months in the placebo group, with an acceptable side effect profiles [169]. Sorafenib was also shown to improve survival in a phase 3 randomized control trial performed in Asian Pacific countries where HBV was the dominant etiology. Overall survival was 6.5 months in the sorafenib group compared with 4.2 months in the placebo group ($p=0.01$) [170]. The most common side effects are diarrhea, fatigue, and hand-foot syndrome, and side effects appear to be more common in patients with advanced cirrhosis [171]. Recent results suggest that patients with amplification of the FGF3/FGF4 locus on chromosome 11, those with lung metastases, and those with more poorly differentiated tumors are more likely to respond to sorafenib [172]. Immunotherapy against glypican 3, a heparan sulfate proteoglycan that is overexpressed in HCCs, has been reported to be effective in improving overall survival in phase 1 clinical trials [173]. Combinations of sorafenib and other targeted agents or locoregional treatment are under active investigation.

12 Unresolved Problems

The disease burden of HCC is increasing worldwide. Since viral hepatitis is the major etiology of HCC, improved prevention and treatment of viral hepatitis should decrease the incidence of HCC. This can be achieved by increasing

HBV vaccination rates, through improved awareness of viral hepatitis by patient education in order to minimize high-risk behaviors, such as needle sharing, and through health-care provider education to minimize iatrogenic transmission. It is also critical to screen high-risk individuals and identify those who have already contracted chronic HBV or HCV infection. There is still a major gap in implementation of HBV and HCV screening, and the majority of patients are diagnosed with viral hepatitis after they develop complications related to liver cirrhosis, when the opportunity to decrease the risk of HCC has largely been lost. Patients with cirrhosis should be enrolled in surveillance programs for the early diagnosis of HCC, since the diagnosis of HCC at an early stage makes them eligible for potentially curative treatments that can result in long-term survival. Unfortunately, the majority of patients are diagnosed with HCC at an advanced stage. The optimal screening strategy for HCC is still controversial. The serum AFP clearly has limitations as a screening test for HCC due to its relatively poor sensitivity for the detection of early-stage disease when used in a single measurement. The repeated assessment of AFP and other markers in the context of a surveillance program by practitioners experienced in recognition of the patterns of nonspecific elevation of AFP can partially offset the concerns about the high rate of false-positive AFP elevations. However, there is clearly a major unmet need to identify sensitive, specific, and inexpensive biomarkers of early HCC. The development of sorafenib as a treatment for advanced HCC was a major landmark in therapy of HCC. However, sorafenib improves overall survival by 2–3 months and is therefore of only moderate efficacy as a single agent. Well-designed clinical trials testing the combination of sorafenib with other targeted treatments, with locoregional treatments, or in patients at high risk of recurrence after surgery or local ablation are needed. There is also the need to develop additional targeted treatments, virotherapy, and immunotherapy approaches to broaden the repertoire of therapeutic choices. Ideally, the development of these approaches will be paired with the identification of key molecular signatures predicting response to specific therapies. Recent major advances in antiviral and anticancer therapeutics and molecular and genetic analyses are very encouraging and give hope for substantial future improvements in the prevention and treatment of HCC.

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Human Herpesviruses: Cytomegalovirus

35

Robert F. Pass

1 Introduction

Human cytomegalovirus, hereafter referred to simply as CMV, is a member of the Herpesviridae, sharing physical and biological properties of this virus family. CMV produces a chronic infection in its human host, with prolonged viral shedding after initial infection and indefinite persistence thereafter in a latent state with perhaps continued low-level replication. Immunosuppression leads to reactivation of latent virus and loss of immune control of replication. Although other members of the Herpesviridae are occasionally transmitted vertically, transmission of CMV from the mother to the newborn or even prenatally is common. It occurs through multiple mechanisms and plays a key role in the epidemiology of CMV infection and in maintaining the prevalence of CMV infection in a population. Cytomegalovirus infection is prevalent in all human populations though the age at acquisition of the virus varies largely due to differences in child rearing and reproductive behavior.

The medical and public health significance of CMV infection is due to the fact that it is the most common cause of congenital infection in developed countries and is a common opportunistic infection in immunocompromised patients. Congenital CMV infection is probably the most common infectious cause of central nervous system (CNS) impairment among children in the United States. It is a leading cause of sensorineural hearing loss and an important cause of mental retardation and cerebral palsy. Cytomegalovirus infection has been particularly troublesome in transplant patients because it can be transmitted with the graft or with blood products and it is reactivated by immunosuppressive medications that are necessary to prevent rejection of the graft. Cytomegalovirus infection is ubiquitous among adults with HIV infection and prior to the advent of highly active antiretroviral treatment (HAART). CMV was a frequent cause of vision loss due to retinitis and damage to multiple organ systems. In addition, CMV is suspected of contributing to the development of chronic vascular disease, autoimmune disease, and immune senescence. Although antiviral treatment is available for CMV infections, it is not completely effective. Prevention of congenital CMV infection through vaccination is an important public health goal, and multiple vaccines are in development.

2 Historical Background

The clinical and histopathologic findings of cytomegalic inclusion disease were recognized for decades prior to the recovery of cytomegalovirus from human tissue in 1955. In 1904, there were two reports describing histopathologic changes consistent with CMV cytopathology (intranuclear inclusion surrounded by a clear zone – the "owl's eye" appearance) in tissues from newborns, although at that time syphilis and parasitic infection were considered likely etiologies [1, 2]. Over the years others described similar histopathologic findings in the salivary glands, lungs, liver, and kidneys from infants and children. The term cytomegaly was used initially in 1921 [3]. It was suggested that the cytopathology seen in some stillbirths was associated with a prenatal insult and that this cytopathology was similar to that seen with varicella zoster and herpes genitalis infection [3, 4]. Subsequently, the cytopathology associated with the fatal prenatal infection was noted in the salivary glands of infants who had died from other causes, suggesting that it could be due to a common agent [5]. In 1950, the term "generalized cytomegalic inclusion disease" was first used to describe infants with multiorgan involvement; diagnosis of this condition by premortem detection of cytomegalic cells in the urine of affected newborns was reported in 1952 [6, 7]. Tissue culture isolation of human CMV occurred in the mid-1950s in three laboratories roughly simultaneously.

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Weller reviewed the history of the initial tissue culture isolation of human CMV [8]. The first reported isolation and propagation of a cytopathic agent from a fatal case of cytomegalic inclusion disease was in 1956 from the laboratory of Margaret Smith [9]. At about the same time, investigators in the Weller laboratory inoculated specimens from a newborn with hepatosplenomegaly, intracranial calcification, and chorioretinitis into tissue culture derived from the embryonic skin and muscle and noted the development of cytopathic changes now considered characteristic of CMV; they concluded that they had isolated the etiologic agent of cytomegalic inclusion disease [10]. In the Rowe laboratory, an agent isolated from the adenoids of children produced cytopathology that was not as expected for adenovirus but was similar to the cytopathic agent isolated by the Smith and Weller laboratories. Rowe et al. developed an antibody assay and determined that the majority of adults had antibody to this agent [11]. The isolation and propagation of CMV opened the door for the development of serological and other diagnostic methods as well as in vitro study of the virus. In less than 10 years from the reported isolation of CMV from humans, seroconversion to CMV was associated with a heterophile-negative mononucleosis, cytomegalic inclusions were reported in the lungs of immunosuppressed adults, and the initial case reports of CMV disease in renal transplant patients were described in a report that anticipated the finding that the transplanted kidney would be a source of infection [12–14]. Early studies of bone marrow transplantation as an experimental treatment for aplastic anemia and acute leukemia in the late 1960s and early 1970s found that CMV was an important cause of interstitial pneumonia that was often fatal [15, 16]. Approximately a decade later cytomegalovirus was recognized as an important opportunistic pathogen in the initial reports of acquired immunodeficiency in homosexual men [17, 18].

3 Methodology

3.1 Mortality

Multisystem disease at birth is evident in approximately 5–10 % of congenital CMV infections; it is occasionally fatal. A recent study used data from US death certificates to estimate mortality due to congenital CMV infection [19]. Death certificates contain basic demographic information and can list up to 20 causes or contributors (identified by ICD-9 or ICD-10 codes). During the study interval from 1990 through 2006, 777 congenital CMV-related deaths were identified; 557 (71.7 %) occurred in infants <1 year of age, 0.11 % of the total deaths among US infants. The annual number of deaths from congenital CMV infection varied from 36 to 56, and there was no trend in either direction during the study interval. Demographic breakdown of CMV-related deaths and adjusted mortality rates are shown in Table 35.1. The mortality rates by race and region roughly parallel overall CMV seroprevalence and congenital CMV infection rates documented in other sources. However, this study was limited by (1) errors in death certificates, both in failing to include congenital CMV infection and in incorrectly concluding that CMV contributed to death, (2) the omission of fetuses that die prior to being recorded as a live birth, and (3) possible failure to diagnose congenital CMV infection.

In the past, CMV infection was an important cause of mortality among bone marrow (stem cell) transplant patients. Antiviral prophylactic and treatment regimens have reduced CMV mortality dramatically in both stem cell and solid organ transplant patients. Similarly, CMV was a frequent opportunistic infection among terminal AIDS patients with very low CD4+ T-cell counts. Where effective antiretroviral therapy has been employed, it has prevented loss of immune function and made CMV disease and mortality uncommon.

Table 35.1 Deaths related to congenital CMV infection and age-adjusted mortality based on data from US death certificates, 1999–2006

Variable		Congenital CMV-related deaths, *n* (%)	Mortality rate[a] (95 % CI[b])
All		777 (100 %)	0.16 (0.15, 0.17)
Sex	Male	340 (43.8 %)	0.15 (0.13, 0.16)
	Female	437 (56.2 %)	0.18 (0.16, 0.19)
Race/ethnicity	Asian	15 (1.9 %)	0.08 (0.04, 0.12)
	African American	199 (25.6 %)	0.27 (0.23, 0.31)
	Hispanic	123 (15.8 %)	0.14 (0.11, 0.16)
	Native American	15 (1.9 %)	0.34 (0.17, 0.51)
	White	425 (54.7 %)	0.14 (0.13, 0.16)
Region	Northeast	82 (10.6 %)	0.10 (0.10, 0.12)
	West	186 (23.9 %)	0.16 (0.14, 0.19)
	Midwest	183 (23.6 %)	0.17 (0.15, 0.19)
	South	326 (42.0 %)	0.19 (0.17, 0.21)

Modified from Bristow et al. [19]; with permission
[a]Age-adjusted mortality per one million person-years
[b]Confidence interval

Fatal CMV disease in a normal host is extraordinarily rare; there are no data upon which to base an estimate of mortality rate. It is possible, however, that CMV influences overall mortality. Studies in older Latinos and in older adults with stable cardiovascular disease have associated CMV antibody level with mortality [20, 21]. Whether CMV infection influences cardiovascular and all-cause mortality was investigated in a representative sample of the US population from the National Health and Nutrition Examination Survey (NHANES) III, 1988–1994, and its linked mortality file, which matched participants with the US National Death Index [22]. After adjusting first for demographic factors (age, gender, race/ethnicity, country of origin, level of education), then for clinical factors (smoking, body mass index, and diabetes), and finally for serum C-reactive protein level, CMV seropositivity remained significantly associated with all-cause mortality (hazard ratio = 1.19, 95 % confidence interval 1.01–1.41). After adjusting for multiple confounders, there was not a statistically significant association between cardiovascular death and CMV seropositivity. These intriguing findings merit further study.

3.2 Morbidity

Because CMV infection and disease are reportable in only a few states, there are no data from public health reports that can be used to provide a population-based estimate of morbidity. Aside from disease in immunocompromised patients, the most important morbidity from CMV infection involves cognitive, motor, and sensory impairments that occur in children with congenital infection; and they are usually not recognized in infancy. Since approximately 90 % of newborns with congenital CMV infection lack signs or symptoms at birth, an accurate estimate of morbidity will require surveillance based on virological screening of newborns. Current estimates of morbidity due to congenital CMV infection and in immunocompromised patients are based on rates from convenience samples.

Although only a minority of infants with congenital CMV infection manifest it clinically, all newborns with congenital CMV infection shed virus in the urine and saliva. Morbidity from congenital CMV infection can be divided into clinical and laboratory abnormalities in the newborn and subsequent central nervous system (CNS) impairments. Neither the abnormalities in the newborn nor their sequelae are specific for CMV; similar morbidity occurs with other infections or diseases. Detection of congenital CMV infection requires screening newborns for the presence of the virus in body fluids. In an analysis of 15 studies (including 117,986 newborns with at least 800 in each study), virological methods detected congenital CMV infection at rates ranging from 0.3 to 1.3 %; the overall rate was 0.7 % of live births [23]. Clinical evidence of disease (symptomatic congenital infection) was present in 12.7 % of the 117,986 newborns with congenital infection [23]. It was estimated that CNS sequelae occurred in 40–58 % of infants who were symptomatic at birth and in 13.5 % of those who had no evidence of disease at birth [23]. The authors estimated that for every 1,000 cases of congenital CMV infection, there would be 5 deaths and 168–188 children with permanent sequelae; it was acknowledged that this could be an underestimate because some studies in their analysis did not provide adequate follow-up assessment of cognitive and vision function. Assuming 4,000,000 US births per year with a 0.7 % congenital CMV infection rate, there are approximately 28,000 newborns with congenital CMV infection born each year. Among those, approximately 140 deaths would occur and 5,000 children would develop permanent sequelae due to CMV infection.

3.3 Serological Methods

Cytomegalovirus infection results in production of IgG antibody to CMV which persists indefinitely; detection of IgG antibody to CMV identifies subjects who have ever had CMV infection and is the basis for seroprevalence studies. Commercially available, automated methods for detection of CMV antibody can distinguish CMV-infected from uninfected persons with a high degree of accuracy and are used to screen blood and organ or tissue donors as well as transplant recipients. Incident CMV infection is usually identified by serological methods including seroconversion from CMV IgG antibody negative to positive. Antibody isotype and avidity can be used to identify recent CMV infection in someone who tests positive for IgG antibody to CMV. After initial (primary) CMV infection, IgM antibody to CMV and low-avidity IgG antibody will be present in serum for around two to 9 months. These methods are now being used in the evaluation of women suspected of having acquired CMV during pregnancy. Serological methods are also useful in evaluating non-immunocompromised patients with mononucleosis-like illness or other symptoms that are compatible with primary CMV infection. Among immunocompromised patients, serological results are not as useful. Although screening for CMV infection prior to organ transplantation or immunosuppression can identify those at risk for primary infection or reactivation after transplantation, serum antibody measurements in immunocompromised patients are not helpful in determining whether or not CMV is causing disease; quantification of CMV viremia is preferred for this determination. Serological methods are not appropriate for diagnosis or surveillance for congenital CMV infection; virus detection by culture or polymerase chain reaction is more accurate.

3.4 Detection of Virus

Viral detection is the most reliable method for diagnosis of congenital CMV infection and for screening newborns (surveillance) for congenital CMV infection. Detection of CMV in body fluids of a newborn is proof of congenital CMV infection, and failure to detect CMV is proof that congenital infection is not present. In most other clinical settings, the presence or absence of CMV in body fluids cannot be as unambiguously interpreted. Cytomegalovirus is shed in body fluids for months to years after initial infection and shedding can recur intermittently afterward. Beyond the newborn period, shedding of CMV in body fluids signifies CMV infection but cannot reliably predict when that infection occurred unless the subject was known to be CMV antibody negative prior to and near the time that viral shedding was found. The presence of viremia is more likely to be associated with recent than past infection in an immunocompetent subject, but viremia can persist for months after primary infection.

Historically detection of CMV was based on tissue culture isolation of virus, a method that involves inoculation of clinical samples onto monolayers of human fibroblasts, careful maintenance of the tissue, with regular observation for 4 or more weeks, and identification of CMV by its characteristic focal cytopathic effect. Tissue culture methods were modified to achieve more rapid results, using monoclonal antibody to immediate early viral proteins to identify CMV in tissue culture (on coverslips in small vials or in 96-well plates) within 24–48 h of inoculation [24, 25]. Polymerase chain reaction (PCR) methods for detection of CMV DNA have a number of advantages over tissue culture-based methods. They are more rapid, making it possible to have results in hours rather than days; PCR is also more sensitive and more amendable to automation than tissue culture methods and can give quantitative results as genome equivalents per unit of sample (volume, number of cells, or mg of DNA). In immunocompromised patients, shedding of CMV in body fluids does not reliably correlate with the presence of disease due to CMV infection. The quantity of virus (or viral DNA) in the blood is more likely to correlate with CMV disease; however, the quantity of virus that is likely to be associated with CMV disease differs for different types of immunocompromised patients (solid organ transplant recipients, stem cell transplant patients, AIDS patients).

4 Biological Characteristics of Human Cytomegalovirus

Human cytomegalovirus is a large, double-stranded DNA virus with physical similarities to other members of the family *Herpesviridae*. CMV is approximately 200–300 nm in diameter with a lipid bilayer envelope through which project spikes of a number of virus-encoded glycoproteins. The envelope surrounds an amorphous tegument; within the tegument is a capsid of 162 capsomeres which contains the double-stranded DNA. The CMV genome is composed of 236,000 base pairs with 167 protein-encoding genes [26]. Reiterated terminal and internal base pair sequences divide the genome into unique long (U_L) and unique short (U_S) regions; specific genes and gene products are identified by their position in the U_L or U_S regions.

The sequence of events that unfold as virus gains entry to the host cell, activates its regulatory programs, copies its DNA, directs assembly of progeny virions, and releases these to the extracellular environment has been the subject of much study. A detailed review of that literature is recommended for the interested reader [27]. Cytomegalovirus attachment and entry are mediated by fusion of specific envelope glycoproteins with cell surface receptors including heparan sulfate, integrins, and platelet-derived growth factor. Cytomegalovirus can also enter cells via an endocytic pathway which is dependent on proteins encoded by the UL128, UL130, and UL131 gene complex. Replication of CMV involves stepwise transcription of genes as occurs with other herpesviruses; immediate early proteins appear within seconds of viral attachment and entry and serve a regulatory role, initiating replication. The delayed early proteins regulate viral DNA replication. Viral DNA replication, protein synthesis, and assembly of nonenveloped particles take place within the nucleus. The late phase of replication involves the synthesis of structural proteins, virion assembly, and release from the cell. The cytomegalovirus envelope appears to be acquired sequentially from the nuclear membrane and endosomal membranes in the cytoplasm and during transport through the Golgi apparatus and egress through the cell surface. The typical cytoplasmic inclusion seen in CMV-infected cells represents the accumulation of nucleocapsids and dense bodies (enveloped tegument without nucleocapsid or DNA) in the Golgi complex. Table 35.2 lists a small number of notable human CMV genes and gene products from a published list that includes information on function for over 100 gene products [27].

5 Descriptive Epidemiology

5.1 Demographics and Prevalence of Infection

Seroepidemiological studies of CMV prevalence have been performed on convenience samples from many different populations and ethnic groups from developed and developing countries. These studies show that CMV infection is endemic in every human population that has been studied,

Table 35.2 Human CMV genes and gene products, selected from a comprehensive published list which includes over 100 genes with assigned functions [27]

Gene	Product location or gene family	Product and function
UL32	Tegument	pp150, nucleocapsid stabilization
UL48	Tegument	Intracellular capsid transport
UL50, UL53	Tegument	Capsid egress from nucleus
UL52	Core	DNA incorporation into nucleocapsids
UL54	Core	DNA polymerase (target of ganciclovir and foscarnet)
UL55	Envelope glycoprotein	gB, multimeric forms, mediates entry
UL73	Envelope glycoprotein	gN, complexes with gM for envelopment
UL75	Envelope glycoprotein	gH, complexes with gL, role in entry when gH-gL complexes with gO or UL128-131A
UL83	Tegument	pp65, abundant tegument protein; suppresses interferon response
UL86	Capsid	Major capsid protein
UL97	Core	Viral protein kinase (phosphorylates ganciclovir)
UL100	Envelope glycoprotein	gM, complexes with gN
UL115	Envelope glycoprotein	gL, complexes with gH
UL123	Regulatory	Major immediate early protein; enhances transcription
UL128, UL130, UL131A	Envelope glycoproteins	pUL128, pUL130, and pUL131A couple with gH-gL to form pentameric complex for endocytic entry into endothelial and epithelial cells

Table 35.3 Prevalence of cytomegalovirus (CMV) infection among women of childbearing age

Study location, date	Population	*N*	% prevalence
Ankara, Turkey [28]	Women 15–49 years	745	99
Cotonou, Benin [29]	Pregnant women	211	97
Seoul, South Korea [30]	Prenatal clinic	575	96
Sendai, Japan [31]	Prenatal clinic	10,218	95
Sao Paulo, Brazil [32]	Pregnant women, middle SES[a]	427	67
	Pregnant women, lower SES	179	84
Northern Italy [33]	Women, pregnant or hospital patients	12,568	77
Helsinki, Finland [34]	Women, prenatal clinics	1,088	71
Birmingham, USA [35]	Prenatal, middle SES	12,140	54
	Prenatal, lower SES	4,078	77
Grenoble, France [36]	Women, prenatal clinic	1,018	52

[a]SES, socioeconomic status

From reference [26] with permission

though there are wide variations in age-related prevalence. Representative data from Europe, North and South America, Africa, and Asia is shown for women of childbearing age in Table 35.3. The prevalence of CMV infection in women of childbearing age is of great interest because of the public health importance of congenital infection. Studies of age-specific prevalence show dramatic differences between populations. For example, in upper- and middle-income groups from the United States and Europe, it is common for CMV acquisition to be delayed until adult years. In contrast, in developing countries prevalence is near 100 % during childhood and prior to sexual maturity. In the United States and Europe, the prevalence of HCMV infection is greater in the lower socioeconomic strata, among nonwhites, women, and immigrants from developing countries [36–38]. Child-rearing practices, particularly breastfeeding and group care of children, can have a major effect on CMV prevalence. In populations where the majority of mothers are CMV antibody positive and nurse their infants, infection rates of 50 % or more in the first year of life have been reported [39]. Group care of young children increases CMV prevalence [40]. Whether children acquire CMV from a maternal source or from other young children, they shed virus for years and will infect other children and susceptible adults. Among teenagers and young adults, sexual contact is an important source of CMV infection; indicators of sexual activity including the number of sex partners and presence of sexually transmitted diseases due to agents such as chlamydia have been associated with higher rates of CMV infection [41–44]. Customs that influence age at onset of sexual activity and sexual mores probably account for differences between populations in CMV infection rates in adolescents and young adults. Studies from developed countries report that prevalence of CMV infection is around 7–10 % higher in women than in men from the same population [38, 45]. Although CMV infection rates are higher in nonwhites than in whites, racial differ-

Table 35.4 Age-adjusted CMV seroprevalence in the noninstitutionalized, civilian population of the United States, aged ≥6 years

Characteristic		*N*[a]	Prevalence estimate, % (95 % CI[b])
Total		21,639	58.9 (57.1, 60.7)
Age, years	6–11	2,679	36.3 (32.8, 40.0)
	12–19	2,918	41.7 (38.3,45.3)
	20–29	3,302	49.3 (45.8,52.8)
	30–29	3,156	54.2 (50.3,58.1)
	40–49	2,483	64.5 (60.6, 68.2)
	50–59	1,800	74.2 (70.7, 77.4)
	60–69	2,257	83.0 (80.3, 85.4)
	70–79	1,721	88.8 (85.7, 91.2)
	≥80	1,323	90.8 (88.4, 92.7)
Sex	Male	10,243	54.1 (52.0, 56.1)
	Female	11,396	63.5 (61.4, 65.5)
Race/ethnicity	Non-Hispanic white	8,212	51.2 (49.2, 53.2)
	Non-Hispanic black	6,228	75.8 (74.7, 76.9)
	Mexican American	6,296	81.7 (80.2, 83.3)
Household income	Low	7,247	70.8 (68.3, 73.1)
	Middle	8,524	60.5 (57.5, 63.4)
	High	3,835	46.6 (44.2, 49.1)

Modified from reference [38]
Based on data from NHANES III, 1988–1994
[a]Actual sample sizes, unweighted
[b]Confidence interval

ences probably reflect differences in exposure related to child rearing, social customs, and crowding. Seasonal variation in occurrence of CMV infection has not been recognized, and epidemics have not been described; but there are reports of very high endemic infection rates in institutional populations such as day-care centers [40].

Although the majority of published studies of CMV prevalence are based on seroepidemiological study of convenience samples, two reports from the United States provide prevalence data from population-based samples. The National Health and Nutrition Examination Survey (NHANES III, 1988–1994) provided demographic data and sera from a randomly selected sample of the US population (≥6 years of age) [38]. Results summarized as age-adjusted seroprevalence are shown in Table 35.4. The overall prevalence of CMV infection was 58.9 %. Prevalence of CMV was higher in females (63.5 %) compared with males (54.1 %). Prevalence was also higher in non-Hispanic blacks (75.8 %) and Mexican Americans (81.7 %) than in non-Hispanic whites (51.2 %). Prevalence estimates increased from 36 % for the 6–11-year age group to 91 % for subjects ≥80 years of age. Factors associated with higher prevalence of CMV infection after multivariate analysis were fewer years of education, residence in the South, larger family size, foreign birth, and health insurance from a nonprivate source. NHANES data (including CMV antibody data) collected during three successive 2-year intervals between 1999 and 2004 were combined into NHANES 1999–2004, and findings were compared with those of NHANES III (1988–1994) [46]. Differences in CMV seroprevalence in the earlier and later periods were not significant. Age-related CMV prevalence estimates by sex, ethnicity, and race from NHANES 1999–2004 are shown in Fig. 35.1.

5.2 Incidence and Mechanisms of Transmission

Cytomegalovirus is acquired by contact with human body fluids that contain the virus. Knowledge of the mechanisms for transmission of CMV is based on deduction from epidemiological studies. The quantity of virus required to initiate infection is unknown. A small challenge study reported inoculation of five healthy CMV-seropositive men subcutaneously with 1×10^3 plaque forming units of a low-passage CMV strain from a newborn with congenital infection; the virus was recovered from only two subjects [47]. One of these subjects had fever, malaise, adenopathy, lymphocytosis, and abnormal liver function tests; the other subjects had milder symptoms and all symptoms cleared by 9 weeks post inoculation. There are no reported challenge studies examining other routes or sites of inoculation or in seronegative subjects. With naturally acquired infection, CMV is present in multiple body fluids for months or longer. Persons with past CMV infection shed virus in the urine, saliva, or genital secretions from time to time. The spread of CMV has been consistently associated with activities that lead to direct contact with body fluids of another person – intimate contact,

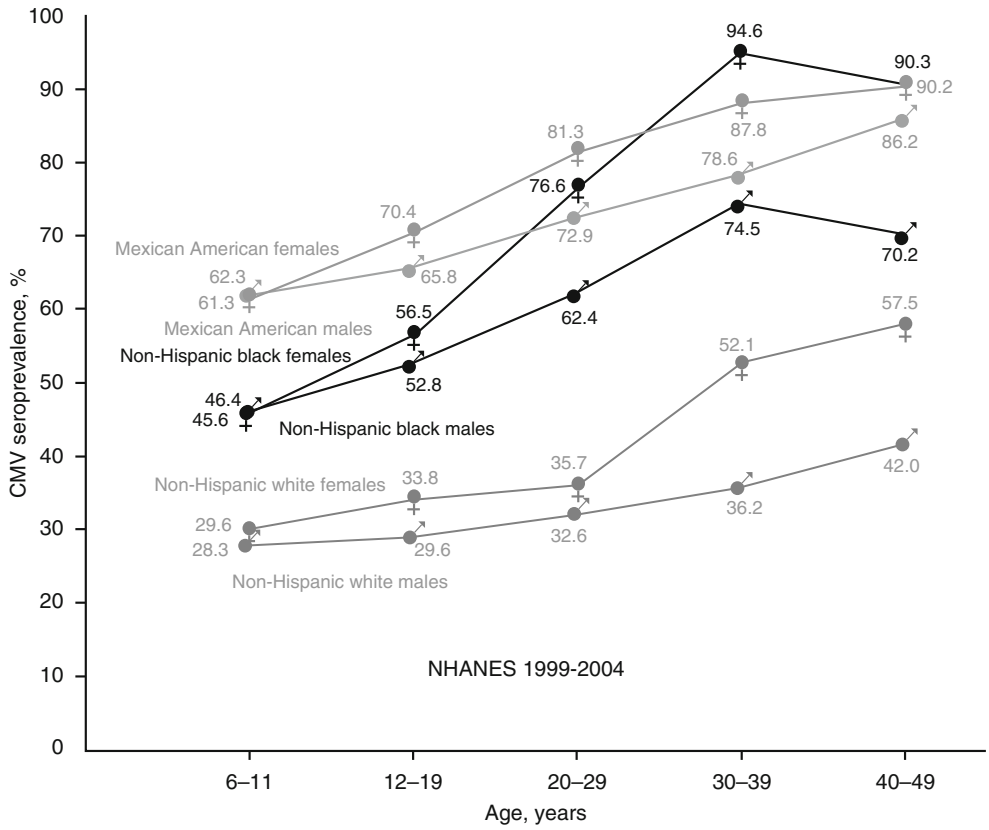

Fig. 35.1 Multivariate adjusted CMV seroprevalence by age group for the United States, based on the National Health and Nutrition Examination Survey (NHANES), 1999–2004. Results are stratified by age, sex, and race/ethnicity. Numbers adjacent to circles are prevalence. Male and female data are shown by their respective icons, ♂ and ♀. For the youngest age group, *circles* were plotted slightly above or below true values in order to separate male and female results (Reproduced with permission from Bate et al. [46])

Table 35.5 Incident CMV infection rates from follow-up of seronegative adults not selected for expected increased risk of infection

Study	Population	Sex	Age, mean years (range)	Race, % nonwhite	*N*[a]	CMV infections	Annualized rate, %/year
Birmingham, AL, USA, 1978–1984 [49]	Pregnant women, upper/middle income	Female	25.4	9.4 %	4,692	77	2.5
	Pregnant women, low income	Female	21.3[b]	89 %	507	19	6.8
Minneapolis, MN, USA, 1986[c] [50]	Nurses, nursing students, blood donors	F:M ≈ 3:1[c]	25.8 (18–59)[c]	<2 %	519	16	1.8
Birmingham, AL, USA, 1993–1998 [51]	Multigravida pregnant women, low income	Female	23.8	83 %	604	142	5.9
Giessen, Germany, 1992–2002 [45]	Blood donors	Female	(18–60)	Not given	6,018	88	0.65
	Blood donors	Male	(18–60)	Not given	7,142	67	0.45

Studies that followed 500 or more seronegative subjects and provided annualized infection rates are included

[a]*N* is the number of CMV-seronegative subjects followed

[b]This is the mean age for 4,078 low-income women screened; the mean age for the seronegative subjects was not given

[c]This study did not provide interval dates for data collection; it was published in 1986. The sex ratio and age listed are for the 943 subjects screened for antibody to CMV; specific data for the seronegative subjects followed was not given

child care, parturition, breastfeeding, blood transfusion, and organ transplantation. Rates for incident CMV infection in young adults are of interest because they can inform estimation of risk of acquiring CMV during pregnancy. Table 35.5 lists cohort studies that have followed 500 or more seronegative subjects, measured infection rates by repeated testing for antibody to CMV, and expressed results as an annualized rate. The study of blood donors from Germany reported higher seroprevalence and incidence of CMV infection in women compared with men [45]. Prevalence data from NHANES III were used to estimate CMV incidence for the US population between 12 and 49 years of age [48]. The overall incidence for seronegative persons in this age range was 1.6 per 100 person-years. The incidence was lower

Table 35.6 Reported high rates of CMV infection among adolescents and adults

Study	Exposure	Rate, %/year
Mothers [52]	Premature infant with transfusion-acquired CMV	47
Parents [53]	Child in group day care	21
	Child attends day care and is shedding CMV	30
Day-care workers, Iowa [54]	Caregiver for preschool-aged children	7.9
Day-care workers, Richmond, VA [55]	Caregiver for preschool-aged children	11
Day-care workers, Toronto [56]	Caregiver for preschool-aged children	12.5
Day-care workers, Birmingham, AL [57]	Caregiver for preschool-aged children	20
Adolescents, aged 12–15 years, Cincinnati, OH [58]	Urban adolescent clinic	13.8
Women, Seattle, WA [59]	Clients of sexually transmitted disease clinic	37

among males (1.5) than females (1.8); it was lower among non-Hispanic whites (1.4) and higher among non-Hispanic blacks (5.7) and Mexican Americans (5.1).

Table 35.6 lists situations in which exposure to CMV is expected and high incidence rates have been reported. Young children or infants who have acquired CMV from a maternal source, from other children, or even from blood transfusions in a hospital newborn nursery shed virus in the saliva and urine for years. Care of young children involves changing diapers and wiping up drool and respiratory secretions; unless great care is taken, contact with body fluids will be a daily occurrence. High rates of CMV infection have been reported in parents of premature babies who acquired CMV from blood transfusion and in parents of children who acquired infection in day-care centers – Table 35.6 [52, 53]. Group care of preschool-aged children can lead to very high rates of child-to-child transmission of CMV. The author has studied day-care centers in which every child in a classroom for 2-year-olds was shedding CMV in the saliva or urine. Not surprisingly high rates of CMV infection have been reported in child-care workers as shown in Table 35.6 [54–57]. In addition, DNA fingerprinting of virus has provided strong evidence for child-to-child transmission in day-care centers as well as child-to-parent and child-to-day-care worker transmission [58, 59]. Cytomegalovirus is present in the saliva, cervicovaginal secretions, and semen in adolescents and adults, especially those with recent primary CMV infection, and high rates of infection have been reported in adolescents and in relation to sexual activity [44, 60, 61].

5.3 Mother-to-Child Transmission of CMV

Mother-to-child transmission of CMV is important medically because of congenital infection with its attendant risk for disability due to central nervous system damage. However, transplacental transmission of CMV is a relatively uncommon event compared with transmission during birth or via breast milk. Although the latter two routes of transmission are not important causes of morbidity (with the possible exception of infection of VLBW premature newborns), they are of tremendous importance epidemiologically.

5.3.1 Intrapartum CMV Transmission

When CMV is present in the cervix or vagina at the time of delivery, the infection rate in newborns delivered vaginally is ~50 % [62]. Newborns infected during birth begin to shed virus in the urine and saliva at around 3–6 weeks of age. In full-term newborns these CMV infections rarely produce any signs of illness and are not associated with cognitive impairment [63]. In VLBW premature newborns, intrapartum CMV infection and breast milk-acquired infection have been implicated as causes of acute systemic illness characterized by a sepsis-like condition with worsening respiratory status and hematologic abnormalities similar to those seen in congenital infection [64, 65]. The occurrence of intrapartum CMV infection is dependent on maternal shedding of CMV in the genital tract. Around 10 % of CMV antibody-positive young women have CMV in the vagina or cervix by virus culture; rates near 30 % have been observed late in pregnancy [35]. In addition, genital tract shedding of CMV is more common among young women and is uncommon in women over 30 years of age [66]. Most studies on genital tract shedding employed virus culture for detection of CMV. Genital tract CMV shedding rates are higher when PCR is used for virus detection; rates of ~20–25 % have been reported in nonpregnant women [67, 68]. The impact of intrapartum transmission on CMV epidemiology depends on the seroprevalence of CMV among women and on maternal age at delivery. In populations in which nearly all mothers are CMV antibody positive, 10–15 % of newborns could acquire the virus during birth. In the United States, the intrapartum infection rate for newborns is probably less than 5 %.

5.3.2 Mother-to-Child Transmission of CMV Through Breast Milk

Cytomegalovirus can be detected in breast milk from around 95 % of CMV-seropositive mothers [69–71]. Colostrum usually has very low levels or no CMV; virus is commonly present in milk collected at weeks 2–12 of lactation [72]. Studies performed in healthy, term newborns prior to the advent of

Table 35.7 Rates of congenital CMV infection from 8 large studies that screened newborns using virus culture or PCR to detect CMV in the saliva or urine

Study	SES	Method	*N*	Congenital infections
Malmo, Sweden 1977–1986 [77]		Urine, VC	16,474	76 (0.46 %)
Birmingham, USA, 1978–1984 [49]	Middle/upper	Urine, VC	8,545	47 (0.55 %)
Canada [78]		Urine, VC	15,212	64 (0.42 %)
London, 1979–1982 [79]		Saliva, VC	14,200	42 (0.30 %)
Birmingham, USA, 1980–1990 [80]	Lower	Urine, VC	17,163	215 (1.25 %)
Sapporo, Japan, 1977–2002 [81]		Urine, VC	11,938	37 (0.30 %)
Sao Paulo, Brazil, 2003–2007 [82]	Lower	Urine or saliva, PCR	8,047	87 (1.08 %)
Eight US cities, 2008–2009 [75]	Mixed	Saliva, PCR, and rapid VC	34,989	177 (0.51 %)

VC virus culture

PCR showed that transmission of CMV through milk was clearly more likely if the milk was positive by virus culture; around 50 % of infants fed with milk that is positive by virus culture acquire CMV. In populations with high rates of both maternal seroprevalence and breastfeeding, the majority of infants acquire CMV during the first year of life [39]. Infants who acquire CMV will shed virus in body fluids for years and will transmit CMV to their peers as well as their caregivers.

5.3.3 Epidemiology of Congenital CMV Infection

Congenital CMV infection is defined by isolation of virus from body fluids collected within 3 weeks of birth. Newborn screening for virus is required to obtain accurate estimates of congenital infection rates. Screening for IgM antibody to CMV or other serological methods are not as sensitive or specific for diagnosis of congenital infection. Clinical identification of cases cannot be used because more than 90 % of newborns with congenital CMV infection will have no detectable abnormalities by physical examination. Testing urine or saliva by PCR or virus culture is the preferred approach to screening [73–75]. Screening of newborn blood or dried blood spots by PCR has received considerable study but does not appear to be as sensitive when used to screen for congenital infection as testing urine or saliva [76]. Congenital CMV infection rates from studies that screened urine or saliva from large numbers of newborns by virus culture or PCR are shown in Table 35.7; rates of congenital CMV infection ranged from 3 to 12.5 per 1,000 live births. Higher rates, around 20–40/1,000, have been reported from smaller studies in Africa and Brazil and in groups of mothers thought to be at increased risk for CMV transmission [83–86]. Congenital CMV infection rates are higher in developing countries and in low-income groups in developed countries. Other maternal characteristics associated with increased risk of congenital CMV infection include younger age, single marital status, and nonwhite race [80, 87].

Although it may seem counterintuitive that congenital infection rates would be higher in locations where rates of maternal immunity to CMV are highest, this finding is due to congenital infections in babies born to mothers who were immune prior to conception. When congenital CMV infection occurs in a mother known to be immune prior to pregnancy, the maternal infection has been referred to as a *recurrent* infection to distinguish it from primary infection. The occurrence of congenital CMV infection in offspring of immune mothers was first reported based on proven congenital infection in consecutive births to the same mother. In the 1970s, congenital infections were reported in a cohort of mothers who were known to have shed virus or to be CMV antibody positive prior to pregnancy [83]. In a more recent cohort study, congenital CMV infection occurred in 28/2,857 (1.0 %) babies born to mothers who were known to be CMV antibody positive at the time of a previous birth, an average of approximately 4 years earlier [51]. Appreciable congenital CMV infection rates (1–3.9 %) have been observed in populations in which the maternal seroprevalence was so high (95–100 %) that one could only conclude that recurrent maternal infections were the source [30, 84–86]. Other studies reported the lack of maternal IgM antibody to CMV and presence of high-avidity IgG antibody as evidence of past infection in a significant proportion of mothers of newborns with congenital CMV infection, evidence that maternal infection occurred prior to the current pregnancy [77, 82]. In low-income populations and in developing countries, in which almost all women acquire CMV prior to reproductive age, most congenital CMV infections occur in women who are immune prior to conception. In contrast, in middle/upper-income populations in the United States and some European countries, congenital infection rates in women known to be CMV antibody positive prior to pregnancy are very low, from 0.14 to 0.24 %, and most congenital CMV infections are the result of primary maternal infection during pregnancy [49, 88–92]. The occurrence of congenital CMV infection in babies born to immune mothers probably reflects reinfection; a role for reactivation or persistent infection cannot be ruled out [93, 94].

The rate of primary maternal infection during pregnancy varies from 0.7 to 4.1 % [35]. Primary infections are more common in younger mothers and in those from a lower socioeconomic

level. The rate of fetal infection with primary maternal infection during pregnancy is approximately 20–75 % [35, 92, 95]. The rate of transplacental transmission is lower when maternal infection occurs in the first trimester, and it increases with increasing gestational age, as has been observed with rubella virus and other causes of congenital infection. Symptomatic congenital infection and CNS sequelae are more likely to occur with first-trimester maternal infection [49, 96–98].

6 Pathogenesis and Immunity

CMV damages tissues by causing cell death, but it can also block host cell death programs temporarily as progeny virions are being produced. The host inflammatory/immune response may also contribute to tissue damage. Damage at the cellular level can directly affect organ function by loss of cells as occurs in the central nervous system or inner ear in congenital infection. Infection of vascular endothelial cells can lead to focal necrosis and organ dysfunction as occurs with retinitis in AIDS patients or in newborns with congenital infection. Key themes in understanding the biology of human CMV infection include its ability to infect vascular endothelium and multiple cell types in many different organ systems, the importance of cell-mediated immunity in controlling its replication and preventing disease, its elaborate mechanisms for evading host innate and adaptive immune responses, its ability to co-opt host cell-signaling pathways and cytokine systems, and its ability to establish latency and persist in the host indefinitely. Infection is probably established by inoculation of virus onto a mucosal surface or into the circulatory system (transfusion, organ transplantation, fetal infection). A plausible sequence of events would include local replication at the site of inoculation followed by viremic spread to other tissues and organs throughout the body. CMV can be found in many different organs and tissues including the salivary glands, kidneys, lung, liver, adrenal glands, vascular endothelium, endometrium, intestinal epithelium, bone marrow, retina, brain, and inner ear. Around 2–4 weeks after exposure, the replication at multiple sites likely leads to a secondary viremia, which is followed by the initial signs of disease in immunocompromised patients or the rare healthy host who develops an illness with primary CMV infection. Although the normal host immune response to CMV is nearly always sufficient to prevent disease, it does not eradicate virus. It takes months for the immune system to control viremia and even longer to terminate viral shedding in body fluids.

6.1 Humoral Immune Response

From studies in transfusion and transplant patients with primary CMV infection, it was found that IgG and IgM antibody to CMV appear 4–6 weeks after exposure. Serum IgG antibody to CMV persists indefinitely after primary infection. Antibodies to multiple CMV proteins are found in human sera after infection. Specific envelope glycoproteins, notably gB, the gH/gL/gO complex, the gM/gN complex, and a pentameric complex of gH/gL/pUL128/pUL130/pUL131A, are the principal targets of neutralizing antibody [99, 100]. Although experience in immunocompromised patients demonstrates that humoral immune response to CMV cannot control ongoing viral replication or prevent disease when cell-mediated immunity is profoundly impaired, there is no question that humoral immunity plays an important role in preventing CMV infection and disease. In transplant patients and newborns with breast milk- or transfusion-acquired infection, cytomegalovirus disease is much more significant among those who were CMV antibody negative prior to infection [101, 102]. Follow-up of seropositive and seronegative young mothers showed that the congenital infection rate in subsequent offspring was reduced by two-thirds among those who were initially antibody positive [103]. A randomized, placebo-controlled trial of a CMV gB vaccine which induces high levels of antibody showed modest efficacy in preventing CMV infection in seronegative young women and CMV disease in seronegative solid organ transplant recipients [104, 105]. In addition, prophylaxis of seronegative recipients of kidneys from seropositive donors with high-titer CMV immune globulin reduced the frequency and severity of CMV disease. There is also substantial evidence from studies in experimental animals that antibody alone can prevent CMV disease.

6.2 Cell-Mediated Immunity

The risk of human CMV disease and the severity of disease increase with impairment of cell-mediated immunity. Cytomegalovirus-specific T lymphocytes appear in the peripheral blood shortly after initial infection in the normal host and remain detectable indefinitely afterward. Cytotoxic T lymphocytes (CTLs) target a number of viral proteins; the tegument protein pp65 (UL83) and the immediate early transcription activator IE1 (UL123) are major targets of CTL [106–108]. Among transplant patients, CMV disease is most likely to occur during the time of greatest immunosuppression and among patients who have received the most potent T-lymphocyte immunosuppressive regimens. Impaired CMV-specific CTLs and CD4+ T-lymphocyte responses have been linked to CMV disease in solid organ and hematopoietic stem cell transplant (HSCT) patients and in AIDS patients with very low CD4+ T-lymphocyte numbers [109, 110]. The administration of autologous CMV-specific CTLs propagated and stimulated by antigen in vitro has been used to control CMV infection [111, 112]. CMV DNA encodes multiple genes with products related to inhibition or modulation of

natural killer (NK) cell responses, evidence that NK cell function is a key component of host defense against CMV [113–115]. There is also evidence that NK cells play a role in preventing CMV reactivation and disease in solid organ transplant patients, and severe infection due to CMV was reported in an adolescent who lacked NK cells [116, 117].

6.3 CMV Evasion of Host Defense Mechanisms

Like many other viruses, CMV has evolved means of modifying or escaping host defenses or using host cell-signaling mechanisms in ways that should promote viral replication and spread within the host and within the community. More than 25 CMV gene products (excluding those that influence the cell cycle or cell tropism) have the potential for these functions, including interference at multiple steps with MHC-I-dependent processing and presentation of antigen, with interferon induction and activity and with NK cell function [27, 118]. In addition CMV encodes host chemokine homologs (IL-10 and CXC), chemokine receptors, multiple genes that inhibit apoptosis, and chemokines that influence leukocyte migration in ways that could facilitate dissemination of virus within the host [27]. The result of all of this viral genomic focus on the host immune and inflammatory systems could well explain why CMV replication and viral shedding in body fluids continue well after antiviral antibody and cell-mediated immune responses are present. However, understanding of how viral functions interact with host defense mechanisms at the level of the whole organism to produce disease or persistent infection is limited.

6.4 Latency

Latency is a characteristic feature of herpesvirus infection. In the broadest terms, it is characterized by the absence of any evidence of viral replication and the persistence of the entire viral genome in host cells. Understanding of CMV latency is incomplete. Review of the molecular and cellular basis for latency is beyond the scope of this discussion but has been reviewed in detail [119]. The latent state is also characterized by limited viral gene expression, with the latency-associated transcripts presumably playing a regulatory role related to maintaining latency and the capability to be reactivated to productive infection in response to specific stimuli. The cellular localization of latent virus may depend on specific cellular functions that allow persistence of viral DNA but restrict viral replication. The leading candidates for the site of latency are myeloid stem cells and bone marrow progenitor cells where CMV DNA and latency-associated transcripts have been found. At the cellular level, it appears that reactivation to productive infection occurs with further differentiation of these cells or in response to inflammatory cytokines or allogeneic tissue. The molecular and cellular evidence for latency is compelling, but in a human with past CMV infection, it is not clear whether the lack of evidence of active infection (viral shedding in body fluids or viremia) is due to latency or persistent infection below the level of detection in limited cell populations such as the intestinal or salivary gland epithelium. It is clear that CMV-seropositive patients will consistently develop reactivation to the point of easy detectability during immune impairment due to drugs or HIV infection or in response to inflammation or allogeneic transplant.

7 Patterns of Host Response

7.1 CMV Infection in the Normal Host

Primary cytomegalovirus infection in healthy adults is clinically apparent in only around 10 % of subjects, and this estimate is based on cohort studies that used interviews to ascertain symptoms near the time of seroconversion. An even smaller proportion of adults with primary CMV infection seek medical attention for illness. Studies of seronegative pregnant women who seroconverted to CMV reported that less than 10 % had an illness suggestive of CMV infection during the interval of seroconversion [49, 120]. A study of 148 blood donors that seroconverted to CMV found evidence of recent infection (CMV DNA in blood) in 13. One of the 13 had clinical and laboratory findings indicative of a mononucleosis-like syndrome. In addition, myalgia was reported by 4/13 donors with primary CMV infection compared with 0/13 in a control group [121]. Symptomatic primary CMV infection in children appears to be distinctly uncommon; two studies in day-care centers where acquisition of CMV infection was common were unable to associate onset of CMV infection with illness [122, 123]. Primary CMV infection is recognized as a cause of a heterophile-negative mononucleosis that cannot be distinguished clinically from the much more common mononucleosis due to Epstein-Barr virus infection. CMV mononucleosis is characterized by fever for up to 2 weeks or longer, pharyngitis, lymphadenopathy, hepatomegaly, and splenomegaly; chills, sweats, abdominal pain, and weight loss have also been reported [124–126]. A study of patients who were referred for laboratory testing because of clinical suspicion of CMV infection and had serological evidence of primary CMV infection (CMV IgM antibody and low-avidity IgG antibody) reported that the average duration of symptoms was 7.8 weeks [127]. Children who have symptoms with primary CMV infection are less likely than adults to have fever and are more likely to have splenomegaly [128]. Rare clinical

manifestations or complications of primary CMV infection include encephalitis, transverse myelitis, Guillain-Barré syndrome, uveitis, hemolytic anemia, thrombocytopenia, vascular thrombosis, myocarditis, and colitis. Severe systemic illness with multiorgan involvement due to CMV infection has been reported [129, 130]. Unusually severe CMV infection in a presumably normal host should prompt careful investigation of the immune system including testing for HIV infection, lymphocyte subsets, and if possible T-lymphocyte and NK cell function.

Primary CMV infection in the normal host is characterized by viremia and prolonged viral shedding in the urine, saliva, and genital fluids [131–133]. Viremia (detected by PCR) is present for weeks to months, and shedding of virus in body fluids can persist for months to over a year. Viral shedding persists even longer in children; those who acquire CMV during the first year of life can shed virus in the urine or saliva continuously for years [134, 135]. Active CMV infection (virus in the blood and body fluids) persists in the presence of antibody to the virus and CMV-specific T-lymphocyte responses.

7.2 Congenital CMV Infection

As previously noted, only around 10 % of newborns with congenital CMV infection have associated clinical abnormalities at birth. The most common abnormalities noted in children with congenital CMV infection are shown in Table 35.8, adapted from Istas et al. [136]. All newborns with any of these abnormalities should be tested for congenital CMV infection unless another diagnosis has been firmly established. Microcephaly, hepatomegaly, splenomegaly, petechiae, and chorioretinitis in a newborn are suggestive of congenital CMV infection. However, accurate diagnosis requires detection of CMV in body fluids to distinguish it from other causes of congenital infection with similar clinical findings. Jaundice, hyperbilirubinemia, petechiae, thrombocytopenia, hepatomegaly, and splenomegaly usually clear spontaneously within weeks of birth [137]. Neurological abnormalities, chorioretinitis, and abnormalities on newborn brain imaging are predictive of subsequent cognitive, motor, and sensory sequelae which occur in around 40–60 % of newborns with symptomatic congenital CMV infection [23, 137–139]. Newborns with congenital CMV infection who have no clinical abnormalities at birth (asymptomatic) do much better; around 7–25 % will have sequelae with sensorineural hearing loss being the most common disability [23, 140–142]. The impact of CNS damage from congenital CMV infection on cognitive, sensory, and motor development can take years to be fully recognized. In addition, hearing loss in congenital CMV infection is often progressive or has onset after the newborn period.

Table 35.8 Clinical findings in 258 infants with congenital CMV infection who were symptomatic at birth, from data reported to the US National Congenital CMV Disease Registry, 1990–1993

Clinical feature	% patients
Non-neurological abnormalities	
Petechiae or purpura	54
Small for gestational age	47
Hepatosplenomegaly	40
Jaundice at birth	38
Hemolytic anemia	11
Pneumonia	8
Neurological abnormalities	
One or more of the following	68
Intracranial calcifications	37
Microcephaly	36
Unexplained abnormality	27
Hearing impairment	25
Chorioretinitis	11
Seizures	11
Neonatal death	9
Laboratory abnormalities	
Platelet count $\leq$75,000/mm^3	48
Direct bilirubin $\geq$3 mg/dL	36
Alanine aminotransferase level >100 U/L	23

Modified from reference Istas et al. [136]

7.3 Postnatal CMV Infection in Newborns

CMV is commonly spread from mother to newborn during birth or by breast milk; these infections are not associated with illness unless they occur in VLBW premature newborns, and even for these patients, there is disagreement as to the clinical impact of CMV infection. Clinical findings similar to those associated with symptomatic congenital CMV infection have been observed in VLBW newborns in temporal association with the onset of CMV infection acquired from the mother's milk or from blood transfusion. Morbidity from transfusion-acquired CMV infection in newborns occurs almost exclusively in those who are CMV seronegative and are VLBW, <1,200 g at birth [101]. It is now common practice to provide the blood for newborns that is from CMV-seronegative donors or that has been filtered to remove CMV. Breast milk-acquired CMV infection does not cause illness in the term, immunocompetent newborn. VLBW premature newborns have lower levels of maternal CMV IgG and may lose serum antibody to CMV within a few weeks of birth. In addition they are more vulnerable because of comorbidities and immunologic immaturity. Significant illness due to transmission of CMV from the mother's milk to VLBW premature newborns has been described [65, 143]. In these infants, onset of CMV infection was associated with worsening of respiratory symptoms, a sepsis-like illness, and thrombocytopenia. Although there has been concern that CMV infection in

VLBW newborns could cause the same sort of sequelae and disability seen in congenital CMV infection, results from different centers are not in agreement on this important point with some reporting no evidence of long-term sequelae and others reporting increased risk for CNS impairments [144–146]. A recent study with 8 years of follow-up reported normal cognitive and motor function for former CMV-infected VLBW premature newborns and a well-matched, uninfected control group. However, cognitive and motor function were slightly better in the uninfected children and differences were statistically significant [147]. With investigators differing on the frequency and importance of morbidity due to CMV from the mother's milk in premature newborns, it is understandable that there is no consensus on whether steps such as pasteurization or freezing to inactivate CMV should be taken to prevent transmission. However, the fact that CMV is frequently transmitted to newborns through the mother's milk and could cause disease should serve as a caution to those who provide banked human milk to premature newborns.

7.4 CMV Infection in Immunocompromised Hosts

Cytomegalovirus infection is one of the most common and persistent opportunistic infections to produce disease in immunocompromised patients. Impaired immunity, especially T-lymphocyte function, leads to reactivation of CMV and active viral infection and with substantial immune impairment, CMV disease. For patients who encounter CMV for the first time while immunocompromised, the risk of CMV disease is greater. This fact is particularly relevant to transplant patients for whom the transplanted organ or tissue or blood transfusions could be the source of a primary CMV infection. The manifestations of and risk factors for CMV disease as well as diagnostic methods and approaches to treatment vary according to the clinical setting and can be quite different for transplant patients compared with AIDS patients.

7.4.1 CMV Disease in Solid Organ Transplant Patients

Cytomegalovirus infection defined by the presence of virus in body fluids or conversion from CMV antibody negative to positive occurs commonly after immunosuppression and transplantation; the infection rate depends largely on the proportion of patients who were CMV seropositive pretransplant, the CMV status of donors, and the intensity of the immunosuppressive regimen. The transplanted organ, blood products, and reactivation of endogenous latent virus are sources of CMV. Cytomegalovirus disease occurs in a variable proportion of CMV-infected transplant patients; reviews by leading experts provide detailed discussion of diagnosis, prevention, and treatment of CMV disease in solid organ transplant recipients [148–151]. Cytomegalovirus disease is common among seronegative recipients of organs from seropositive donors, but severe disease can also occur in solid organ transplant recipients who are CMV seropositive pretransplant and profoundly immunosuppressed by chemotherapy aimed at preventing rejection of the graft. Cytomegalovirus disease increases the length of hospitalization and significantly increases the cost of organ transplantation [152–155].

Viremia and viral shedding in the urine and other body fluids usually occur about 4–6 weeks after transplantation. Signs of CMV disease may accompany onset of viremia and usually occur within the first 12 weeks after transplantation. Common clinical and laboratory signs at the onset of CMV disease are often referred to as *CMV syndrome* and include fever, malaise, arthralgia, rash, leukopenia, and elevation of hepatic transaminases. More severely affected patients (those with primary infection and/or more profound immunosuppression) could have pneumonitis, retinitis, or gastrointestinal ulceration. Cytomegalovirus infection in solid organ transplant patients has also been associated with coinfection by other opportunistic pathogens, notably *Pneumocystis carinii*, *Aspergillus*, and other fungi, and antiviral prophylaxis aimed at preventing CMV disease has reduced the rate of secondary bacterial and candida infections [156, 157]. Cytomegalovirus infection in solid organ transplant patients has also been linked to inflammation or rejection of the donor organ – graft rejection or glomerular injury in renal transplant patients, hepatitis in liver transplant patients, pneumonitis in lung or heart-lung transplants, and coronary artery restenosis in heart transplants.

7.4.2 CMV Disease in Hematopoietic Stem Cell Transplantation

Prior to the use of antivirals to prevent and treat CMV infection, CMV was a major cause of mortality in HSCT patients largely due to its association with pneumonitis in patients with graft-versus-host disease. In the early days of bone marrow transplantation, CMV pneumonia (often in association with graft-versus-host disease) occurred in 20–40 % of HSCT recipients and had a mortality of around 85 % [158]. A study from Sweden found that CMV disease rates in HSCT patients fell to 2.2 % and mortality to 0 following implementation of effective antivirals [159]. Sources of CMV infection for HSCT patients include donor cells, blood products, and reactivation of their own latent virus. Blood products are of particular concern because transfusions of blood and platelets are often needed prior to engraftment of the transplanted stem cells. The clinical manifestations of CMV infection in HSCT patients include the CMV syndrome at the onset of infection which may resolve without further problems. Pneumonitis and gastrointestinal disease (esophagitis, gastritis, colitis) are the most common manifestations of more serious CMV

disease; encephalitis, retinitis, and hepatitis have also been attributed to CMV. The risk of opportunistic infections is also increased in HSCT patients with CMV infection. Graft-versus-host disease is one of the factors associated with severe CMV disease; other clinical variables associated with CMV disease in HSCT patients are transplantation of stem cells from a CMV-positive donor to a CMV-seronegative recipient, donor-recipient histoincompatibility, myeloablative conditioning regimens, and immunosuppressive regimens which produce lymphocyte depletion or profound T-lymphocyte functional impairment (high-dose steroids, mycophenolate mofetil, T-cell depletion, anti-CD52, and antithymocyte globulin) [160, 161].

Although the use of antivirals has reduced the rate of CMV disease in the first 90–120 days after HSCT transplant, a significant proportion of patients develop CMV disease within a few months after antiviral prophylaxis or treatment is discontinued. A report from a major US HSCT center revealed that among patients who received either prophylactic or preemptive antiviral therapy for CMV in the first 100 days post transplant, 17.8 % experienced late-onset CMV disease at an average of 5.5 months after transplant [162]. Patients with late-onset CMV disease had increased mortality, 46 %, and CMV disease recurred in 38 % of survivors. Late-onset CMV disease in HSCT patients is widely recognized as a persistent problem for which there is not yet an ideal solution. Prolongation of antiviral treatment is an option though it raises concern for development of antiviral resistance.

7.4.3 CMV Infection in Acquired Immunodeficiency Syndrome

Prior to the use of HAART, CMV disease was common among adult AIDS patients, especially those with CD4 cell counts of less than 50/mm^3. The widespread use of HAART has reduced the proportion of HIV-infected patients with profound immune impairment and dramatically reduced the occurrence of CMV disease in them. Retinitis is the most common manifestation of CMV disease in adults with AIDS; without antiviral treatment, CMV retinitis progresses to blindness [163]. Other manifestations of CMV disease in AIDS patients include gastrointestinal inflammation and ulceration involving any part or multiple sites of the digestive tract. Less common effects of CMV include encephalitis, peripheral neuropathy, polyradiculoneuritis, pneumonitis, hepatitis, adrenalitis, pancreatitis, and sialoadenitis [164, 165].

Cytomegalovirus disease is much less common in children with AIDS in developed countries but has similar manifestations when it occurs. Prior to the control of HIV infection with antiretroviral drugs, coinfection with CMV was associated with more rapid progression to AIDS in some studies in children [166, 167]. Similar to experience in adults, the use of HAART in HIV-infected children in the United States made CMV disease distinctly uncommon; data from the Perinatal AIDS Collaborative Transmission Study showed a more than tenfold reduction in nonocular CMV disease and a similar dramatic reduction in retinitis in the era of HAART compared with pre-HAART [168]. Congenital CMV infection is a potential complication of maternal HIV/AIDS. One would intuitively expect congenital infection rates to be higher among babies born to HIV-infected mothers. However, a Brazilian study of HIV-infected mothers who did not have advanced HIV infection found the rate of congenital CMV infection in their babies (2.7 %) was similar to that in babies born to mothers without HIV infection (2.9 %) [169]. Evidence from other studies suggests that maternal HIV infection does increase the risk of congenital CMV infection. A study from Spain reported an overall congenital CMV infection rate of 4.6 % in infants of HIV-infected mothers; the rate was 9.2 % prior to 1997 compared with 1.3 % afterward, and the rate was higher in mothers who did not receive zidovudine during pregnancy [170]. A report from the French Perinatal Cohort indicated that the rate of congenital CMV infection in HIV-1-uninfected infants born to HIV-1-infected mothers decreased from 3.5 % in the pre-HAART era to 1.2 % in the post-HAART era [171].

8 Treatment and Prevention

8.1 Antiviral Agents

Antivirals licensed for systemic use in the United States for treatment of CMV infection are ganciclovir, valganciclovir, foscarnet, and cidofovir. The mechanisms of action and clinical use of these antivirals have been reviewed [172]. Ganciclovir, a synthetic analog of deoxyguanosine, interferes with viral DNA replication after intracellular phosphorylation by preventing incorporation of deoxyguanosine triphosphate into viral DNA [172]. Ganciclovir has been the most widely used antiviral for CMV, but it is poorly absorbed from the gastrointestinal tract and requires intravenous administration. Valganciclovir, a valyl ester of ganciclovir, with much better oral bioavailability, is converted to ganciclovir after absorption and is often used in situations where prolonged administration of drug for treatment or prevention is necessary. Pharmacokinetic studies have established dosages for valganciclovir which will achieve systemic ganciclovir levels similar to those achieved with IV administration [173]. Foscarnet is also used to treat and prevent CMV infections in immunocompromised hosts. It is preferred in some centers in transplant patients for preemptive therapy in order to avoid the marrow-suppressive effects of ganciclovir because it is less likely to be associated with neutropenia due to bone marrow suppression [174]. Foscarnet is also used when antiviral resistance to ganciclovir is a concern and at

times in combination with ganciclovir. Cidofovir has been used primarily to treat CMV retinitis in AIDS patients. Cytomegalovirus resistance to ganciclovir is associated with prolonged antiviral use and treatment failure [175]. Antiviral resistance has been reported with each of the antivirals that are licensed for CMV treatment or prevention; a review that covers the epidemiology, molecular basis, diagnosis, and management of CMV antiviral resistance is recommended for the interested reader [176].

8.2 Antiviral Treatment of CMV Disease

Cytomegalovirus disease in immunocompetent hosts does not merit antiviral treatment; the expected outcome, with rare exceptions, is spontaneous recovery. Although no antiviral is currently licensed for treatment of symptomatic congenital CMV infection, ganciclovir has been shown to decrease the risk of progression of hearing loss and improve developmental outcome when given to newborns with severe symptomatic congenital CMV infection intravenously for 6 weeks [177, 178]. The use of ganciclovir to treat these newborns has been endorsed by the Committee on Infectious Diseases of the American Academy of Pediatrics; the use in less severely affected newborns was not recommended [179]. The majority of newborns with congenital CMV infection have no clinical abnormalities at birth and will not have any sequelae of infection. Whether antiviral treatment will reduce the risk of hearing loss or other sequelae in newborns with congenital CMV infection who have no abnormalities at birth is not known. The use of valganciclovir orally in the place of IV ganciclovir for treatment of congenital CMV infection is currently being investigated [180].

Antivirals are used for treatment of CMV disease defined by clinical and laboratory abnormalities in immunocompromised patients. When the patient treated for CMV disease is expected to remain profoundly immunocompromised, treatment of the acute illness is often followed by maintenance antiviral therapy over an extended period of time. In addition to treatment of the established disease, antivirals are used in transplant patients to prevent disease. Prophylactic administration of antiviral, usually ganciclovir, from the time of transplant for several months is used in situations in which the perceived risk of CMV disease is high enough to counterbalance the potential toxicity of the antiviral or the risk of antiviral resistance, for example, in CMV-negative recipients of a solid organ transplant from a CMV-seropositive donor. In centers in which there is laboratory capability for rapid and frequent measure of the quantity of CMV in the blood, a preemptive approach to prevention is often used. Preemptive strategies are based on frequent laboratory monitoring of the quantity of CMV in the blood (likely using quantitative real-time PCR) with administration of antiviral triggered by detection of a quantity of CMV DNA in the blood associated with a significant risk of disease. Reviews of CMV disease in solid organ and HSCT patients by experts and consensus guidelines sponsored by professional organizations consider the evidence for antiviral strategies and provide recommendations on antiviral use in transplant patients [150, 151, 160, 181–183]. There are similar guidelines for HIV/AIDS patients, though the effective use of antiretroviral agents to control HIV infection and prevent development of immune impairment has dramatically reduced the occurrence of retinitis and other manifestations of CMV disease [167, 184].

8.3 Prevention of Maternal and Congenital CMV Infection

Prevention of CMV infection in pregnant women was recently reviewed [185]. The two most important sources of maternal CMV infection are contact with young children and sexual contact. Because CMV infection in the normal host is almost always clinically silent and shedding of CMV in body fluids can continue for months to years after initial infection, control efforts must be based on the assumption that the saliva, urine, or other body fluids from anyone are a potential source of CMV. Aside from transfusion and organ transplantation, it is rare for the circumstances of CMV exposure to be identified in time for postexposure prophylaxis to be considered. There is currently no role for postexposure prophylaxis with immune globulin or antivirals as there are no data on their efficacy or safety. Hand washing and careful attention to hygiene are recommended to prevent CMV infection in pregnant women. The Centers for Disease Control and Prevention has made specific recommendations for prevention of CMV infection for pregnant women and persons who care for infants and children which are aimed at interrupting exposure to body fluids, shown in Fig. 35.2. Some prenatal care providers screen pregnant women for antibody to CMV during pregnancy though this practice is uncommon in the United States. Such screening could be used to identify those with serological evidence of primary infection (CMV IgM antibody positive with low-avidity CMV IgG antibody) for counseling and prenatal diagnosis. In addition it would be possible to target women who were seronegative with information aimed at decreasing their risk of infection. Studies in pregnant women demonstrated that rates of CMV infection could be reduced by intensive efforts to educate seronegative women on risks and consequences of CMV infection during pregnancy and on recommended methods for prevention [186, 187]. Screening of pregnant women for CMV infection or susceptibility has not been recommended by obstetric organizations [188–191]. Screening of pregnant women for CMV will merit further consideration when it is clear that accurate tests for primary CMV infection are widely available, evidence for the

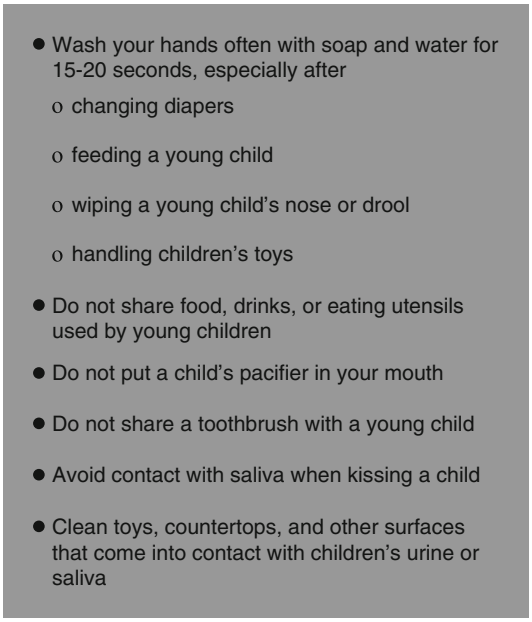

Fig. 35.2 Centers for Disease Control and Prevention of the United States Public Health Service recommendations for pregnant women to decrease risk of maternal and congenital CMV infection [32]

effectiveness of prevention measures is stronger, and interventions other than termination of pregnancy if fetal infection is found are available. The American College of Obstetricians and Gynecologists does recommend counseling women with young children or those who work with young children on the use of gloves and rigorous hand washing after contact with diapers or respiratory secretions [188].

Passive immunization with CMV immune globulin preparations has been studied for prevention and treatment of fetal CMV infection. In 2005, a study of CMV immune globulin in women with primary infection early in pregnancy reported a fetal infection rate of 16 % in women who received serial injections of CMV immune globulin compared with 40 % in those who chose no treatment [192]. A retrospective study reported higher frequency of adverse outcome 16/37 (43 %) for infants of women with primary CMV infection early in gestation during years when CMV immune globulin was not being used, compared with infants of women enrolled in later years who were given a single treatment of 200 Units per kilogram of CMV immune globulin 4/31 (13 %) [193]. A randomized, placebo-controlled, observer-blind clinical trial found no statistically differences between CMV immune globulin recipients and placebo recipients in the rate of fetal infection or the clinical outcome of congenital infection [194]. In addition there was not a significant difference in neutralizing antibody or antibody avidity between women who transmitted virus to the fetus and those who did not. A large, randomized, placebo-controlled, phase 3 clinical trial of CMV immune globulin for prevention of congenital infection through treatment of women with primary gestational CMV infection is enrolling subjects [195].

8.4 Prevention of Healthcare-Acquired CMV Infections

Cytomegalovirus infections that occur as a result of hospitalization or as a complication of medical care can cause significant morbidity in organ transplant recipients, VLBW newborns, pregnant women, patients with congenital and acquired immunodeficiencies, and patients receiving immunosuppressive medications. Cross infection from healthcare worker to patient or from patient to patient through fomites or healthcare workers is rare, probably because it is prevented by hygiene practices that are a routine part of hospital care. For hospitalized patients known to have CMV infection, standard precautions are recommended. Transmission of CMV in the healthcare setting is a potential complication of blood transfusions, assisted reproductive technology, transplantation of organs or tissue, and provision of banked human milk to premature newborns. The latter two have been discussed in Sects. 8.4 and 8.3, respectively.

8.4.1 Prevention of Transfusion-Acquired CMV Infection

Transmission of CMV through blood transfusion is prevented by limiting vulnerable patients to transfusion with the blood from donors who have never been CMV infected (CMV IgG negative) or by filtering the blood through commercially available cotton wool filters, which remove white blood cells and have been shown to reduce the risk of transfusion-transmitted CMV infection [196]. Selection of seronegative donors is more effective for preventing transfusion-acquired CMV infection and disease in HSCT patients than is leukoreduction [197]. A meta-analysis of studies that compared selection of seronegative donor blood to leukoreduction concluded that the former was associated with a 58 % reduction in risk of CMV infection [198]. However, a survey of the American Association of Blood Bank physicians in 2010 found that 65 % of respondents considered leukoreduction and selection of CMV-negative donors to be equally effective for prevention of CMV infection [199]. Practices differ among institutions as to which approach is used and whether blood products from CMV-seronegative donors are provided to specific high-risk groups of patients.

8.4.2 Prevention of CMV Transmission Through Assisted Reproductive Technology

It is common to find CMV in the semen, though published rates vary widely depending on the population studied and

method used for viral detection. Using cell culture, CMV was found in the semen of 3/54 (5.6 %) young adults in North Carolina [200]. A study in HIV-uninfected subjects in Paris reported detection of CMV by cell culture in the semen from 7/111 (6.3 %) of male partners of women with human papillomavirus infection [201]. A study of 635 cryopreserved semen samples from 231 donors in France found that 1.57 % overall were culture or PCR positive as were 3.3 % of CMV-seropositive donors [202]. A study in an infertility clinic in Crete reported 50/172 (29 %) semen samples were positive for CMV by PCR [203]. A study of couples from an in vitro fertilization program in Texas reported 25 % of 53 semen samples positive for CMV by PCR; none of 568 oocytes and embryos not suitable for cryopreservation were positive for CMV [204]. There is justifiable concern over the possibility for transmission of CMV to recipients of donor sperm, especially to CMV-seronegative recipients, because they could potentially develop a primary CMV infection at the time of conception. It was estimated from CMV seroprevalence in recipients and donors in an infertility clinic in France that the risk of insemination of a CMV-seronegative woman by the semen from a CMV-seropositive donor would be 21 % if there was no matching of seronegative women with the semen from seronegative donors [205]. In the United States, the Food and Drug Administration (FDA) regulates transplantation of human cells and tissue, including the semen and oocytes, and requires establishments that transfer human cells to follow written protocols to prevent transmission of infections [206]. The FDA also lists commercial antibody assays that have been cleared for screening for IgG and IgM antibody to CMV [207]. The American Society for Reproductive Medicine has published guidelines for assisted reproductive technology aimed at preventing transmission of CMV and other infectious agents. The guidelines recommend screening of recipient women and semen donors for CMV IgG and IgM antibody and state that the semen from CMV-seropositive donors should be restricted to seropositive recipients [208].

8.4.3 Prevention of Transmission of CMV from Patients to Healthcare Workers

Transmission of CMV from patients to healthcare workers is very unlikely if standard hospital hygiene and infection control practices are followed. The rates of incident CMV infection among healthcare workers with patient contact do not appear to be different from those among adults of similar age [50, 209]. It is important to recognize that most patients who have CMV in body fluids will not be identified, especially in pediatric wards, because acquisition of CMV is a normal childhood occurrence and CMV-infected children shed virus for years. Therefore, rather than singling out patients known to have CMV infection for special precautions or restricting pregnant healthcare workers from caring for them, healthcare workers should consider all patients as potential sources of CMV. The Hospital Infection Control Practices Advisory Committee of the CDC has recommended standard precautions, the same precautions used for of all hospitalized patients, for patients with CMV infection [210]. Screening healthcare workers for CMV antibody was not recommended.

8.5 Cytomegalovirus Vaccine

An expert panel assembled by the Institute of Medicine concluded that the development of a vaccine to prevent congenital CMV infection should be a top priority for the United States for the twenty-first century [211]. The first clinical trial with a CMV vaccine was conducted in the early 1970s, and there have been numerous clinical trials with a small number of different CMV vaccine platforms in the intervening 40 years. Immunization with the Towne CMV vaccine, a live virus vaccine attenuated by serial in vitro passage, reduced the severity of disease due to primary CMV infection in seronegative renal transplants who received kidneys from seropositive donors [212]. However, it did not prevent infection in transplant patients or in parents whose children had acquired CMV in day-care centers [213]. A randomized, placebo-controlled, phase 2 clinical trial with a subunit vaccine composed of recombinant CMV envelope glycoprotein B with MF59 adjuvant showed 50 % efficacy for prevention of CMV infection in seronegative young mothers, the first evidence for vaccine prevention of CMV infection [104]. A randomized clinical trial with the same vaccine in solid organ transplant patients showed that vaccine recipients were less likely to develop viremia or require preemptive antivirals than placebo recipients [105]. A more recent clinical trial with a plasmid DNA vaccine showed that immunization of HSCT patients could reduce the frequency and magnitude of viremia, a surrogate marker for CMV disease [214]. A number of novel CMV vaccines are now in development [215]. Although there is optimism about the development of a CMV vaccine that can prevent maternal or congenital infection and reduce disease burden in immunocompromised patients, no phase 3 clinical trials are yet underway, and any prediction of when a licensed vaccine will be available would be speculative.

9 Unresolved Problems

The importance of CMV as a fetal and neonatal pathogen causing morbidity, mortality, and disability is well established as is the role of CMV as an opportunistic infection in

immunocompromised patients, especially transplant recipients. The knowledge of routes of transmission of CMV and the use of antiviral drugs have contributed to prevention and treatment of CMV disease in these patient groups. Although substantial effort and expense are currently devoted to preventing and treating CMV disease, it remains a major problem for immunocompromised and especially transplant patients. Antiviral treatment of newborns with congenital CMV infection appears to have a very modest effect on outcome, and prevention of congenital infection is more of a hope than a reality. The biggest unresolved problem in control of CMV infection is the development of vaccines that can prevent fetal infection and CMV disease in immunocompromised patients.

There is growing interest in a possible role for CMV infection as a cause of a wide variety of chronic diseases. It has been linked to all-cause mortality and proposed as a factor in immune senescence, an explanatory term for the decreased ability of the aged to combat infections [22, 216]. Immune senescence is characterized by reduced numbers of naïve T lymphocytes, decreased diversity of naïve T-lymphocyte receptor repertoire, and decreased diversity and function of CD4+ and CD8+ T cells [217]. Biological evidence suggesting a role for CMV in immune senescence comes from studies documenting the broad immunogenicity of CMV proteins and the remarkable domination of T-lymphocyte responses in CMV-seropositive subjects with around 10 % of CD4+ and CD8+ memory cells committed to CMV, a level of immunodominance not seen with other infections [218]. Cytomegalovirus infection has been associated with decreased immunogenicity of influenza vaccine in subjects aged greater than 60 years, in whom an increase in late-differentiated CD4+ T cells was also associated with decreased immune response [219]. It has been hypothesized that the preoccupation of the immune system with CMV diminishes its ability to deal with other infections as a result of accumulation of terminally differentiated T lymphocytes specific for CMV and increased levels of inflammatory cytokines [220].

CMV has also attracted interest as a possible factor in development of chronic vascular and autoimmune disease. The virus infects vascular endothelial cells and induces angiogenesis; abnormal neovascularization promoted by CMV infection has been proposed as a mechanism for induction of atherosclerosis [221, 222]. The association between CMV infection and coronary restenosis in cardiac transplant recipients and accelerated graft rejection in other solid organ transplant patients is well established. Furthermore, a study in non-immunocompromised adults reported an association between the presence of serum IgG antibody to CMV and impaired in vivo response to endothelium-dependent vasodilators [223]. However, two large seroepidemiological studies failed to confirm an association between CMV infection and atherosclerotic diseases [224, 225]. Although Guillain-Barré syndrome, immune thrombocytopenia, and autoimmune hemolytic anemia are generally accepted as rare complications of primary CMV infection, the association between CMV and other autoimmune diseases is more controversial. A role for human CMV infection has been proposed in the onset or progression of systemic lupus erythematosus, systemic sclerosis, and type 1 diabetes [226–230]. It will be challenging to sort out what role CMV infection plays in these and other specific chronic diseases. Inflammation and immunosuppression used to treat autoimmune diseases can trigger reactivation of human CMV, and the virus encodes a number of genes which reflect its complex interaction with the host inflammatory system. The associations with autoimmune diseases could be due to epidemiological, pathogenic, or host genetic features that are not fully understood at this time [228, 231]. If it becomes clear that CMV plays an etiologic or even facilitating role in atherosclerosis or autoimmune disease, the current view of CMV as a major problem for the fetus and immunocompromised persons and a relatively benign, silent commensal for others would be radically changed. It would then be necessary to intensify vaccine development and to formulate strategies aimed at preventing CMV infection in everyone rather than at preventing disease in vulnerable groups.

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Human Herpesviruses: Herpes Simplex Virus Types 1 and 2

36

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1 Introduction

The term *herpes* has been used in medicine for at least twenty-five centuries [1]. The word έρπης, from the verb έρπειυ (to creep), was used by the ancient Greeks to describe spreading cutaneous lesions of varied etiology [2]. The "herpetic eruptions which appear about the mouth at the crisis of simple fever" were first described around 100 AD by a Roman physician, Herodotus. About 1,600 years later, herpes of the genital tract was first reported by a French physician, Astruc. By the nineteenth century, the generally accepted use of the term *herpes* was restricted to certain diseases associated with vesicular eruptions. Early in the nineteenth century, Willan and Bateman first suggested that herpes labialis, herpes genitalis, and herpes zoster could be differentiated from each other based on clinical features [1]. By the latter part of that century, a recognition of cytopathological differences permitted discrimination between infections of the pox and the herpes groups. In the early part of the twentieth century, epidemiologic and experimental studies further supported the distinction between herpes zoster and oral or genital herpes. The studies of Gruter and other European workers showed that specimens obtained from zoster lesions could not be transmitted to the rabbit cornea, in contrast to those obtained from the other two herpetic conditions. Around 1920, a German physician, Lipschutz, maintained that, although biologically related, orolabial herpes and genital herpes were etiologically different.

Over the ensuing 40 years, the experimental host range of herpes simplex viruses was widened to include other laboratory animals, chick embryos, and ultimately cell cultures. The clinical spectrum of HSV infections was expanded to include gingivostomatitis, encephalitis, meningitis, Kaposi's varicelliform eruption, and neonatal disease. Data also accumulated to show that HSV infections could recur in the presence of demonstrable levels of serum antibodies.

In the early 1960s, Schneweis [3] in Germany and Plummer [4] in England reported antigenic differences among HSV strains. By 1967, Nahmias and Dowdle [2] in the United States had demonstrated that the large majority of genital and newborn infections are caused by HSV-2 and that most nongenital infections are caused by HSV-1, relating these clinical findings to the usual mode of transmission of the two virus types. By the 1970s, strain differences of the two HSV types were illuminated at the molecular level by restriction endonuclease analysis of their viral DNAs [5]. In more recent times, the use of type-specific serologic tests and polymerase chain reaction (PCR) methods has allowed for the rapid detection and accurate typing of HSV infection.

2 Description of Agent

Herpes simplex viruses belong to the Herpesviridae family, a collection of more than 300 DNA viruses that infect a wide range of species from fungi to man [6, 7]. The other human herpesviruses are cytomegalovirus (CMV), varicella zoster virus (VZV), Epstein–Barr virus (EBV), and human herpesviruses 6A (HHV-6A), 6B (HHV-6B), 7 (HHV-7), and 8 (HHV-8). All of these viruses have the capacity to persist in their natural host, either in neural cells, e.g., HSV and VZV, or in nonneural cells, e.g., CMV, EBV, HHV-6, HHV-7, and HHV-8. The sequence and positional relationships of multiple genes suggest that the human herpesviruses were derived

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from a common ancestral herpesvirus that existed, perhaps, 400 million years ago [8]. The high prevalence in primitive societies of antibodies to the nonsexually transmitted human herpesviruses, in contrast to the low prevalence of antibodies to nonpersistent viruses [9], emphasizes the survival advantage conveyed by viral persistence and supports the notion of an early origin of the herpesvirus family. HSV-1 and HSV-2 are closely related alpha herpesviruses that share about 50 % nucleic acid homology and 83 % amino acid sequence identity indicating these two viruses evolved from a common ancestor [7]. Based on amino acid sequence analysis, it has been estimated that the two viruses diverged between 8 and 10 million years ago [10, 11]. It has been hypothesized that divergence occurred as a consequence of bipedalism, the acquisition of continual sexual attractiveness by the ancestral human, face-to-face mating, and increased microbiological separation of the respective sites of infection [9, 11]. It is also possible that HSV-1 is more closely related to the ancestral herpesvirus and that HSV-2 represents recent evolution of the family.

Despite an early observation [12] that HSV-1 is more likely to be associated with a nongenital infection and that HSV-2 has a predilection for genital infection, both HSV-1 and HSV-2 can cause oral and genital primary infections. However, the viruses do differ in their propensity to cause recurrent infections, with recurrent oral and ocular infections more likely to be due to HSV-1 and recurrent genital infections more likely to be caused by HSV-2 [12, 13]. It has been proposed that the two viruses evolved in part to facilitate reactivation from the distinct sensory ganglia: HSV-1 from trigeminal ganglia and HSV-2 from sacral ganglia. Animal model studies using chimeric mutant viruses suggest that the unique latency-associated transcript (LAT) regions of the two viruses may confer the anatomic site-specific reactivation profile [14]. There are some other interesting biological differences between HSV-1 and HSV-2 including differences in susceptibility to some antiviral agents [15], differences in lability to temperature (HSV-2 is more labile at 39 °C than HSV-1), and the apparent ability of fever to trigger recurrent fever blisters (labial herpes) but not genital herpes.

Herpes simplex viruses consist of four major morphological components: a centrally located core surrounded by three concentric structures—the capsid, the tegument, and the envelope (Fig. 36.1).

The core contains DNA coiled around proteins arranged in the form of a barbell. The icosahedral capsid contains 162 capsomeres and measures around 100 nm. Between the capsid and the envelope is the tegument, composed of fibrillar material. The envelope, derived from nuclear and, occasionally, other cell membranes, confers a diameter of 150–200 nm on the complete virus particle. The lipid composition of the envelope makes the virus particularly susceptible to ether and other lipid solvents. Herpes simplex viruses are labile outside the host, losing infectivity within minutes to hours [17, 18]. Virus infectivity is lost more quickly at extremes of temperature and pH and with exposure to ultraviolet and ionizing radiation or surface active agents such as nonoxynol-9 [19].

The HSV-1 virion contains a linear double-stranded DNA molecule of 152 kilobase pairs (kbp). The genome of HSV-2 is slightly larger at 155 kbp. About half of the HSV-1 and HSV-2 DNA sequences are homologous, and they share approximately 83 % amino acid sequence identity. Ongoing work is defining the biological roles of the 74 viral genes encoded by each virus. In addition to encoding proteins required for viral replication, the viruses encode several proteins and microRNAs (miRNAs) that facilitate immune evasion [20, 21]. HSV-1 and HSV-2 have a wide in vitro and in vivo host range, being able to infect a wide variety of cell types of human or animal origin as well as numerous types of experimental animals, ranging from mice to monkeys [22–26]. HSV-1 infection does not protect against HSV-2, but it may decrease the risk of symptomatic seroconversion [27].

HSV-1 encodes for at least 11 glycoproteins, of which gB, gD, gH, gK, and gL are essential for productive infection in cell culture [28, 29]. HSV gG is type-specific, and gE and gI can act as a receptor for the Fc portion of IgG. HSV gB, gD, and the heterodimer gH/gL are involved in viral entry into the cell [30].

3 Laboratory Methodology Involved in Epidemiologic Analysis

3.1 Serology

Development of radioimmunoassay-based gel methods [31], then, more convenient Western blot methods, allowed accurate discrimination of antibodies to HSV-1 from those directed against HSV-2 antigenic targets [32, 33]. Because HSV-1 typically is acquired early in life, later acquisition of HSV-2 induces anamnestic responses to type-common epitopes, effectively masking the type-specific HSV-2 response in tests based on the relative strength of neutralization or binding of serum antibodies to crude HSV-1 or HSV-2 antigen mixtures. Studies of patients who were seroconverting to HSV-1 or to HSV-2 showed that the Western blot ("HSV-WB") was uniquely able to overcome the anamnestic response to detect type-specific antibody production in those with prior HSV-1 infection [32, 34]. HSV-WB remains the gold standard test for confirming the presence of HSV-2 antibodies [35].

Two other reference tests, the immunodot enzyme assay (IEA) developed at Emory University [33] and the gG-2 monoclonal antibody blocking assay developed by the Central Public Health Laboratory (CPHL) in London

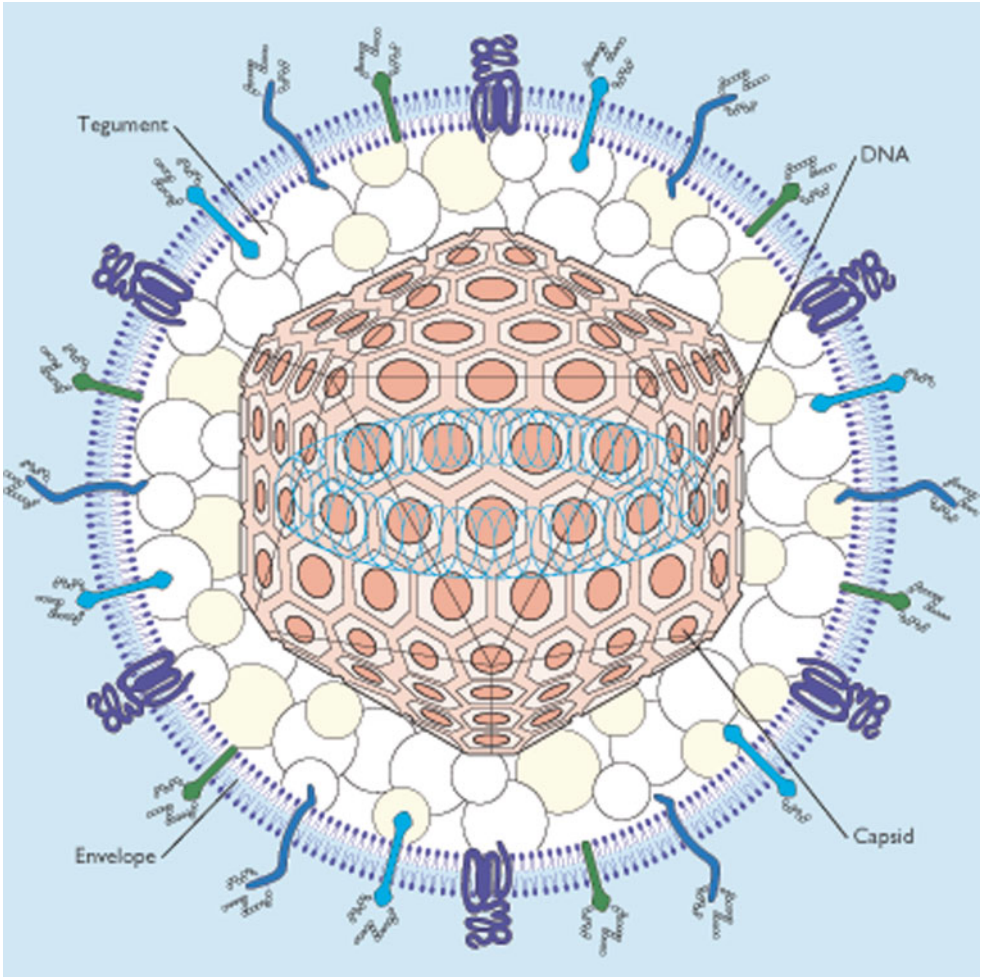

Fig. 36.1 Schematic of HSV virion (Adapted from Johnston et al. [16])

[36, 37], were extensively validated against HSV-WB in populations with well-characterized, virologically defined infections. The IEA has played a key role in tracking the prevalence of HSV-2 over time in the United States [38–41]. The CPHL test has been used to determine HSV-2 prevalence in European countries [42].

In the 1990s, HSV-WB was used to characterize the performance of tests based on a single type-specific glycoprotein, gG-1 from HSV-1 and gG-2 from HSV-2, for detecting antibodies to HSV-1 and HSV-2, respectively. Most gG-based tests have simpler, enzyme immunoassay formats, and a number of these EIAs are now available commercially (Table 36.1). The HerpeSelect HSV-2 IgG EIA from Focus Technologies is the most widely used, FDA-approved, commercial type-specific serology in the United States for diagnostic testing, with results calculated as an index value. The test operates well in the high positive range (index value >3.0), but care must be taken in interpreting low-positive results, due to possible antibody cross-reactivity with HSV-1 resulting in a false-positive result. In one large study, only 35 % of low-positive results were confirmed as positive by HSV-WB [43]. The EIAs developed by Alere, EUROIMMUN, and Trinity are all FDA approved but have had limited published performance data [44, 45].

At least one commercial EIA (from Focus Technologies) can be successfully applied to antibodies eluted from dried blood spots, a potential advantage for seroprevalence studies in areas without serum storage or laboratory testing capacities [46]. Other gG-based tests such as the "Biokit HSV-2 Rapid Assay" use membrane and lateral flow technologies to provide near-patient or "point of care" testing outside of laboratory settings [47]. While commercially available, the relatively high cost of the Biokit rapid assays has outweighed its potential value in determining seroprevalence of HSV-2 in underserved areas of the world. However, the Biokit rapid assay has been useful in some settings as an alternative to limited-availability reference tests to confirm the results of EIA-positive tests in serum samples [43, 48].

Within 10 years of the development of type-specific serologic tests for HSV, commercial and reference tests became critical research tools for the descriptive epidemiology of genital HSV-2 in populations around the world, revealing HSV-2 prevalence that varied widely by region, gender, and age [49]. The CPHL monoclonal antibody blocking test was

Table 36.1 Examples of HSV type-specific serology tests

Test format	Examples	Advantages	Limitations for seroepidemiologic studies
Enzyme immunoassay, FDA approved	HerpeSelect IgG ELISA (Focus) HSV Captia (Trinity) EUROIMMUN Alere/Wampole	Commercially available	Population-specific performance issues of some tests
Enzyme immunoassay	Kalon HSV-2 EIA	Commercially available Better specificity in African populations than other EIAs	Lower sensitivity than other EIAs for early seroconversion
Membrane rapid assays	Biokit HSV-2 rapid assay	Near-patient format Commercially available Possible utility as confirmatory assay	More expensive per test than EIA
Western blot	HSV Western blot (University of Washington, USA)	Validated against clinical and virologic criteria	No commercial formats, limited availability
Reference tests	Immunodot enzyme assay (IEA) (Emory and CDC,USA) gG monoclonal antibody inhibition assay (CPHL, UK)	Validated against clinical and virologic criteria or UW HSV Western blot	No commercial formats, limited availability
High-throughput tests	Bio-Rad HSV IgG	Multiplexed, FDA approved	Limited availability

used to demonstrate the low specificity of most commercial enzyme immunoassays in sera from sites in Africa [45]. There is now an extensive literature using either the CPHL test or the HSV-WB as gold standards that document poor specificity of the Focus Technologies HSV-2 EIA in sera from sub-Saharan Africa [50, 51] and the Republic of South Africa [48]. A selective, further loss of specificity reported in the HIV-positive subpopulations in these areas was not replicated in a large study in Kampala, Uganda [52]. To a lesser extent, the EIA from Kalon and the rapid assay from Biokit also exhibit lower specificity than in North American populations [52–54]. The combinations of tests and changing cutoffs for positive results may mitigate the specificity problems [48, 51, 54], but until the underlying cause is discovered and serologic assays are developed to overcome the problem, seroepidemiologic outcomes for HSV-2 should take into account the method used and any confirmatory testing used in the study.

The availability of accurate type-specific serology also led to a modification in the definition of genital herpes to recognize the high proportion of patients who become infected without recognizable symptoms. The understanding that genital herpes is often unrecognized by clinicians based on clinical presentation alone [55, 56] led to recommendations for laboratory testing, in general, and for type-specific serology, specifically, in high-risk populations [57]. It is not recommended to use HSV IgM testing to diagnose HSV. While IgM can be more sensitive than IgG for individuals who are seroconverting to HSV, IgM is also produced in many individuals during the course of recurrent shedding episodes. Thus, even type-specific IgM is a poor marker of first-episode HSV infection [58]. Avidity tests have been developed to discriminate between first and subsequent HSV-2 episodes, but these tests have not been adapted for widespread commercial use [59].

Because no serologic assay has yet been described that can discriminate between HSV-1 antibodies elicited by oral versus genital infection and because the most widely used commercial HSV-1 type-specific serology tests may lack sensitivity [60, 61], current estimates of genital HSV-1 prevalence may be conservative.

3.2 Virus Detection Methods

Direct observation of virus in patient or subject samples provides strong causal inference of symptom etiology. Methods that have been well described for HSV diagnosis include those that detect type-common or type-specific HSV antigens, viral culture, or various viral nucleic acid detection techniques. Each approach has advantages and limitations as applied to studies intended to define the role of HSV in clinical syndromes or conditions for transmission of the virus (Table 36.2). DNA amplification methods such as polymerase chain reaction (PCR) and viral culture share the advantage of amplifying the target virus or nucleic acid before detection and are, therefore, far more sensitive than antigen detection methods.

Early studies of HSV shedding and transmission were generated using viral culture-based methods [56]. Since HSV viability depends on collection and transport methods, use of viral culture may underestimate presence of virus [62, 63]. Once a specimen arrives in the laboratory, cell culture isolation methods can markedly affect the ability to detect HSV by its characteristic effects on cell morphology or cytopathic effect (CPE). Choice of host cells and length of time that cul-

Table 36.2 Advantages and limitations of HSV-specific detection methods as used for epidemiology studies

Method	Advantages	Limitations
Antigen detection	Can simultaneously detect and type as HSV-1 or HSV-2 Inexpensive, fast to perform (2–4 h) Less vulnerable to virus degradation than culture	Lack of sensitivity
Viral isolation in cell culture	Virus is amplified via several replication rounds before detection More sensitive than antigen detection	Virus may lose viability in transport Recognizing CPE is subjective and requires training for expertise Requires antigen detection to definitively identify CPE as being due to HSV Slow to perform (3–10 days)
Modified viral dulture	Faster than culture (16–48 h) Some viral amplification provided greater sensitivity Less expensive than culture Objective endpoint allows readout by less experienced personnel	Viable virus required Less sensitive than standard culture
ELVIS™ HSV ID	Faster than culture (24 h), more sensitive than other modified culture methods Objective, colorimetric readout	Binding virus required Less sensitive than some culture techniques Much less sensitive than PCR
DNA amplification and detection (PCR)	Most sensitive of all testing methods Not vulnerable to loss of virus viability if samples collected into extraction buffer Rapid (2–4 h) Objective readout	Older methods vulnerable to contamination May be more expensive Current lab-based methods differ from lab to lab Limited commercial kit availability

tures are held for observation are the major factors that affect the rate of positivity across laboratories. Modified culture methods that provide a short (16–48 h) amplification period in culture followed by "blind" detection of viral antigen before CPE is seen can increase sensitivity and allow simultaneous typing of isolates. A second, well-characterized, modified viral culture method, "ELVIS™ HSV ID," uses genetically modified host cells in which an HSV structural protein induces expression of an enzyme reporter gene [64].

Studies since the late 1990s have depended on nucleic acid amplification methods because of their proven superiority in sensitivity over all other methods and high specificity [65]. Closed systems such as the LightCycler (Roche Diagnostics) [66] and TaqMan (Applied Biosystems Inc.) [67] methods greatly reduced the contamination of specimens with resulting amplicons or with exogenous DNA from other sources in the laboratory, making PCR both reliable and sensitive for the detection of low levels of virus in specimens. Commercial reagents have been available for HSV PCR, but most major labs have developed their own PCR tests, making comparisons of studies based on PCR difficult. Recently, the US Food and Drug Administration (FDA) approved an alternative test, BD ProbeTec HSV Q^x, an automated system that amplifies and simultaneously detects type-specific regions of the glycoprotein G gene [68]. Because the primers and probes for PCR tests can be selected to target only HSV sequences, the test has inherently high specificity and has been used to good effect to demonstrate that HSV is a predominant (although often not the sole) pathogen present in genital ulcers in the United States [69], Europe [70], and Africa [71].

PCR is now the accepted method to diagnose neonatal herpes [72] and HSV encephalitis from cerebrospinal fluid specimens [73]. Its increased sensitivity has been instrumental in revealing HSV shedding and localization patterns after infection, and it is now the method of choice for defining epidemiologic patterns of oral, ocular, and genital shedding.

4 Descriptive Epidemiology

4.1 Seroepidemiology

Since the realization of antigenic differences in HSV-1 and HSV-2 in the 1960s [4] and the development of serologic assays to differentiate between HSV-1 and HSV-2 as discussed above, seroepidemiology studies have revealed distinct patterns of incidence and prevalence of HSV-1 and HSV-2.

4.1.1 HSV-1 Incidence

HSV-1 is typically acquired during childhood through nonsexual contact via infected oral secretions [74]. While few studies have measured seroincident HSV-1 infection directly, incidence can be inferred more indirectly through serial cross-sectional studies. The 1999–2002 US National Health and Nutrition Examination Survey (NHANES) found that HSV-1 seroprevalence in children increased from 26.3 % in 6–7-year-olds to 36.1 % in 12–13-year-olds, suggesting rapid acquisition during the childhood years [61].

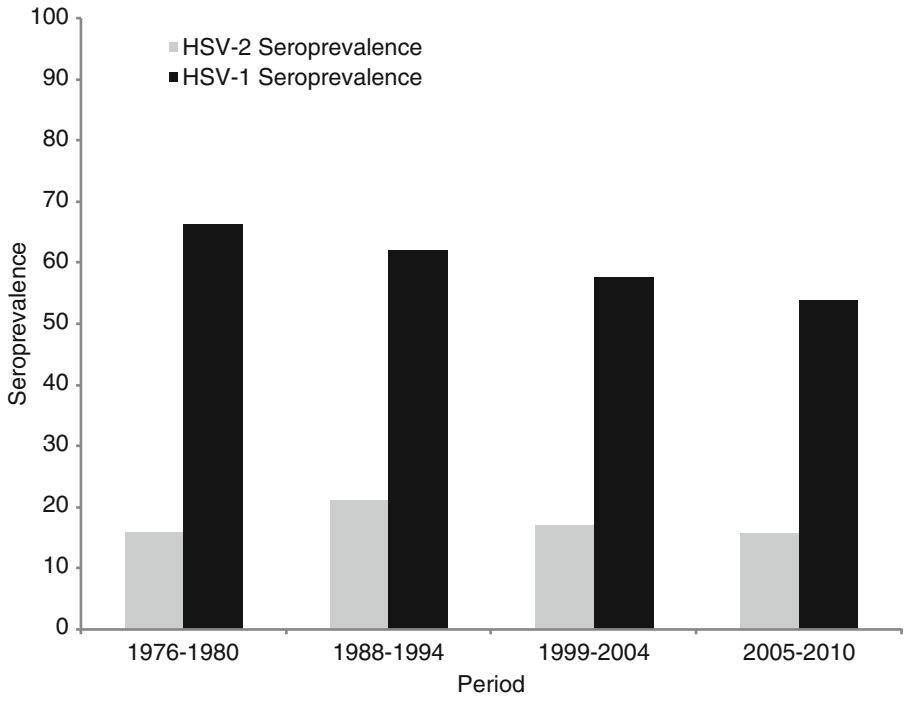

Fig. 36.2 Age-adjusted seroprevalence HSV-1 and HSV-2 in the United States, NHANES Data 1976-2008 [39]

4.1.2 HSV-1 Prevalence

HSV-1 seroprevalence is highest in developing countries, where it reaches nearly 100 % and increases with age [49]. In the United States, HSV-1 seroprevalence has been declining over the past three decades, with 2005–2010 NHANES revealing that 53.9 % of persons ages 14–49 are HSV-1-seropositive, a 6.8 % decline since 1999–2004 (Fig. 36.2) [39, 75].

The largest decline in seroprevalence was found in persons ages 14–19, from 46 to 39 %, a relative decrease of 14.7 % [39]. In parallel with the declining HSV-1 seroprevalence in adolescents, sexual acquisition of HSV-1 is becoming more common, and HSV-1 has surpassed HSV-2 as the leading cause of first-episode genital herpes in women <25 years of age and men who have sex with men [76–79]. HSV-1 seropositivity is significantly associated with increasing age, birth outside of the United States, and poverty [39, 61]. Rates of HSV-1 seropositivity are nearly twofold higher in non-Hispanic blacks and Mexican Americans as compared to non-Hispanic whites.

4.1.3 HSV-2 Incidence

HSV-2 is most often acquired after initiation of sexual activity, through intimate genital contact. In the United States, prospective studies performed in family planning clinics and STD clinics have shown an incidence of 7.8 and 11.7 per 100 person-years, respectively [80, 81]. Similarly, US military populations have an HSV-2 incidence of 6.2 per 100 person-years [82]. In adolescent women (ages 12–15) initiating sexual activity in the United States, the HSV-2 incidence was 4.4 per 100 person-years [83]. Again, incidence has been estimated indirectly in settings with high HSV-2 seroprevalence, such as sub-Saharan Africa, where seroprevalence rates increase markedly during adolescence, from 7.3 % in 15-year-olds to 33.5 % at 24 years of age in women [84]. Similar increases are seen in men, from 3.1 % at age 15 to 14.4 % at age 24 [84], with up to 43 % of uncircumcised men infected by age 24 in some populations [85]. As of 2003, 536 million people aged 15–49 were estimated to be infected with HSV-2 worldwide, with incidence of 23.6 million per year (Table 36.3) [86].

4.1.4 HSV-2 Seroprevalence

HSV-2 seroprevalence is twofold higher in women compared to men and increases with age and the number of lifetime sexual partners, reaching nearly 100 % in cohorts of female sex workers [49, 86]. Country-specific HSV-2 seroprevalence has been estimated using random sampling and population-based surveys in antenatal clinics and in high-risk populations such as HIV-seropositive persons, commercial sex workers, and persons attending STD clinics [49]. The most comprehensive review of global HSV seroprevalence was performed by Smith et al. [49]. HSV-2 seroprevalence is highest in sub-Saharan Africa, with >80 % of men and women ≥35 years of age infected [49]. In Central and South America, seroprevalence in women ranges from 33 % at ages 25–29 to 45–50 % at age 40–50. Seroprevalence is 4–40 % in Europe, with highest prevalence in Greenland (74 % of women age 25–39) and Scandinavia (15–35 % among women age 25–35). Data from the Middle East and

Table 36.3 Global seroprevalence and seroincidence of HSV-2 infection in men and women, by region, in 2003 [86]

	Seroprevalence (in millions)			Seroincidence (in thousands)		
Region	Men	Women	Total	Men	Women	Total
North America	11.9	17.8	29.7	466	641	1,107
Latin America and the Caribbean	15.1	38.6	53.7	820	1,714	2,534
North Africa and the Middle East	8.6	9.6	18.2	195	388	583
Sub-Saharan Africa	45.5	78.2	123.7	2,575	3,547	6,122
Western Europe	7.2	13.7	20.9	340	373	713
Eastern Europe and Central Asia	12.3	28.9	41.2	624	755	1,379
Eastern Asia	47.8	61.8	109.6	2,341	2,957	5,298
Japan	0.7	4.1	4.8	32	28	60
Pacific	0.5	0.3	0.8	23	17	40
South Asia	29.4	33.2	62.6	1,589	991	2,580
Southeast Asia	41.2	27.6	68.8	1,776	1,305	3,081
Australia and New Zealand	0.4	0.9	1.3	21	41	62
Total	220.7	314.8	535.5	10,802	12,757	23,559

Adapted from Looker et al. [86]

Table 36.4 Epidemiology of clinical manifestations of HSV infection

	Incidence/prevalence	Population affected
Genital ulcer disease	50–80 % due to HSV-2 (prevalence)	Sexually active persons
Gingivostomatitis	Not available	Children
HSV encephalitis	2.2 cases/million PY	Children, sixth decade of life
Ocular HSV	11.8/100,000 PY	Mean age 43
Neonatal HSV	4–31.3/100,000 PY	Neonates
Eczema herpeticum	~6/100,000 pediatric hospital admissions	Children, median age 1
Herpetic whitlow	2.4/100,000 PY	20 % in children, 41 % in young adults

North Africa are limited, but in antenatal and obstetrics clinics, seroprevalence ranges from 0 % in Syria to 12 % in Bangladesh to 32 % in Egypt [87]. In Northeast India, women in antenatal clinics had an overall seroprevalence of 8.7 %, although this varied from 2.7 to 15 % depending on the region [88]. In Asia, seroprevalence is lowest in Japan and the Philippines, with estimates at 7–9 %. Women in antenatal clinics in Australia had seroprevalence ranging from 4.3 % in 15–19-year-olds to 20 % in >35-year-old. In all populations studied, HSV-2 seroprevalence was higher in commercial sex workers and STI clinic attendees than the general population [49].

In the United States, the 2005–2008 NHANES showed that 16.2 % of persons age 14–49 were HSV-2 seropositive (Fig. 36.2) [89]. In the 1988–1994 NHANES, there was an increase in HSV-2 seroprevalence to 21.9 % [38]; in the most recent survey, this trend seems to have reversed and seroprevalence rates have stabilized [39]. As with HSV-1, race is associated with seroprevalence; non-Hispanic blacks have rates three- to fourfold higher than non-Hispanic whites or Mexican Americans [89]. Most people with HSV-2 infection have not been previously diagnosed; in the NHANES, 81.1 % of people were not aware of their infection [89]. HSV-2 seroprevalence rates are higher in STD clinic populations, with overall rates of 40 % in the United States [90]. HSV-2 seroprevalence is also higher in HIV-infected persons, with 50–90 % seroprevalence in this population [84, 91–95].

5 Patterns of Host Response: Clinical Manifestations and Epidemiology of HSV Infections

Most people with HSV infection are asymptomatic or have mild disease that is undiagnosed [39]. Therefore, persons who present to clinical care represent a minority of those infected. However, HSV causes a wide range of manifestations on mucosal surfaces and skin and in the central nervous system (Table 36.4).

5.1 Genital Infections

HSV is the leading cause of genital ulcer disease (GUD) worldwide. Studies using PCR in the United States [96], Africa [71, 97–99], and Asia [100] show that 50–80 % of genital ulcers are due to HSV. The predominance of HSV-2 has increased as the etiologic agent of GUD appears to have increased over time. For instance, in a South African mining community, HSV-2 prevalence in genital ulcers increased

from 17 % in 1993 to 36 % in 1998 [101] and in Botswana from 23 % in 1993 to 58 % in 2002; over the same period, *Haemophilus ducreyi* declined from 25 % in 1993 to 1 % in 2002 [97]. In response to this epidemiologic shift, antiviral therapy (acyclovir) is now included in the World Health Organization (WHO) recommendations for syndromic management of genital ulcer disease [102]. Genital herpes has also been an increasing cause of visits to physician offices in the United States over the last four decades, with 232,000 clinic visits in 2010, up from 17,000 in 1970 and 57,000 in 1980 [103].

5.1.1 Primary Genital Infection

HSV primary infection is defined as acquisition of virus in the absence of antibody to either HSV-1 or HSV-2. In a large series of 209 persons with laboratory-documented primary genital herpes, the most recent sexual contact was 5.8 (range 1–45) days prior to symptoms [104]. Patients typically develop discrete vesicles or pustules on genital skin and mucosal surfaces, which ulcerate and coalesce to form painful wet ulcers. Genital symptoms and viral shedding are greatest during this wet ulcer stage. As the skin reepithelializes, a dry crust forms, viral shedding ceases, and the lesions resolve. Symptoms last a median of 19 days during primary infection in the absence of antiviral therapy [104]. Primary genital HSV may cause significant local signs and symptoms, including severe bilateral ulcerations, cervicitis or proctitis, dysuria or urinary retention, vaginal or urethral discharge, and lymphadenopathy [104]. In addition, ~25 % of persons with symptomatic primary infection have evidence of HSV viremia [105], and this may be associated with more systemic symptoms such as fever and myalgias. Up to 1/3 of women develop signs of aseptic meningitis during primary symptomatic genital infection [104]. Primary genital HSV-1 infection may be associated with more lesions and longer duration than primary genital HSV-2 [104].

5.1.2 First-Episode Infection

First-episode genital HSV is defined as the first recognized clinical outbreak of genital HSV, and it may occur either in the absence of HSV antibody (primary) or in the presence of antibody to HSV-1 or HSV-2 (non-primary). Symptoms of non-primary HSV are less severe than primary HSV, possibly due to the presence of neutralizing antibody [104] or acquired immunity. The differentiation between first-episode and recurrent disease has implications for patient management, as higher doses of antiviral agents and longer duration of therapy are required to treat first episode.

5.1.3 Recurrent Genital Infection

Clinical recurrences of genital HSV recurrences are very heterogeneous: some patients may have monthly genital recurrences, while others may never have a recognized recurrence after their first episode. In a large series of 457 patients with laboratory-documented symptomatic primary genital HSV-2 diagnosed before suppressive antiviral therapy was widely used, the median number of recurrences during the first year after primary genital infection was 4; 20 % of patients had more than 10 recurrences [12]. After the first 2 years of infection, the median number of recurrences decreased to 2 episodes/year [106], although some people continued to have much more frequent recurrences. In contrast, HSV-1 genital recurrence is less frequent, with a median of 1.3 recurrences the first year after symptomatic primary and 0.7 recurrences/year in the second year [107]. Recurrent genital HSV infection may have a prodrome of neuralgia, tingling, or itching prior to the appearance of genital vesicles/pustules or ulcers in 60 % of patients. Symptoms during recurrent infection are generally localized to the genital tract and are more mild than first episode, although they are more severe in women, lasting a mean of 6 days, as compared to men (mean 3 days) [104].

5.1.4 Other Genital Symptoms

HSV is the most common cause of gram stain-negative proctitis in men who have sex with men (MSM) [108] and causes about 25 % of cases of proctitis overall in this population [109]. HSV proctitis may present with fever; severe, burning rectal pain and tenesmus; perianal ulcerations; and urinary retention [109]. HSV is shed from the cervix in most women with first-episode genital herpes; the cervix may have ulcerative lesions or may be diffusely friable [104].

5.1.5 Subclinical Acquisition and Infections

The placebo or control arms of randomized clinical trials of HSV vaccines and antiviral agents have provided the opportunity to prospectively evaluate the epidemiology of HSV acquisition. In one vaccine study that evaluated 2,393 high-risk persons, only 57 (37 %) of 155 new HSV-2 infections were symptomatic; the remaining 98 (63 %) persons had subclinical seroconversion [27]. Other prospective studies reported similar findings [110]. In the control arm of the Herpevac trial, which tested a glycoprotein D2 subunit vaccine in HSV-1-/HSV-2-seronegative women, the incidence of HSV-2 was 1.1/100 person-years; 63 % of seroconversions were subclinical [111]. The HSV-1 incidence was 2.5/100 person-years and 74 % of seroconversions were subclinical.

5.1.6 Genital Herpes in the Immunocompromised Host

Severe genital HSV may be found in immunocompromised persons. Chronic genital ulcerations due to HSV-2 were one of the first recognized manifestations of AIDS [112] and remain an AIDS-defining condition. Persons with advanced HIV infection have frequent HSV recurrences; the risk is greatest for those with CD4 count <100 cells/mm not on

highly active antiretroviral therapy [113]. Atypical presentations such as hypertrophic lesion may occur in immunosuppressed patients [114–118]. In addition, persons undergoing solid organ or stem cell transplantation are at increased risk for recurrent HSV. Immunocompromised persons may develop acyclovir-resistant herpes [119, 120], a rare occurrence in immunocompetent hosts [121]. Continuous anti-HSV suppressive therapy prevents both HSV recurrences and acyclovir resistance in immunocompromised hosts [122].

5.2 Oral Infections

Similar to genital disease, oral HSV may be classified as primary and recurrent disease. In contrast, however, oral disease is caused almost exclusively by HSV-1, with few case reports of HSV-2 causing oral disease.

5.2.1 Gingivostomatitis

This typically occurs in young children and is due to primary acquisition of HSV-1. Children present with fever, severe labial and oral ulcerations, and pharyngitis or esophagitis, with difficulty eating due to pain [123]. Lesions last for a mean of 12 days, with persistent viral shedding for a mean of 7 days [123]. Similar to primary genital HSV-2 infection, 34 % of patients with symptomatic primary gingivostomatitis are viremic [124].

5.2.2 Recurrent Oral Infections

Oral HSV recurrences, often called "cold sores" or "fever blisters," present as a single labial ulceration, most often involving the lower lip. Risk factors for recurrence include fever, illness, and exposure to sunlight [125, 126]. Although the recurrence rate of oral HSV-1 is not well defined, in one prospective study of 12 persons after primary concurrent oropharyngeal and genital HSV-1, orolabial recurrences occurred in 42 % of persons, with a recurrence rate of 0.12 recurrences/month [13]. Similar recurrence rates were reported in persons with a history of symptomatic oral HSV-1 [125].

5.3 Neurologic Infections

5.3.1 Encephalitis

Herpes simplex encephalitis (HSE) is a significant cause of HSV-related morbidity and mortality. HSV-1 is the leading cause of sporadic viral encephalitis worldwide, causing a third of viral encephalitis in the California encephalitis project [127]. In a prospective study from England, 38 (19 %) of 203 patients with encephalitis had HSV infection [128]. There is a bimodal age distribution, with 1/3 of cases occurring in children >6 months and <18 years of age and half of cases occurring in adults over age 50 [129]. Encephalitis may occur during primary infection but occurs during reactivation in about 2/3 of cases [130]. In a retrospective study conducted over 12 years in Sweden, 236 cases of laboratory-confirmed HSV-1 encephalitis were diagnosed, for an incidence of 2.2 cases per million persons per year [131]. Similar findings have been described in the United States [132]. HSV encephalitis was equally distributed in men and women [131]. Despite antiviral therapy, the 1-year mortality was 14 % [131]. Patients may present with febrile confusion and focal neurologic signs. Cerebral spinal fluid (CSF) typically shows a pleocytosis, consisting predominantly of lymphocytes; it may be hemorrhagic, with slightly decreased glucose and markedly increased protein. Brain imaging typically reveals temporal lobe involvement, sometimes with hemorrhage requiring craniotomy. Seizures and neuropsychiatric sequelae occur in nearly a quarter of patients after recovery from acute encephalitis [131]. A significant advance in the field is the ability to accurately diagnose HSV encephalitis with HSV PCR from cerebrospinal fluid, a faster and less invasive approach than viral culture from brain biopsy material [133]. HSV-2 may also cause encephalitis but this is less common. Intravenous acyclovir is the gold standard therapy for HSE. Without therapy, mortality is 70 % [132]. HSV may also cause ascending myelitis [134, 135], although this is rare.

5.3.2 Meningitis

HSV may cause recurrent aseptic meningitis ("Mollaret's meningitis"), with fever, headache, and stiff neck. This is commonly caused by HSV-2 and may or may not be accompanied by a genital herpes outbreak [136]. In a series of 40 persons with HSV-2 meningitis, almost half had confirmed or suspected neurologic recurrence during a 1-year follow-up [137]. A recent trial of suppressive valacyclovir 500 mg BID did not prevent recurrences [138]. Higher doses of oral antiviral therapy may be required to suppress recurrences of Mollaret's meningitis.

5.3.3 Possible Role in Alzheimer's Dementia

HSV-1 has long been hypothesized to have a pathogenic role in the development of Alzheimer's dementia (AD) [139], based on the following evidence for biological plausibility: (1) the frontal and temporal lobes and hippocampus are affected in AD and HSV-1 encephalitis [140]; (2) HSV-1 is a risk factor for developing AD in persons with apolipoprotein E ε4 (APOE4) isoform [141, 142], and this allele is associated with symptomatic oral HSV-1 lesions [143] with transgenic mouse models showing that APOE4 mice have significantly higher number of HSV1 genomes in brain tissue as compared to APOE3 mice, suggesting an interaction between HSV-1 and APOE4 [144]; and (3) in neuronal cell culture, HSV-1 infection causes β-amyloid

[145] and amyloid precursor protein accumulation [146] as well as tau phosphorylation [147], hallmarks of the pathogenic changes of AD. In Vero cells, HSV-1-induced β-amyloid and abnormally phosphorylated tau protein accumulation is reduced in the presence of antiviral agents such as acyclovir [148]. HSV-1 DNA has been found in brain tissue from persons with AD [141] and in amyloid plaques [149], but it has also been found in normal age-matched controls [141]. Further research will be required to determine whether HSV-1 infection contributes to the pathogenesis of AD.

5.4 Ocular Infections

HSV-1 and HSV-2 can cause keratitis, blepharitis, conjunctivitis, iridocyclitis, or retinitis [150]. Herpetic keratitis is the leading cause of corneal blindness in the developed world [151]. HSV infection produces corneal ulcerations that may be recurrent and can lead to scarring and neovascularization. In a longitudinal study conducted in Rochester, Minnesota, the incidence of ocular HSV was 8.4/100,000 person-years [151]. In this series, the first episode most commonly involved the superficial cornea (63 %) or lids and conjunctiva (54 %); the deep cornea was involved in only 6 % of patients [151]. The Moorfields hospital in London has followed 108 patients with primary HSV ocular disease over a period of 2–15 years; of these 32 % had recurrent disease [152]. Nearly half of these had 2–5 recurrences, and 11 % had more than 6 recurrences. In the Rochester series and others, the recurrence rates increase with the years of follow-up [151]. Most recurrences involved the conjunctiva or eyelid; few had corneal involvement [152]. HSV-1 and HSV-2 may also cause acute retinal necrosis, which can lead to vision loss. Acute retinal necrosis may occur years after neonatal herpes [153].

5.5 Neonatal Infections

Exposure to either HSV-1 or HSV-2 during labor and delivery can lead to severe infection in the neonate [73]. Although neonatal herpes is not a reportable disease [154], several studies have provided estimates of incidence in the United States and Canada. Using inpatient hospital admission data, the incidence of neonatal HSV in the United States is 9.6 cases/100,000 live births [155]. Incidence estimates using population-based methods and hospital discharge records [156, 157] or ICD-9 codes [158, 159] involving a number of cities in the United States range from 4 to 13.3 cases/100,000 live births [160]. A prospective, active-surveillance study in Canada showed similar rates, with 5.9 cases/100,000 live births [161]. In this study, 40 % of women had no history of genital HSV and only 2 % of cases had intrapartum lesions noted [161]. Rates up to 31.3/100,000 have been found in prospective studies at research centers with active surveillance [162]. Maternal acquisition of HSV near the time of delivery is associated with higher risk of neonatal herpes [163]. In the largest study of risk factors for HSV transmission to the neonate, the detection of HSV from the genital tract at the time of delivery and first-episode genital herpes near delivery were significant risk factors for transmission [162]. In addition, HSV-1 infection, HSV isolation from the cervix, the use of invasive monitoring, maternal age <21 years, and delivery before 38 weeks were associated with neonatal herpes [162]. Women who have genital ulcers at the time of delivery have a markedly reduced risk of transmission if delivered by cesarean section [162]. However, C-section is not recommended for HSV-2-seropositive women who do not have ulcers or prodrome at the time of delivery [164]. In women who are HSV-2 positive with a history of genital herpes, suppressive antiviral therapy is recommended from week 36 to delivery [164]. This practice decreases the risk of genital herpes recurrences requiring cesarean section [165, 166], reduces viral shedding [166–168] at the time of delivery, and presumably decreases the risk of HSV transmission, although this has not been demonstrated in clinical trials [166, 167]. Of note, cases of neonatal HSV have been described even in babies whose mothers received suppressive therapy [169]. While 85 % of neonatal HSV transmission occurs during the peripartum period, HSV can be transmitted in the postnatal period (10 %) or in utero (estimated 5 %) [72].

Neonatal HSV is classified into three types: (1) skin, eye, and mucous membrane (SEM) accounts for 45 % of infection; (2) CNS disease accounts for 30 % of infection; and (3) disseminated disease accounts for 25 % of infection [170]. Patients with SEM or disseminated disease present at 10–12 days of life, and those with CNS disease present at 16–19 days of life [72, 170]. Infants typically present with skin vesicles, although up to 40 % of patients with disseminated or CNS disease may not have any vesicles present [170]. Lethargy and fever occur in ~50 % of patients with CNS or disseminated disease, seizures occur in nearly 60 % of patients with CNS disease, and pneumonia and DIC occur in nearly 40 % of patients with disseminated disease [170]. SEM disease is associated with minimal morbidity and mortality. Although antiviral therapy has reduced morbidity and mortality due to CNS or disseminated disease, even with a 21-day course of high-dose IV acyclovir (60 mg/kg/day) [171], there is 30 % mortality with disseminated disease [170]. A 6-month course of suppressive oral acyclovir appears to be safe and may result in improved neurodevelopmental scores in patients with neonatal herpes [172]. HSV viral type does not affect neonatal herpes morbidity or mortality.

5.6 Cutaneous HSV Infections

HSV-1 or HSV-2 can cause a variety of syndromes involving the skin.

5.6.1 Eczema Herpeticum (Kaposi's Varicelliform Eruption)

Eczema herpeticum is a rare complication of childhood atopic dermatitis, occurring in 3–6 % of patients [173]. It is a vesicular rash that starts within areas of eczema but can spread to involve normal skin. A prospective Canadian study identified 79 cases of eczema herpeticum between the years of 2000 and 2009. The mean age of patients was 4.3 years, one-third of patients were <1-year-old, and nearly 10 % had recurrent infection [174]. Fever or bacterial infection occurred in over half of the patients, and keratoconjunctivitis occurred in 10 %. A retrospective study in the United States described 1,331 children hospitalized with eczema herpeticum, accounting for approximately 6 in 100,000 admissions. The median age in this study was 1 year (range 1–4). Prompt initiation of acyclovir was associated with decreased hospital length of stay [175].

5.6.2 Herpetic Whitlow

Recurrent digital vesicles/ulcers occur after autoinoculation, often in children. Occupational HSV acquisition can occur in persons who have frequent contact with oral mucous membranes, for instance, nurses [176], dentists/dental hygienists [177], or anesthesiologists. In the largest series describing 79 cases, the incidence was 2.4 cases per 100,000 persons, with 20 % of cases in children <10 years of age and 41 % in young adults aged 21–30 [178]. All cases in children were HSV-1. Recurrent episodes have been described with both HSV-1 and HSV-2 infections. Although herpetic whitlow usually involves the fingers, rare cases of toe involvement have been described [179, 180].

5.6.3 Herpes Gladiatorum (HG)

Outbreaks of HSV-1 have been described during wrestling events [181], likely due to contact of abraded skin with HSV-1 from infected oral secretions. The face, neck, and arms are the most commonly affected areas. An estimated 17 % of 330 participants in a 28-day camp acquired HG, which likely underestimated the transmission rate, as the HSV serostatus of participants was unknown [182]. Some authors advise the empiric use of valacyclovir prophylaxis for wrestlers' events, although the efficacy and cost-effectiveness of this approach are unknown.

5.6.4 Herpes-Associated Erythema Multiforme (HAEM)

HSV is the most common infectious trigger of erythema multiforme. Oral and skin lesions are caused by a cell-mediated response to HSV. Patients with HAEM have HSV detected in CD34+ cells in blood and in skin biopsies by PCR [183]. Some patients may also have recurrent EM [184].

Disseminated HSV Infections. Rarely, HSV may cause disseminated disease in adults resulting in fulminant hepatitis, sometimes requiring liver transplantation [185], pneumonia [186], and high levels of viremia. Cases have been described in pregnant women [187, 188], immunocompromised hosts [189–191], or healthy, immunocompetent adults [192–195]. Intravenous acyclovir should be used in cases of proven or suspected disseminated HSV.

6 Pathogenesis

The pathophysiology of primary mucocutaneous HSV infection is schematically represented in Fig. 36.3.

In the susceptible host, primary infection begins when HSV is transmitted to mucosal or cutaneous surfaces generally in the oral cavity or genital tract and occasionally other cutaneous sites. HSV entry into cells is a complex process involving attachment and membrane fusion. At least five glycoproteins are involved in the process, gC, gB, gD, and the heterodimer gH/gL [197]. Two viral glycoproteins, gB and gC, are responsible for attachment of the virion to cell surface proteoglycans [198]. Following attachment the virion envelope glycoprotein gD binds to specific cell surface receptors, including the herpesvirus entry mediator (HVEM) on fibroblasts and ocular cells; nectin-1 on neurons, keratinocytes, and epithelial cells; and 3-O-sulfotransferease-modified heparan sulfate. Subsequent interactions with gB and gH/gL trigger fusion events that lead to entry [199]. During the fusion process, the viral envelope is lost, the capsid and associated tegument enter the cytoplasm, and the capsid travels along microtubules via the dynein and kinesin motors using retrograde transport to the cell nucleus [200]. In the neuronal nucleus, the viral DNA either enters a latent state or is replicated to generate progeny virions. Subsequent reactivation of latently infected neurons also results in viral replication and generation of progeny virions. Genome replication, capsid assembly, and genome encapsidation appear to occur in the nucleus with subsequent transport to the cytoplasm for envelopment and posttranslational modification of envelope glycoproteins. The enveloped virions are moved along the microtubules via a kinesin motor-driven anterograde transport process to the nerve endings where they are released into the extracellular milieu. Viral replication occurs in epithelial cells at the portal of entry, but HSV also rapidly enters the nerve endings and moves via axoplasmic transport processes to the nucleus of the neurons located within the sensory ganglia [196, 201, 202]. Animal studies have shown that, following replication in ganglion

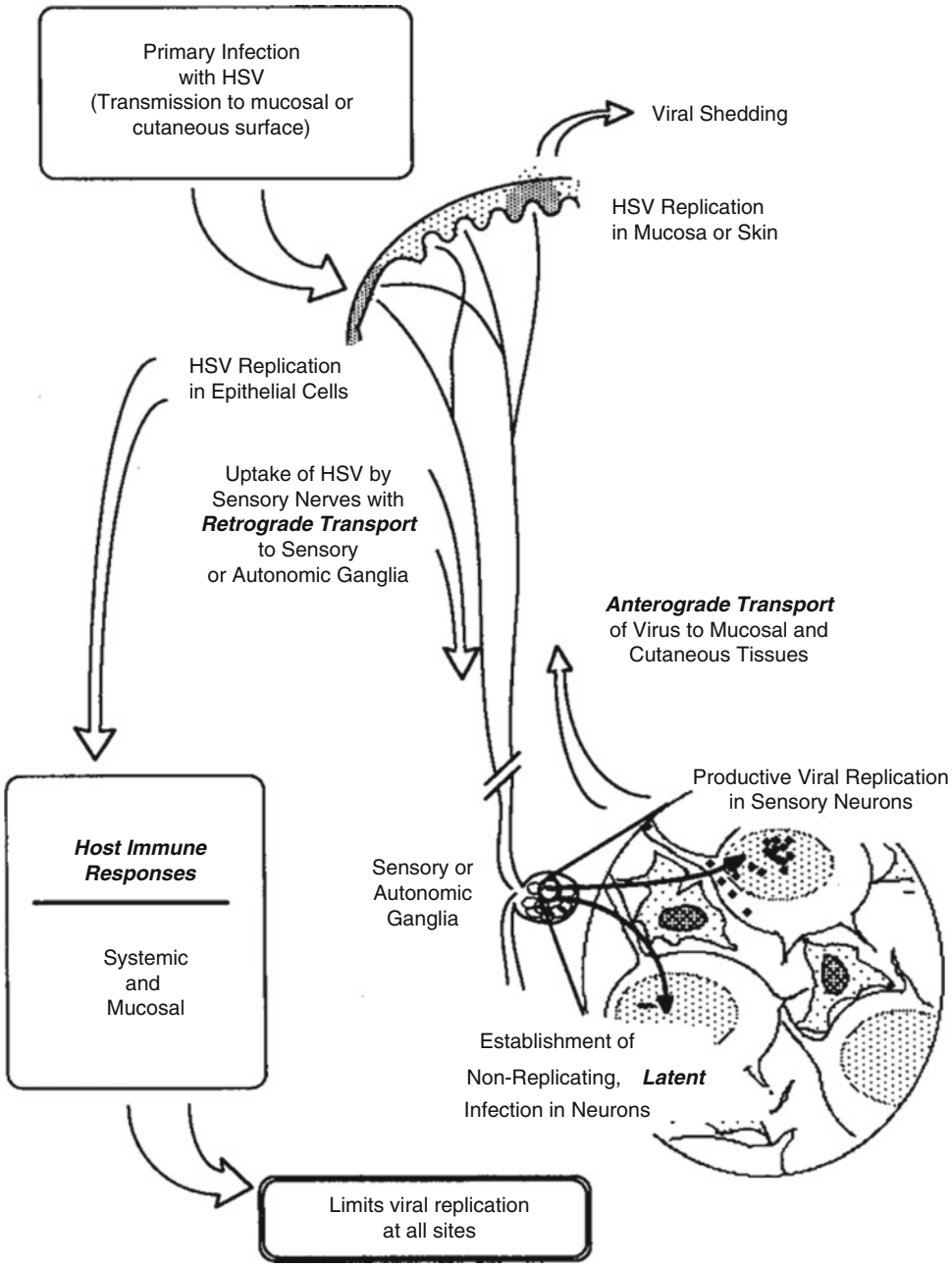

Fig. 36.3 Schematic representation of the pathogenesis of primary mucocutaneous herpes simplex virus infection (Adapted from Stanberry [196])

cells, virus is transported through unmyelinated C-type sensory nerve fibers back to mucosal and cutaneous sites where further replication produces the characteristic vesicles of primary mucocutaneous HSV infection [203, 204]. The intraneuronal spread and amplification of virus at the level of the ganglia appear necessary for the development of clinically apparent mucocutaneous HSV infection [196]. The distinction between the individual who experiences subclinical primary infection and the patient who suffers severe symptomatic disease probably relates to the extent to which virus replicates in sensory ganglia and spreads to multiple mucocutaneous locations. The pathogenesis of recurrent mucocutaneous HSV infection is schematically presented in Fig. 36.4. The current paradigm is that superinfection by a second strain of HSV-2 is uncommon [205, 206], although it has been described [207–209].

HSV has evolved a novel survival strategy; it is able to persist within the sensory neurons in a nonreplicating state throughout the life of the host. The establishment, maintenance, and reactivation of the latent infection involve IFNγ and granzyme B secretion by CD8+ T cells and viral factors including the latency-associated transcript (LAT), viral miRNAs, and immediate early proteins [210, 211]. In humans and some experimental animal models,

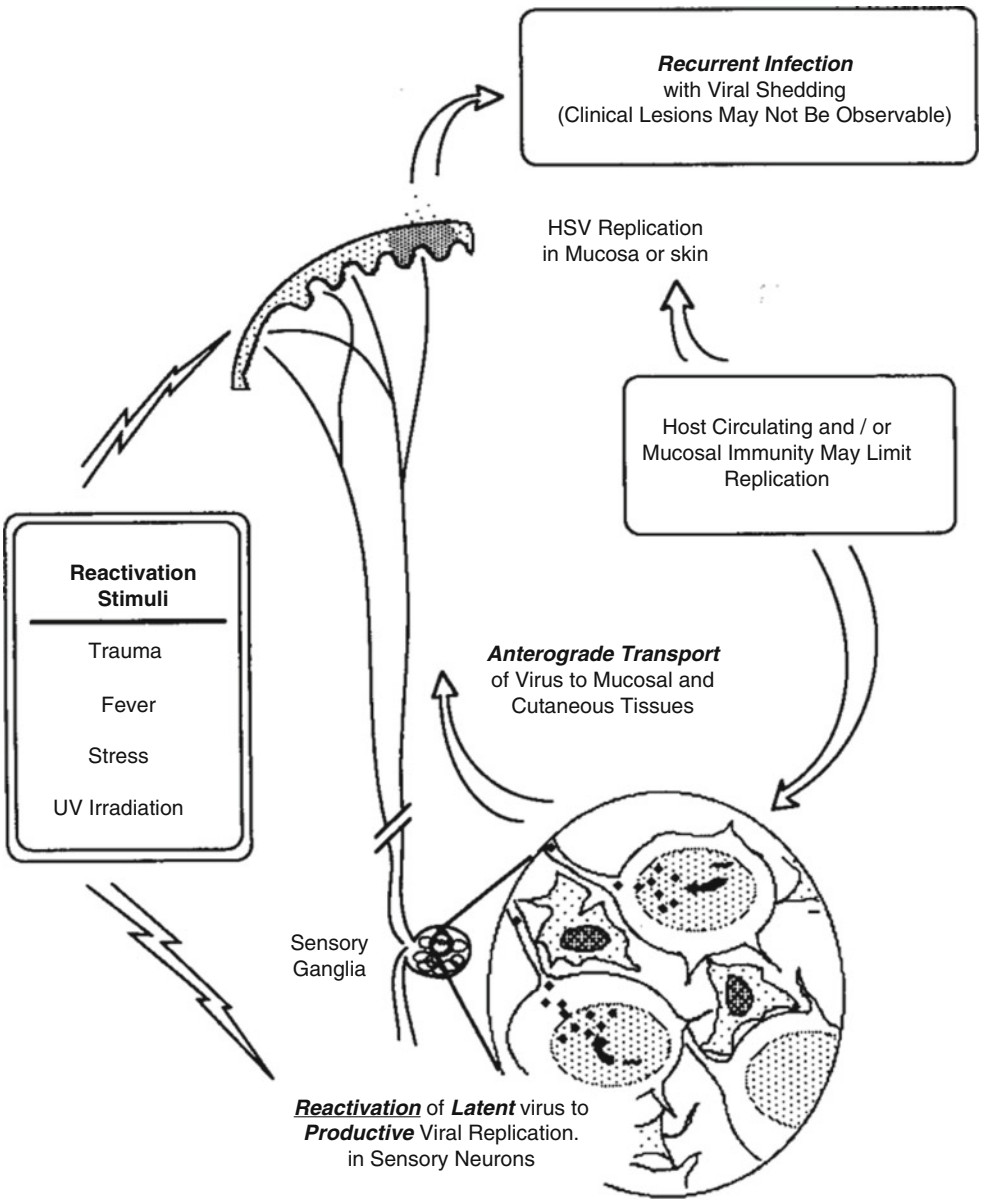

Fig. 36.4 Schematic representation of the pathogenesis of recurrent mucocutaneous herpes simplex virus infection (Adapted from Stanberry [196])

the nonreplicating latent virus can periodically reactivate to a replication-competent state, resulting in the production of infectious virions. Stimuli such as ultraviolet radiation, trauma, or stress may trigger the reaction process. Following reactivation, virus is transported via selected sensory neurons back to the periphery where further replication in epithelial cells results in recurrent mucocutaneous symptoms or subclinical viral shedding. It was previously believed that reactivation events are infrequent or rare and that virus was transmitted only in the presence of oral or genital lesions. However, studies that have used daily sampling of oral and genital secretions for HSV culture or PCR have shown that HSV is often detected on mucosal surfaces and skin ("shed") in the absence of symptoms [56, 212]. Clinical and virologic phenotypes of HSV infection are driven by interactions between anatomic site of infection (trigeminal or sacral ganglia) and the type of HSV [13]. HSV-1 appears to have better tropism for the trigeminal ganglia, leading to increased shedding and reactivation from the oral mucosa, while HSV-2 establishes a more robust infection in the sacral ganglia. Disrupting the HSV-2 LAT [213] or replacing the HSV-2 LAT with HSV-1 LAT in the guinea pig model leads to less frequent genital recurrences, suggesting that tropism may be due to individual type-specific proteins [14, 214].

HSV infections of the central nervous system (encephalitis) and of the eye have special pathogenic mechanisms by which virus causes disease [215, 216]. Brain and mouth isolates taken concurrently in patients with herpes encephalitis have been found to be identical in some patients but dif-

ferent by restriction enzyme analysis in others. These data suggest that persons previously infected with HSV-1 can develop a central nervous system herpetic infection from their old strain or from a newly acquired HSV-1 infection. Additionally, serologic data demonstrating concomitant primary oral and central nervous system infection support the concept that herpes encephalitis outside the newborn period can be a primary infection [217]. In the case of HSV infection of the posterior segment of the eye, it is recognized that, in large part, an immunopathologic process produces the retinal injury [215].

Unlike HSV infections in adults, neonatal herpes is rarely asymptomatic. Animal model studies suggest that the pathogenesis of infection may be different for SEM, CNS, and disseminated disease [218, 219]. Factors that probably influence the extent and severity of neonatal disease include the portal of virus entry, viral inoculum, neurovirulence of the virus strain, immunocompetence of the neonate, and perhaps genetic determinants of susceptibility to HSV infection. For neonatal infection the portals of entry may include the umbilical cord, the eye, the oral and nasal orifices, and the skin if damaged by trauma or scalp electrode placement. If host and viral factors restrict HSV replication to the site of inoculation, the infant may develop infection limited to the skin, eye, or mouth. Encephalitis without evidence of disseminated disease probably results from intraneuronal spread of virus from mucosal sites in the eye, nose, or mouth to sites within the central nervous system. Disseminated infection with multiple organ involvement including the brain is probably due to hematogenous spread, viremia having been documented to occur in some cases of neonatal infection [220, 221]. As with other HSV infections, neonatal herpes results in the establishment of a latent infection that may be reactivated to cause recurrent cutaneous infections and likely affects the long-term developmental outcomes of the infected infants [172, 222].

Host immune responses, particularly cellular responses, are important in limiting HSV infection. Patients with immature or compromised immune function generally have more severe HSV infections. These patient populations include newborns; severely malnourished children; those with measles or HIV infection; those with severe burns; those receiving immunosuppressive therapy; patients with cancers; persons with selected immune deficiencies, such as the Wiskott–Aldrich syndrome; and rarely pregnant women [223–228]. While viremia does not appear to play an important role in the pathogenesis of infection in the normal host, in the immunocompromised patient, including the newborn, virus may disseminate to internal organs via hematogenous spread or extend to contiguous tissues due to swallowing or aspiration of contaminated secretions resulting in esophagitis, tracheitis, or pneumonia [186, 220, 228, 229].

7 Immunity

With primary infection, innate and adaptive immune responses develop in a coordinated fashion to control viral replication and promote healing [202]. Despite these responses, HSV establishes a lifelong latent infection in the sensory ganglia and can subsequently reactivate to cause symptomatic or subclinical recurrent infections [230]. In mucosal tissues innate responses prompt the production of antimicrobial factors such as β-defensins and trigger toll-like receptor (TLR) activation, which promotes inflammatory cytokine production with subsequent natural killer cell activation and maturation of dendritic cells [231]. Germline mutations that impair innate immune responses have demonstrated that innate immunity is critical in the control of HSV infections [232].

In individuals with primary HSV infection, IgG, IgA, and IgM antibodies can be detected within 1–3 weeks by a variety of assays [233–237]. Reactivation of latent infection with subsequent symptomatic and subclinical recurrences can result in anamnestic IgG as well as IgM responses. The role of antibodies in the control of HSV infections is unclear. Some animal studies suggest that B cells and antibodies are not essential for resolution of HSV infection [238, 239], while others suggest they may be important in protecting against infection [240]. A study in newborns suggested that high levels of maternally derived HSV neutralizing antibody could protect the infant from neonatal herpes infection [241]. It is worth noting that patients who lack the ability to produce antibodies are able to control HSV infections, suggesting at the very least that effective control of HSV infections can be mediated through non-antibody mechanisms.

The discussion of assays best suited for epidemiologic studies and the challenges of distinguishing HSV type 1 and HSV type 2 antibodies is presented in Sect. 3.

CD4 and CD8 T cells play critical roles in the control of primary, latent, and recurrent HSV infection [202, 242]. Animals lacking CD4 and/or CD8 cells are diminished in their ability to limit HSV replication in epithelial and neural tissues [243]. Likewise, patients with impaired T-cell immunity can have more severe and prolonged clinical disease, greater likelihood of hematogenous dissemination with visceral organ involvement, and more frequent recurrent infections [220, 244–247].

There is some evidence to suggest that host immunogenetics and differential immune responses can influence infection phenotype. For example, infected individuals with CD4 T cells reactive to glycoprotein B peptides HSV1:$gB_{166–180}$ and HSV1:$gB_{666–680}$ were found only in asymptomatic subjects, while those with CD4 T cells reactive to the epitope HSV1:$gB_{661–675}$ were found only in symptomatic subjects [248]. With regard to recurrence phenotype, lower levels of IgG1 and IgG3 antibodies that mediate antibody-dependent

cellular cytotoxicity were found in subjects who experienced recurrent infections, suggesting these antibodies play a role in controlling recurrences [249]. Most HSV-1-infected patients do not develop central nervous system infection; however, herpes encephalitis does develop in individuals with toll-like receptor 3 deficiency [250].

8 Transmission

HSV is transmitted through intimate contact with infected oral or genital secretions, during exchange of saliva, sexual contact, or during labor and delivery (neonatal

HSV) [251]. HSV is most frequently transmitted during periods in which virus is detected on mucosal surfaces in the absence of signs or symptoms of HSV (subclinical viral shedding).

8.1 HSV Genital Shedding

It is well established that both HSV-1 and HSV-2 can be shed from genital and perigenital sites.

8.1.1 HSV-2 Genital Shedding

Studies of daily self-collected genital swabs for HSV culture and PCR in association with recording of daily symptoms and lesions have allowed for characterization of the frequency, patterns, and symptoms associated with genital HSV shedding [56, 212, 252, 253]. HSV-2 genital shedding rates are highest during the first year after first-episode genital herpes [56, 212, 254], with HSV detected by HSV PCR on 34 % of days [254]. HSV shedding rates decline to ~20 % of days 1 year after first-episode genital herpes (Fig. 36.5).

Shedding is persistent over time, with shedding rates of 17 % in persons ≥10 years post first-episode infection [254] (Table 36.5). Persons with asymptomatic genital HSV-2 shed less frequently than those with symptomatic disease, with virus detected on 10 % of days (Fig. 36.5) [253]. Men and women shed HSV from the genital tract at similar rates [252]. Genital HSV-2 shedding is associated with symptomatic genital infection, number of recurrences per year, and white race [253]. In a cohort of 27 women with recurrent HSV-2 infection, 100 % shed when followed over a 100-day period, suggesting that most people shed virus [255].

Episodes of shedding are clustered together and last a median of 3 days, with longer shedding episodes in the presence of a lesion, lasting a mean of 6.8 days (Fig. 36.6) [56, 256].

However, shedding occurs most frequently in the absence of genital symptoms or lesions ("subclinical shedding") [56, 253]. Subclinical shedding occurs more frequently in persons with a history of symptomatic as compared to asymptomatic infection (13.1 % of days vs. 8.8 % of days) [253]. Importantly, HSV can be transmitted during periods of subclinical shedding [257]. HIV-infected persons shed more frequently that HIV-negative men [258] and women [244], with increased risk of shedding in those with lower CD4 counts.

Intensive genital and oral shedding studies have provided insight into the pathogenesis of HSV infection. Collection of oral and genital swabs every 6 h has shown that most HSV reactivations are of very short duration, with 60 % of shedding episodes cleared within 12 h [259, 260]. Shedding

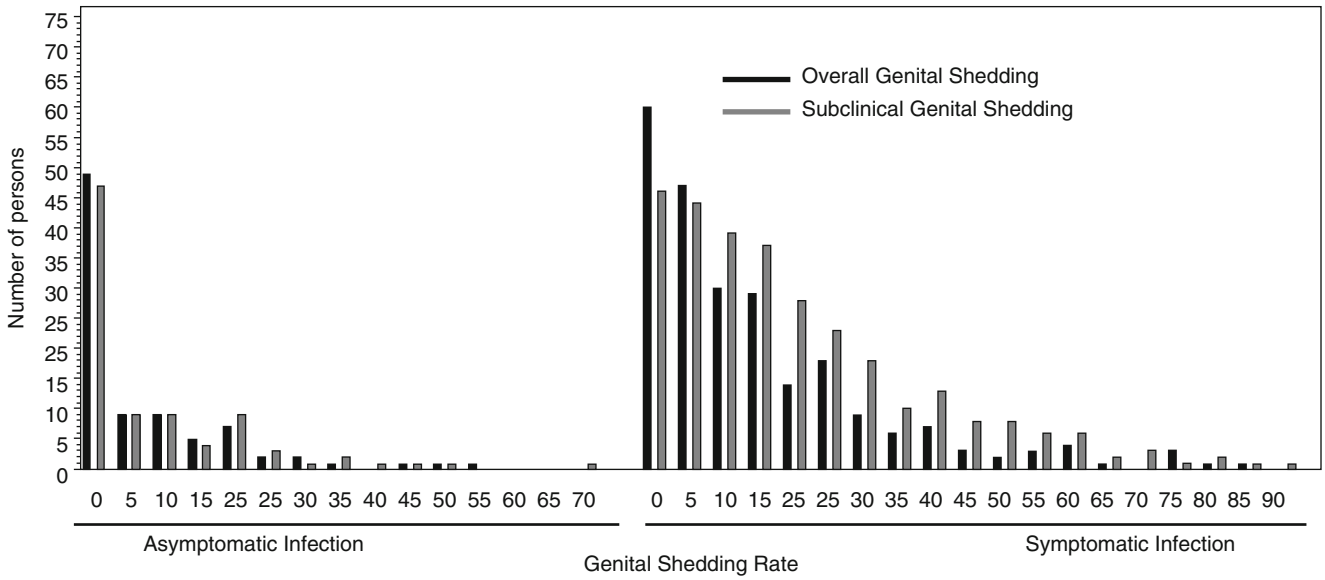

Fig. 36.5 Distribution of genital HSV-2 shedding rates among 88 persons with asymptomatic infection and 410 person with symptomatic infection (Adapted from Tronstein et al. [253])

Table 36.5 Overall shedding rates measured by PCR, by site and HSV type, and by time after primary infection, where available

	HSV-1 (%)	HSV-2 (%)
Oral	33.3	1.3
Genital, overall	N/A	20
Genital, <1 year after 1st episode	2[a]	33.6
Genital, 1–9 years after 1st episode	<1[a]	20.6
Genital, >10 years after 1st episode	N/A	16.7
Genital, asymptomatic	N/A	10

[a]Data available using culture only

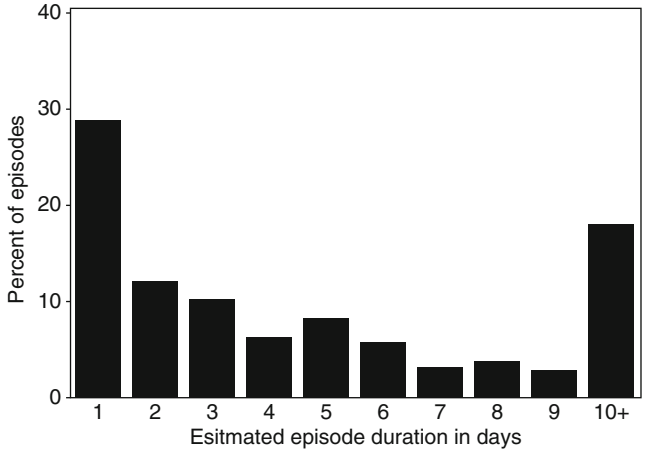

Fig. 36.6 Duration of 1695 genital HSV shedding episodes identified by PCR (Adapted from Schiffer et al. [256], p. 557)

does not follow a circadian pattern, with episodes occurring at equal rates throughout a 24-h period. These studies have also revealed that HSV reactivations are more frequent than previously appreciated and suggest rapid clearance of HSV, perhaps via a resident immune response in the oral and genital mucosa [260]. Mathematical models predict that HSV is produced at a rate of 50 particles/day from the ganglia to produce observed shedding patterns [261] and that increased quantity of CD8+ T cells limits the length and symptoms of reactivations [262]. In addition, genital HSV shedding may involve the bilateral genital tract, particularly in the setting of a genital lesion [263, 264].

8.1.2 HSV-1 Genital Shedding

There are few data characterizing genital HSV-1 shedding rates after first-episode genital HSV-1 infection, and these studies have used HSV culture [56]. For 14 women with symptomatic genital HSV-1 infection, genital HSV was detected on 1.9 % of days; subclinical shedding was detected <1 % of days sampled. Further studies with more participants with both symptomatic and asymptomatic genital HSV-1 infection and using PCR will be necessary for a more definitive description of genital HSV-1 shedding patterns.

8.2 HSV Oral and Ocular Shedding

8.2.1 HSV-1 Oral and Ocular Shedding

HSV-1 oral and ocular shedding was studied in 50 asymptomatic persons over a 30-day period. HSV was detected from 37.5 % of oral swabs and 33.5 % of ocular samples [265]. Only one participant did not shed HSV-1 from the eye or saliva during the follow-up period. In a review of published HSV-1 oral shedding studies, 6 % of people were shedding HSV when sampled with a single specimen [266]. HSV-1 was detected in 31.9 % of participants sampled more than 5 times per week; the overall rate of shedding was 33.3 % of samples in persons who collected at least 3 specimens.

8.2.2 HSV-2 Oral Shedding

In a study of HIV-negative men, the oral HSV-2 genital shedding rate measured by PCR was 1.3 % of days [267]. Oral shedding was always asymptomatic and was often concurrent with genital HSV-2 shedding or lesions [267]. The rate of oral HSV-2 shedding was higher in HIV-infected men than in HIV-negative men.

8.3 Genital HSV Transmission in Sexual Relationships

Prospective studies of HSV-2-discordant couples have demonstrated that 70 % of HSV-2 transmissions occur in the absence of symptoms in the source partner [257]. A study of 199 persons with newly acquired, laboratory-documented genital herpes showed that the median duration of the sexual relationship was 3.5 months, with a median of 40 sexual acts prior to transmission [268], suggesting rapid transmission in new sexual partnerships.

9 Treatment and Prevention

9.1 Treatment

Three oral antiviral agents are available to treat HSV infections. Acyclovir, valacyclovir (prodrug of acyclovir), and famciclovir (prodrug of penciclovir) are guanosine nucleoside analogues that require initial phosphorylation by the HSV-encoded thymidine kinase, followed by phosphorylation by cellular kinases [269]. The nucleotide triphosphates are preferentially incorporated by viral DNA polymerase into the growing DNA chain, resulting in chain termination [269]. These drugs are inactive in uninfected cells and have an excellent safety profile, including well-documented safety in pregnant women [270, 271]. These oral antiviral drugs are used to treat genital and labial herpes either at the time of a recurrence (episodic therapy) or daily to prevent recurrences

(suppressive therapy). Episodic therapy is recommended for first-episode and recurrent genital herpes and should be started at the first sign of a clinical recurrence [57]. The use of oral antiviral drugs during first-episode or recurrent genital herpes decreases the duration of lesions, symptoms, and viral shedding [272–274]. High-dose, single-day oral regimens of valacyclovir and famciclovir can be used episodically for oral or genital HSV recurrences [275]. Suppressive therapy prevents recurrences of genital HSV-2 [276–279], decreases the frequency and titer of HSV shedding from anogenital sites [110, 212, 280], and improves quality of life [281]. In HSV-2-discordant couples, daily suppressive therapy for the infected partner also reduces the risk of genital HSV-2 transmission [110]. However, breakthrough shedding and lesions can occur on suppressive therapy, even when high doses are used [282]. Suppressive therapy can also be used to prevent oral HSV recurrences [283]. Dosages and schedules for the oral antivirals were reviewed by Cernik and colleagues [284], and recommendations for treatment of genital herpes are available from the US Centers for Disease Control and Prevention (CDC) [57].

Intravenous acyclovir achieves high levels of CNS penetration and is the treatment of choice for CNS disease [132], neonatal HSV [171], disseminated HSV infection, and severe primary genital infection [285]. Ocular drops of acyclovir, vidarabine, trifluridine, or ganciclovir may be used for HSV keratitis [286]. Foscarnet is available for acyclovir-resistant herpes [287]. Helicase-primase drugs [288] and microbicides [289] are in early development, as are other novel antiviral delivery methods, such as vaginal acyclovir rings [290].

9.2 Prevention

9.2.1 Vaccine Strategies

The optimal intervention for prevention of HSV infection and disease would be a preventive or therapeutic HSV vaccine, a tool that thus far has eluded scientists [242]. Most HSV vaccines tested in humans have been based on glycoprotein targets on the surface of the HSV virion. Several vaccines have been efficacious in mouse and guinea pig models but have failed to prevent infection or disease in human trials [242]. One promising exception was an HSV-2 glycoprotein D (gD-2) subunit vaccine with alum and 3-O-deacylated monophosphoryl lipid A adjuvant tested in persons with high-risk sexual behavior and in HSV-2-discordant couples [291]. Overall, the vaccine was not effective, but in a subgroup analysis of HSV-1-/HSV-2-seronegative women, the vaccine was ~75 % effective [291]. A more recent, larger trial involving ~8,300 HSV-1-/HSV-2-seronegative women disappointingly showed that this vaccine did not protect against HSV acquisition, despite stimulation of robust neutralizing antibody [292]. We do not know immune correlates of protection against HSV, and we will likely need to understand more about the effective immune response against HSV before we will be successful in creating an effective vaccine [242]. Recent studies from the mouse model have suggested that systemic vaccination to stimulate memory T-cell responses ("prime") followed by local recruitment of T cells through application of topical genital agents ("pull") approach to stimulate resident memory T-cell responses in the genital tract may be an effective immunization strategy [293]. Live-attenuated [294], replication-competent [295], and replication-incompetent vaccine constructs have also been effective in animal models [296, 297] but will require further testing in humans. Future HSV vaccine development will require consideration of whether a vaccine needs to protect against both HSV-1 and HSV-2.

9.2.2 Available Prevention Strategies

There are four available strategies for the prevention of genital HSV transmission; however, no strategy is 100 % effective. In heterosexual HSV-2-discordant couples, daily suppressive antiviral therapy for the infected partner decreases HSV-2 transmission by nearly 50 % [110]. Consistent condom use decreases the risk of transmission by ~30 %, with similar efficacy for men and women [298]; condoms are likely less effective for HSV-2 than other sexually transmitted infections because areas of viral shedding may not be covered by the condom. In a time to event study, disclosure of HSV serostatus to sexual partners decreased time to HSV-2 acquisition in susceptible partners [268]. Randomized trials of male circumcision for prevention of HIV acquisition have demonstrated that circumcised men have a 25 % lower risk of acquiring HSV-2 and a lower risk of GUD than uncircumcised men [299, 300]. However, circumcision status does not affect the risk of HSV-2 transmission to female partner [301].

10 Unresolved Epidemiologic Issues

Whether and who to screen for HSV-2 infection has been a contentious issue. While some experts advocate screening individuals in STD clinics, persons with high-risk sexual practices, or women in the prenatal setting to identify those at risk of transmitting neonatal HSV [302], screening is currently not recommended by the US Preventive Services Task Force [303], due to concerns about false-positive tests in populations with low prevalence, cost-effectiveness, lack of proven interventions to improve health outcomes, and potential harm in learning that one is HSV-2 seropositive. Despite concerns about psychological effects of screening for herpes, studies have shown that there is no longstanding harm to HSV-2 screening and diagnosis in asymptomatic persons [304].

Neonatal HSV is reportable in only 9 US states, and even in these states, there is no clear case definition for reporting disease [160]. The resulting inability to determine the true incidence and morbidity/mortality of disease has led to a call for the CDC to institute national reporting, but this has not been implemented, to date [154].

Despite improved understanding of viral pathogenesis and host immune responses and the availability of effective antiviral agents for HSV, the patterns of occurrence of HSV infection have changed little in recent years, and the epidemiology of the highly evolved, ubiquitous herpes simplex virus is likely to remain substantially unaltered, without an effective vaccine.

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Human Herpesviruses: Human Herpesvirus 6

37

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1 Introduction

Over the last quarter of a century since its discovery, human herpesvirus 6 (HHV-6) has been recognized as the most rapidly and ubiquitously acquired virus known to infect humans. During this period HHV-6 has been primarily known as the "roseola virus," but recent advances in technology have markedly expanded the spectrum of its mein to include diseases associated with both primary infection and reactivation. In addition, new data on HHV-6 transmission, its integration into human chromosomes, its latency, and congenital infections have added to our understanding and to the conundrums of its epidemiologic and biologic behavior.

2 Historical Background

The techniques developed for the in vitro cultivation of human peripheral blood mononuclear cells (PBMCs) led not only to the discovery of human retroviruses but also to the identification of HHV-6. The new virus was detected within the peripheral blood lymphocytes of adults with lymphoproliferative diseases and/or AIDS in 1986 [91]. Based upon preliminary cell typing experiments, the virus was initially called human B-lymphotropic virus (HBLV). Further characterization of the virus and its proclivity to infect CD4+ T lymphocytes resulted in its being renamed the sixth member of the human herpesvirus family. Two years later when this same virus was detected by Yamanishi and colleagues [116] in the lymphocytes of four infants with roseola, a whole new spectrum of its potential disease associations and clinical importance were evoked, and these continue to be explored.

For over 200 years roseola-like illnesses have been described among young children under multiple sobriquets, including exanthem subitum, rash rose of infants, and most prophetically, sixth disease. The specific disease now recognized as roseola was first separated from other exanthematous illnesses by Zahorsky with his description of 15 cases in 1910 [127]. The first prospective study by Breese [17] 30 years later provided the descriptive epidemiology of roseola, which was confirmed as a manifestation of infection with HHV-6 a half century later.

3 Methods for Epidemiologic Analysis

Initially serologic surveys were the primary means of describing the epidemiology of HHV-6 infection, particularly its acquisition and duration. These studies demonstrated the ubiquity of infection with HHV-6 by detecting high rates of antibody in all populations examined [26, 42, 115]. New molecular techniques for detection and determining viral state have further defined its epidemiologic patterns according to age, presence in body sites, replicative state, and new or reactivated infection [20, 33, 67, 111] (see section Diagnosis, Table 37.1).

4 Biologic Characteristics Affecting the Epidemiologic Pattern

The viral structure of HHV-6 is typical of herpesviruses and includes a 160–162 kb linear double-stranded DNA genome contained within a nucleocapsid with icosahedral symmetry [16]. A tegument composed of proteins and RNA surrounds the nucleocapsid which is in turn enclosed in a lipid envelope embedded with viral glycoproteins, together forming the

†Deceased

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Table 37.1 Diagnosis of HHV-6 infections

		Type of infection diagnosed				
Assay[a]	Specimen	Acute primary	Recent active	Past/latent	Reactivated	Comments
Serology[b]						
Antibody titers	Serum/plasma					
IgG titers		+	+	+	±	Sensitive. Seroconversion indicative of primary infection
						HHV-6A and HHV-6B not differentiated
						Limited HHV-6 and HHV-7 cross-reactivity depending on assay used
						Results not available during acute illness as requires testing of 2 or more serial specimens
						Single specimen, if antibody negative, DNA positive, suggests early, primary infection
						Maternal antibody may be present <6 months of age and confuse results
IgM titers		+	+			Not detected in all primary infections
						May be detected in small percent of normal individuals and in some reactivated infections
Avidity titers		+	+	+		Single specimen testing indicates acute infection within 6 weeks (low avidity) or >6 weeks (high avidity)
DNA/RNA detection						
DNA PCR	Mononuclear cells in blood and other specimens	+	+	+	+	DNA detection may indicate acute and/or past infection, requires serology and/or RT-PCR for clarification
						Differentiates HHV-6A and HHV-6B
	Serum/plasma or whole blood	+			+	Lysis of peripheral blood mononuclear cells can produce appreciable proportion of false-positive results[a] [2, 20]
Real-time DNA PCR	Serum/plasma or whole blood	+			+	Quantitative, very sensitive, costly
						Lysis of peripheral blood mononuclear cells can produce appreciable proportion of false-positive results[a] [2, 20]
RNA PCR						
RT-PCR	Mononuclear cells	+			+	Very sensitive, semiquantitative
						Differentiates HHV-6A and HHV-6B, costly
Real-time RT-PCR	Mononuclear cells	+			+	Very sensitive, quantitative, costly
Viral growth	Mononuclear cells of blood and other specimens	+			+	Available only in research laboratories
						HHV-6A and HHV-6B can be differentiated
						Results usually not available during acute illness
Viral antigen detection	Blood, secretions	+			+	Suggests active replication, semiquantitative
						Differentiates HHV-6A and HHV-6B
						Sensitivity and specificity variable

Used with permission from Hall [43]

[a]CI-HHV-6 may confound interpretation of diagnostic tests; see text on Congenital Infections

[b]Multiple serologic tests are available including immunofluorescent assays (IFA), neutralizing antibody assays, enzyme immunoassays (EIA), immunoblots, anticomplement assays [33, 111, 122]

fully mature ~200 nm virion particle [26]. Additionally, the overall genome organization of HHV-6 is similar to other herpesviruses with a large, central unique region (U) of approximately142–145 kb size bounded on both ends by identical direct repeat (DR) elements of 8–13 kb [30, 39, 55]. The unique region of the genome also contains three

intermediate repeat elements [26]. Based upon an identified tropism for lymphocytes, HHV-6 was initially thought to be a γ herpesvirus similar to Epstein-Barr virus (EBV). However, studies of the genomic structure combined with sequencing data suggested that the unique region of HHV-6 was similar to human cytomegalovirus (CMV) and is colinear with human herpesvirus 7 (HHV-7). This led to HHV-6 being classified along with CMV and HHV-7 into the *Betaherpesvirinae* subfamily [26, 79]. Further reports have confirmed that HHV-6A and HHV-6B are is most closely related to HHV-7, and these viruses share the genus *Roseolovirus* [16].

The genome of HHV-6 is predicted to contain 115–119 open reading frames (ORFs) coding for approximately 97 unique genes [30, 39, 55], including seven conserved gene blocks shared by all human herpesviruses and found in a similar alignment in HHV-6, HHV-7, and CMV [16, 26]. HHV-6 also encodes a set of beta genes found exclusively in HHV-6, HHV-7, and CMV, as well as several unique genes not found in other herpesviruses [26].

Two species of HHV-6 have been identified, HHV-6A and HHV-6B, based upon differences in genome coding content, epidemiology, and cell tropism [38]. Prototypic isolates of both HHV-6A and HHV-6B have been completely sequenced with an overall nucleotide sequence identity between them of 90 %. The most conserved regions of the HHV-6 genome are found in open reading frames U2 through U85 [30, 55]. Recent studies have confirmed stable genetic differences between HHV-6A and B, adding credence to the move to separate them into two distinct species [33]. Further diversity in HHV-6 isolates was initially suggested by finding that HHV-6B could be subdivided into two subgroups with 96 % sequence identity between the groups based upon a region of an immediate early gene [23]. However, subsequent analyses of the regions encoding two major HHV-6 glycoproteins, gB and gH, have demonstrated that single isolates may contain regions characteristic of both subgroups, and thus, this distinction is not uniform [3].

Recent reports have highlighted the importance of the direct repeat (DR) segments of the HHV-6 genome in the virus life cycle. Cleavage and packaging motifs as well as imperfect and perfect human telomere repeat sequences (TAACCC) are present in the DR regions [75]. Preliminary studies have suggested that the perfect telomere repeat segments (TRS) of the virus mediate integration of the entire HHV-6 genome into the telomeres of human chromosomes in a subset of the population [8]. Multiple chromosomes have been shown to harbor integrated HHV-6, usually one site per person, and always at the telomere, suggesting that the telomere provides the specificity for integration [76]. Although the exact mechanism has not been fully elucidated, integration is postulated to occur via homologous recombination and may be facilitated by the U94 protein of HHV-6 [7]. U94 is unique to HHV-6 but shares homology with the adeno-associated virus 2 (AAV-2) Rep 68/78 gene [107, 108] and has been demonstrated to play a role in HHV-6 latency [90]. Viral integration into the host telomere has also been described in animal herpesviruses, specifically Marek's disease virus (MDV), a pathogen of chickens that is associated with the development of T-cell lymphomas [33]. Whether chromosomally integrated HHV-6 (ciHHV-6) causes any human diseases is currently unknown. Furthermore, yet to be determined is whether ciHHV-6 virus can replicate in vivo and produce infectious viral particles, as has been demonstrated in vitro and in MDV [8].

Infection with HHV-6 is followed by latency or persistence of the virus for the life of the host. Primary lytic infection with HHV-6 principally occurs in mature CD4+ T cells in vivo and is associated with characteristic cytopathic effects and cell lysis in vitro [101]. Monocytes and macrophages have been demonstrated to be the predominant cell types harboring latent HHV-6 in the peripheral blood [60] with more recent data suggesting that CD34+ stem cells may also be a site of viral latency and a reservoir for reactivation [6, 57]. Although all isolates of HHV-6 can be propagated in vitro in primary cord blood mononuclear cells, HHV-6A and HHV-6B can be distinguished in part by their differential growth in established cell lines [27, 28]. Santoro and colleagues determined that the human complement regulatory protein CD46 is a receptor for HHV-6 and co-immunoprecipitates with glycoprotein H (gH) of the virus [92, 93]. During infections with HHV-6A, the complex of gH/gL/gQ1/gQ2 glycoproteins was found to be the viral ligand for CD46, but not for HHV-6B with CD134 recently identified as a specific receptor for HHV-6B, perhaps helping to explain the differences in tropism between HHV-6A and HHV-6B [73, 104]. The presence of CD46 on all nucleated cells of the body may also help to explain the finding that HHV-6 can infect many cell types including fibroblasts, epithelial cells, endothelial cells, astrocytes, oligodendrocytes, microglia, dendritic cells, and natural killer cells [5, 19, 22, 27, 31, 66, 68, 95]. These in vitro findings correlate with the ability to detect HHV-6 DNA in saliva, cervical swab samples, peripheral blood mononuclear cells, and brain tissue in clinical specimens and suggest these sites harbor HHV-6 in a latent or persistent state following primary infection.

5 Descriptive Epidemiology

Serosurveys have demonstrated that infection with HHV-6 is ubiquitous and occurs quite rapidly after the fall in maternal antibody at 4–7 months of age [126]. Essentially all children are seropositive by 2–3 years of age with the peak time of acquisition of primary HHV-6 infection at a median 8 months of age (Fig. 37.1) [48]. In general, no seasonal pattern of

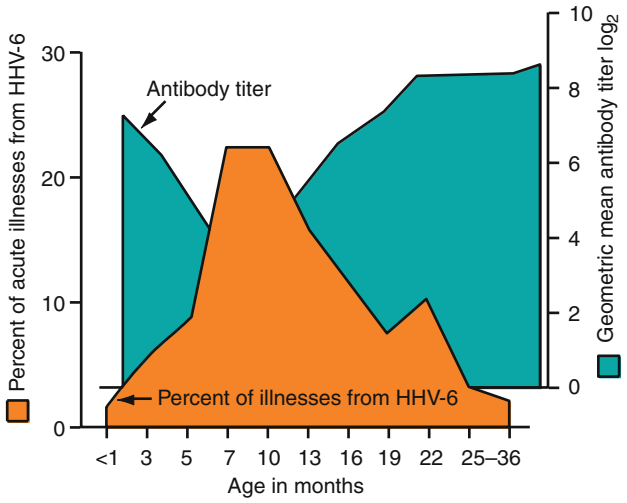

Fig. 37.1 Changes in HHV-6 antibody titers and percent of illness due to primary infection by age Used with permission from Hall [43]

roseola has been identified, and most cases are sporadic with rare outbreaks reported [83].

HHV-6B is responsible for 97–100 % of primary infections in North America, Europe, and Japan [29]. However, HHV-6A has been reported recently to cause the majority of primary infections in one region of sub-Saharan Africa [12, 13]. The significant cross-reactivity between HHV-6A and HHV-6B in standard serologic assays has confounded broader study of the epidemiology of the two species. HHV-6 A and HHV-6B specific immunoblot assays have been developed, but determining their validity has been hampered by the lack of available sera from individuals with only HHV-6A infection [50, 105]. The frequency of infection with HHV-6A is unclear, but currently HHV-6A has not been definitively associated with recognized clinical findings, and HHV-6A DNA is not readily detectable in PBMC or saliva in adults from North America or Europe [36, 45]. These data are in contrast to the relative frequency of HHV-6A and HHV-6B noted in studies of ciHHV-6 in the United States in which HHV-6A is identified in approximately 33 % of cases [47]. The variations in the detection of HHV-6A and HHV-6 B DNA may relate to differences in tissue tropism, mode or age of acquisition, or the geographic location of the population. The complete understanding of HHV-6A awaits further study.

6 Mechanisms and Routes of Transmission

Information on the transmission of HHV-6 has been limited by the typical absence of contact with similar clinical cases by the affected individual and, second, by the lack of a feasible animal model. In 1941 Breese [17] unsuccessfully attempted to recover a filterable virus from the secretions of infants during the preeruptive febrile phase using multiple means of inoculation to infect a variety of animal models and tissue cultures. No other laboratory investigations of transmission occurred until Kempe and coworkers in 1950 [59] demonstrated that the sera and secretions obtained during the febrile phase of a child with roseola contained an infectious agent that could transmit the clinical disease after an incubation period of 10 days in humans and when transmitted to monkeys produced a characteristic febrile illness after about 5 days. Further investigations to determine the definitive incubation period of HHV-6 have not been done.

6.1 Horizontal Postnatal Transmission of HHV-6B

HHV-6B is assumed to be acquired from the secretions of asymptomatic close contacts based on the observations of the rapid acquisition of infection in the first months of life and the lack of exposure to clinically similar cases [48]. Although the data are limited [24, 109], children who acquired HHV-6 prior to their first birthday have been reported as being more likely to practice saliva-sharing behaviors [89]. HHV-6 DNA may be detected in saliva, but in contrast to HHV-7, infectious virus rarely is identified [15, 16]. HHV-6 DNA has also recently been identified in 60 % of nasal mucous specimens from healthy volunteers suggesting that respiratory droplet transmission may also be possible [49].

6.2 Perinatal HHV-6 Infections

Primary infection has been documented in the perinatal period which suggests that horizontal transmission may also occur at or shortly after birth [18, 48]. HHV-6 DNA has been detected in 3.3–10 % of cervical swab samples in nonpregnant women [21, 63] and in 7.5–25.5 % of cervical swab specimens of pregnant women [21, 71, 84]. HHV-6 DNA has not been detected in breast milk [32].

6.3 Congenital HHV-6 Infections

Congenitally acquired HHV-6 infections have been shown to occur by the detection of HHV-6 DNA in cord blood [24, 44]. Although the prior assumption was that all HHV-6 congenital infections were due to transplacental infection from maternal viral reactivations or reinfections, similar to that of CMV, HHV-6 intrauterine infections have been shown to result from either transplacental passage or from the unique mechanism of germline passage of the chromosomally integrated genome of HHV-6 [21, 25, 47]. However, the great majority (86 %) of

congenital infections appear to be caused by chromosomal integration [47]. In all cases of ciHHV-6, the viral genome is integrated into the telomeres of human chromosomes, as described above [7]. Both HHV-6A and HHV-6B have been shown to be integrated, and ciHHV-6 has been estimated to be present in 0.2–1 % of populations from Japan, the United Kingdom, and the United States [65, 85, 103].

A recent report suggests that mothers with ciHHV-6 also may pass their integrated virus via a transplacental mechanism [46]. In this study, children with congenital HHV-6 infection not due to chromosomal integration and their parents were examined. None of the fathers had ciHHV-6, but all of the mothers had ciHHV-6, and the mother's species type was the same as that identified from her infant. These findings suggest that ciHHV-6 may replicate and raise the possibility that nearly all transplacental congenital infections could be from replicating maternal ciHHV-6.

6.4 Diagnosis of ciHHV-6 Passed by the Germline

Because ciHHV-6 is in germline cells, each nucleated cell will contain one or more ciHHV-6 genomic copies. Thus, the diagnosis of ciHHV-6 is presumed if HHV-6 DNA is (1) consistently detected in all nucleated cells and (2) in cells of different lineages, such as white blood cells and hair follicles and (3) viral loads are persistently high (≥1 genomic copy/cell or about one million HHV-6 DNA copies per ml blood) [113].

7 Pathogenesis and Immunity

Primary infection with HHV-6 induces a coordinated immune response from the host. In the first 2 weeks of infection, enhanced natural killer (NK) cell activity is detected in children with roseola along with elevated serum levels of interferon (IFN)-α, IFN-γ, interleukin (IL)-2, IL-4, and monocyte chemotactic protein (MCP)-1 [61, 100, 123]. Concurrently, IgM and IgG antibody responses to primary HHV-6 infection are produced with viral-specific neutralizing antibodies identified within 1 week which are temporally associated with clearance of the virus [11, 98, 119]. The IgM response is nonspecific and lasts approximately 1–2 months, while specific IgG antibodies are maintained with the levels declining in later adulthood [120, 126]. HHV-6 infection also induces T-cell proliferation with viral-specific CD4+ T-cell epitopes mapped to regions of abundant virion proteins [78]. Evidence for the importance of both antibody- and cell-mediated responses to HHV-6 is found in the generally apparent protective effect of maternal antibody in the newborn period and the recognition that disease associated with HHV-6 reactivation occurs most commonly in immunocompromised hosts with T-cell deficits, secondary to either medications or disease.

In addition to the host response to infection, HHV-6 interacts with the immune system in important ways suggesting that the virus has acquired several strategies for immune evasion. Following infection, HHV-6 induces downregulation of the T-cell receptor (TCR)/CD3 complex in PBMCs via inhibition of receptor recycling [99]. MHC class I molecules are also diverted from the cell surface during HHV-6 infection [37, 52], while CD4 transcription is upregulated [34, 69]. HHV-6 also can infect antigen-presenting dendritic cells, but the functional impact of infection is unclear [14, 96, 102]. In addition to the typical cell lysis which follows infection [40, 54, 118], HHV-6 infection induces apoptosis of T cells, primarily naïve and central memory CD4+ and CD8+ cells. The HHV-6 genome encodes two homologs of β-chemokine receptors [56, 72] as well as a protein, identified as a CCR-2 chemokine agonist [70, 132], that can induce monocytic cell migration. These myriad interactions with the immune system are postulated to enhance viral replication in infected cells, recruit susceptible cells to the site of infection, or circumvent the host immune response to infection.

Despite the rapidly increasing knowledge of the basic biology of HHV-6, how the infection induces disease remains unclear. Early reports found that the duration of symptoms in primary infection was associated with the degree and persistence of viremia suggesting that the virus has direct effects on the host [9]. HHV-6 DNA also has been detected in most, but not all, of cerebrospinal fluid (CSF) samples of patients with encephalitis, implying either direct viral pathogenesis or possibly an immune-mediated process [4, 53, 124]. However, proving HHV-6 to be the direct cause of disease based solely upon the detection of HHV-6 DNA is confounded by its known capacity for latency or persistence in the central nervous system (CNS) and by the knowledge that viral DNA can be detected in asymptomatic individuals with ciHHV-6 [77]. Further studies and diagnostic methods are needed to clarify the pathogenesis of HHV-6.

8 Patterns of Host Response

8.1 Clinical Features of HHV-6 Infection

8.1.1 Primary Infection

Manifestations of primary HHV-6 infection are variable and may show geographic differences. In Japan, initial disease is primarily roseola (exanthem subitum), whereas in the United States the clinical findings are nonspecific or have findings suggesting other illnesses [10, 41].

Roseola typically presents with the abrupt onset of fever which usually reaches 39–41°C and lasts 3–5 days, and with defervescence the rash appears. It is usually maculopapular

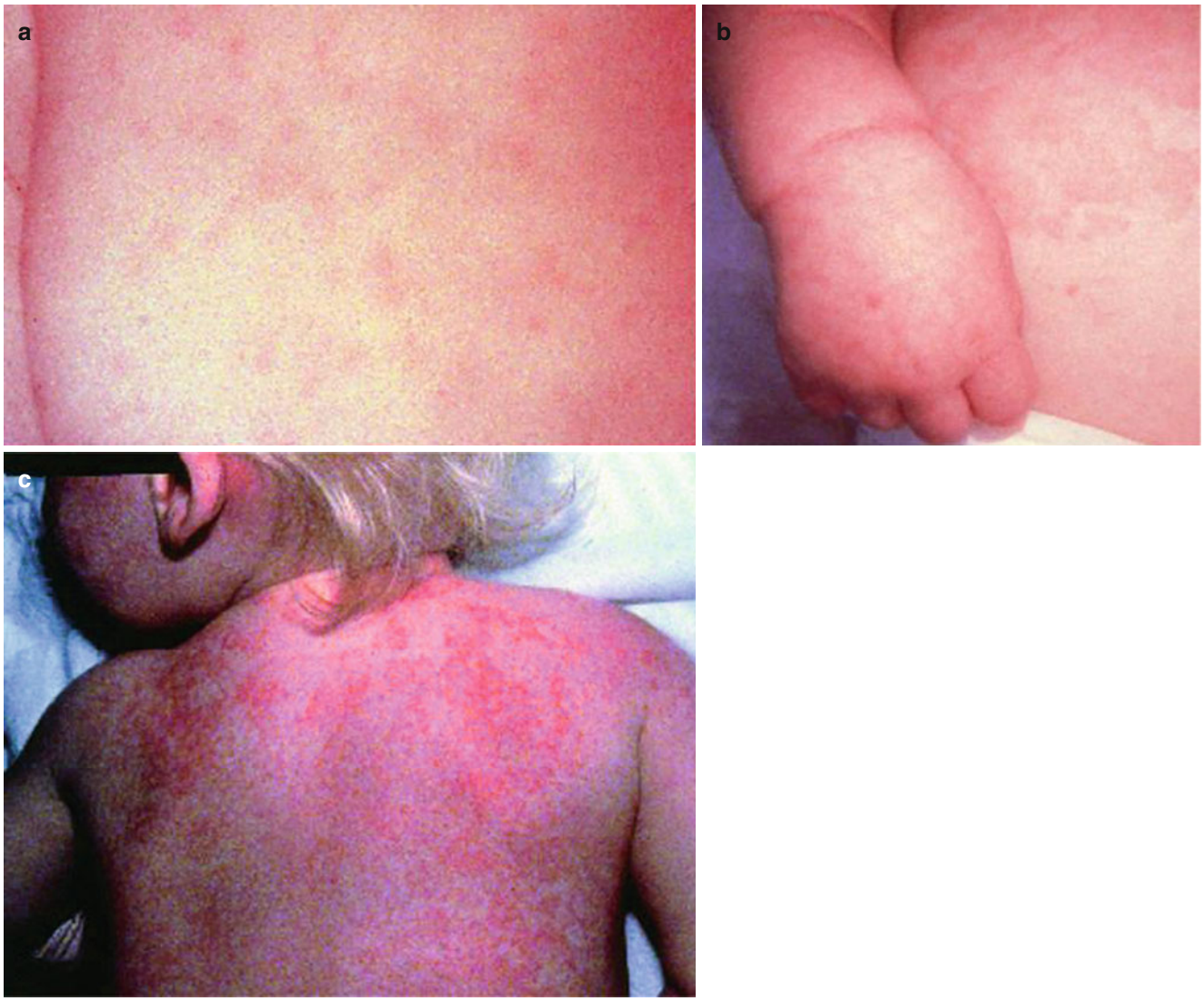

Fig. 37.2 The rash of roseola in a child with primary HHV-6 infection (Used with permission from Hall [43]

and varies in intensity of color and number of lesions. The trunk tends to be most involved, but the back of the neck, forehead, and extremities may also be affected (Fig. 37.2).

In the United States, the initial presentation in about 30 % of primary illnesses is a highly febrile disease without localized findings (Fig. 37.3) [48]. Only about 20 % of children later develop a rash and are diagnosed with roseola. Approximately 10 % of children with proven HHV-6 infection are diagnosed at presentation with presumed bacterial sepsis, and about one-half are initially diagnosed with a febrile respiratory or gastrointestinal illness. Otitis media is frequently diagnosed, based primarily on tympanic membrane erythema. Whatever the initial diagnosis, however, the most characteristic findings of primary illness are the abruptness and height of fever (average 40 °C), the development of postoccipital lymphadenopathy, and the decline in the white

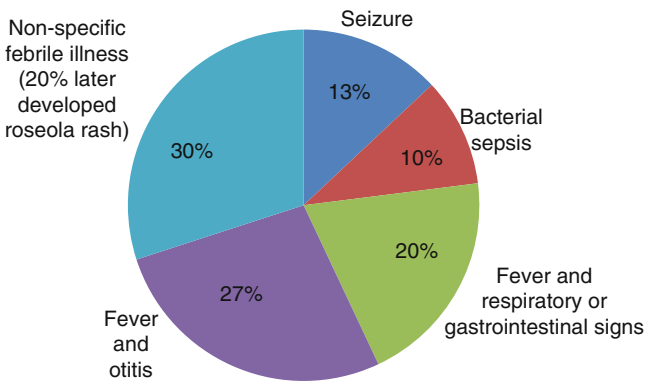

Fig. 37.3 Diagnosis at presentation of 354 US children with proven primary HHV-6 infection (Used with permission from Hall [43])

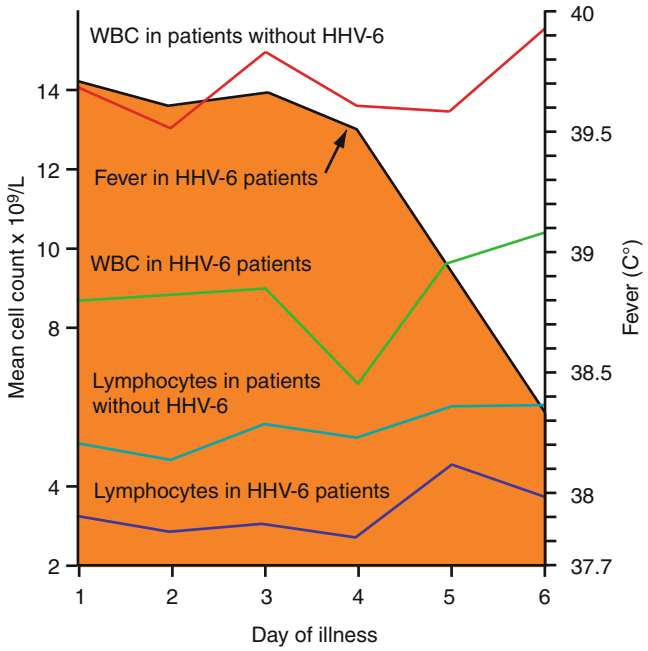

Fig. 37.4 Fever and hematological parameters in children with proven primary HHV-6 infection (Used with permission from Hall [43])

blood cell count after 2–3 days, which is subsequently followed by a rise in the proportion of lymphocytes (Fig. 37.4) [17, 87].

Of note is that the great majority of primary HHV-6 infections appear to be symptomatic but self-limited and the usual outcome is complete recovery. Asymptomatic infection has been estimated to occur in only about 5 % of primary infections [131].

Seizures, occurring in 13 % of children with primary HHV-6 infection, are the most common complication and sometimes have features that are atypical for classical febrile seizures including higher frequencies of partial seizures, prolonged and repeated seizures, and postictal paralysis [97]. Among young children identified as having primary infection with HHV-6 or HHV-7 and suspected to have encephalitis, status epilepticus was the most common presentation [112]. A recent nationwide Japanese survey estimated that the annual rate of encephalitis or encephalopathy complicating exanthem subitum (roseola) was 6 per 100,000 cases of roseola [125]. A little over half of these children developed significant neurologic sequelae, which were associated with abnormal findings on computed tomography (CT) during the acute phase of illness [125]. CSF findings in children with primary HHV-6 infection and encephalitis or encephalopathy are usually normal or show only mild elevations in cell counts or protein [58]. Additionally, the viral load in the CSF has been found to be less than 100 copies per milliliter in a small number of children with encephalitis complicating primary HHV-6 infection, and CSF concentrations of IL-6 have been shown to be significantly elevated compared to controls, suggesting that inflammatory cytokines may have a role in the pathogenesis of HHV-6 disease [53, 58].

8.1.2 Congenital HHV-6 Infections

No specific clinical findings currently are associated with congenital HHV-6 infection [44]. Most infants with congenital HHV-6 infection are asymptomatic and do not have findings which are clinically different from those of controls without congenital HHV-6 infection. Unknown, however, is whether subsequent developmental effects will occur.

8.1.3 HHV-6 Reactivation

In addition to the association between primary HHV-6 infection and seizures noted above, studies also have suggested a link between reactivated or persistent HHV-6 infection of the CNS and chronic seizure disorders that develop later in childhood or beyond. Best described is medial temporal lobe epilepsy (MTLE), which may follow prolonged febrile seizures or status epilepticus and is associated with mesial temporal sclerosis [106]. HHV-6 DNA has been identified in the hippocampus and lateral temporal lobes of a significantly greater number of brain samples from patients undergoing surgery for MTLE, and with higher viral loads, compared to samples from subjects with other types of epilepsy [31, 35]. HHV-6 protein was also identified in the affected areas by immunohistochemistry, suggesting active viral replication. Furthermore, the possible connection between HHV-6 and epilepsy is strengthened by preliminary data suggesting that HHV-6 infection interferes with a glutamate transporter protein in the CNS which may be a possible mechanism for the induction of seizures [35].

Reactivation of HHV-6 has been repeatedly demonstrated among immunocompromised hosts, especially among patients undergoing hematopoietic stem cell transplant (HSCT). Prospective studies have demonstrated reactivation of HHV-6 in the peripheral blood of 35–65 % of patients, most often from 14 to 28 days after transplantation [80, 81, 114, 121]. HHV-6 reactivation in HSCT is consistently associated with fever and rash and sometimes with CNS disorders [121, 129]. In particular, a specific syndrome of posttransplant acute limbic encephalitis (PALE) has been identified and characterized by altered mental status, insomnia, amnesia, and seizures, with temporal lobe disease evident on imaging [51, 110]. This syndrome occurs in approximately 1–4 % of patients, usually associated with the detection of HHV-6B, and higher viral loads in plasma than that observed in patients without CNS disease [74, 81, 82, 128]. Additionally, umbilical cord blood transplantation appears to confer a significantly higher risk for these complications than other types of HSCT and is associated with high mortality and long-term morbidity [51, 74]. HHV-6 reactiva-

tion has also been implicated in many but not all studies as causing delayed engraftment of platelets and neutrophils, graft failure, graft-versus-host disease, and myelosuppression in patients following HSCT [26].

Among patients with solid organ transplantation, the rate of HHV-6 reactivation varies from 20 to 55 % [62]. However, disease has been noted in only about 1 % [88]. Described consequences of HHV-6 reactivation include fever, rash, hepatitis, colitis, encephalitis, renal dysfunction, and graft rejection [62, 88]. Nonetheless, the specific impact of HHV-6 reactivation on solid organ transplantation is not yet clear. Routine monitoring for HHV-6 reactivation during transplant is not recommended nor is antiviral prophylaxis [88].

Little information exists on the role of ciHHV-6 among transplant recipients. Preliminary studies have suggested that patients with ciHHV-6 who have liver transplants have significantly higher rates of bacterial infections than those without ciHHV-6 [64]. Further evidence to determine whether the chromosomal integration of HHV-6 adversely affects the outcome of solid organ transplantation will require study of many more patients with ciHHV-6.

Among the immunocompetent adult population, an association between HHV-6 and multiple sclerosis has been suggested, but this link remains controversial [117]. HHV-6 reactivation also has been observed among patients with the drug-induced hypersensitivity syndrome, also known as drug rash with eosinophilia and systemic symptoms (DIHS/DRESS). However, if and how HHV-6 contributes to the pathophysiology of DIHS/DRESS is unclear [1].

8.1.4 Laboratory Diagnosis

Multiple serologic and molecular assays have been developed to detect and characterize infection with HHV-6 (Table 37.1).

Many of these tests are difficult to perform reliably and not available outside of research laboratories. Polymerase chain reaction (PCR) is readily available and has been used to identify the presence of the HHV-6 genome in multiple body fluids and tissues. The identification of HHV-6 DNA in plasma or other cell-free fluids by qualitative PCR was initially thought to be a reliable marker for active viral replication, due to either primary or reactivated infection [94]. However, recent data suggest that the detection of HHV-6 DNA in plasma is only 84 % specific compared to viral isolation [20]. Additionally, essentially all plasma samples from individuals with ciHHV-6 have detectable HHV-6 DNA in every sample which further confounds the interpretation of plasma results. Quantitative PCR and reverse transcription PCR can be used in addition to standard qualitative DNA detection assays to help discriminate between latent virus, actively replicating virus, and ciHHV-6 [20, 85].

9 Prevention and Control

HHV-6 infection is ubiquitous among humans, rapidly acquired in infancy or early childhood, and present for the life of the host. Despite the substantial gains in our understanding of HHV-6, many gaps remain, and the full clinical impact of infection has yet to be characterized completely. These limitations have prohibited significant progress toward the prevention of infection with HHV-6. Additionally, in view of the recent recognition that the complete viral genome is integrated into human chromosomes and can be passed vertically from parent to child in approximately 1 % of infants, complete prevention is unlikely to be possible.

Efforts at controlling disease manifestations due to primary HHV-6 infection or reactivation also remain preliminary. The antiviral drugs, ganciclovir, foscarnet, and cidofovir, have all been shown to have in vitro activity against HHV-6. They have been used either alone or in combination among small numbers of patients with presumed HHV-6 disease, but their effect on decreasing viral replication has been variable [26, 33, 130]. However, no drug has been studied in controlled clinical trials with clinically relevant endpoints, and none is currently licensed for the treatment or prevention of HHV-6 infection or disease.

10 Unresolved Problems

Although HHV-6 infection has been associated with several different clinically distinct diseases, the full extent of the pathology induced by infection or reactivation of the virus is still unknown. Because HHV-6 infects essentially the entire population and induces lifelong latency or persistence, a major challenge is determining when disease is due to the direct or indirect effects of the virus versus when the viral DNA is detected but not involved in disease pathogenesis. Significant steps needed to understand HHV-6 infection include deciphering the extent and timing of infection with HHV-6A, its clinical manifestations compared to those of HHV-6B infection, and whether an interaction between the two exists.

The recognition of ciHHV-6 has significantly increased our recent understanding of HHV-6's pathophysiology, and much of the prior information on HHV-6 needs to be reevaluated in light of the potential effects of ciHHV-6. The recently described role of ciHHV-6 in congenital infections has been particularly revealing but has simultaneously engendered an intriguing list of further questions. Does ciHHV-6 have the ability to replicate and create fully mature viral particles? Does ciHHV-6 cause disease, and, if so, what is the pathogenesis? Could ciHHV-6 interfere with normal cell development by disrupting the human genome or by producing viral genetic elements or proteins even in the absence of fully mature virions? [86] Among individuals with ciHHV-6 does

disease subsequently develop after birth and in different tissues than those usually seen with typical primary HHV-6 infection? Does a difference exist between infection due to chromosomal integration and infection from HHV-6 acquired postnatally that can be explained by differences in biology, immunity, or tolerance to one or both? The coming chapters in the biography of HHV-6 will be intriguing and important.

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Suggested Reading

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Epstein-Barr Virus (EBV): Infectious Mononucleosis and Other Non-malignant EBV-Associated Diseases

38

Karen F. Macsween and Ingólfur Johannessen

1 Introduction

Epstein-Barr virus (EBV), a member of the human gammaherpesvirus subfamily, was discovered in African lymphoma cell lines by Epstein, Barr and Achong in 1964 following the initial description by Burkitt, a British surgeon working in Africa, of an endemic malignant tumour affecting the jaw of children in Uganda [1–3]. Thus, EBV was the first candidate human tumour virus to be identified. The malignancy, affecting African children (Burkitt's lymphoma, BL), is now known to be of B lymphocyte origin, and EBV is considered to be an essential cofactor in the outgrowth of the endemic form of the tumour. Furthermore, EBV is the causative agent of infectious mononucleosis (IM) [4, 5], and the virus is aetiologically associated with a variety of epithelial and lymphoid lesions in the immunocompetent and immunosuppressed host, although its role in some tumours is not yet entirely clear [6] (see Table 38.1).

Of lymphoid malignancies, EBV is found in 96 % of endemic (African) BL (eBL; reviewed in [15]). In Europe and the USA, around 20 % of sporadic BL (sBL) is associated with EBV (reviewed in [16]) as well as approximately 50 % of Hodgkin's lymphoma (HL) (reviewed in [17]), and around 90 % of non-HL (NHL) arising in organ transplant recipients (post-transplant lymphoproliferative disease, PTLD; reviewed in [18]). Additionally, EBV is found in subsets of T/natural killer (NK) cell lymphomas (reviewed in [19]). Furthermore, EBV is detected in almost 100 % of undifferentiated (non-keratinised) and 30–100 % of differentiated (keratinised) nasopharyngeal carcinoma (NPC) as well as up to 10 % of gastric carcinomas (reviewed in [20]).

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2 Historical Background

The discovery by Peyton Rous at the Rockefeller Institute for Medical Research (later, the Rockefeller University) in New York City in 1911 of a cell-free transmissible agent that caused sarcoma in chickens (later identified as a retrovirus – the Rous sarcoma virus) was a watershed in the understanding of tumour development since it was the first oncovirus to be described [21]. Also, it provided evidence for the role pathogens might play not only in animal but also human tumour development.

Anthony Epstein (knighted in 1991), a British doctor working in pathology at the Middlesex Hospital Medical School in London (UK) in the early 1960s, had carried out research on Rous sarcoma virus and acquired expertise in electron microscopy (EM) under the supervision of George Palade (awarded the Nobel Prize in Physiology and Medicine in 1974 for innovations in EM and cell fractionation) at the Rockefeller Institute. By chance, Epstein noticed a poster at the Middlesex Hospital in early 1961 that advertised an open lecture on Wednesday 22 March in the Hospital's Courtauld Lecture Theatre on the most common childhood cancer in tropical Africa given by Denis Burkitt who was then working in Uganda. At the lecture, the first one Burkitt gave in the UK on what is now known as BL, Epstein assessed that the tumour epidemiology presented (high incidence in a geographical belt with high temperature and rainfall) was consistent with a viral cause of disease that was carried by an arthropod vector. Following his first encounter with a child with BL in 1957, and realising how common the tumour was in African children, Burkitt had embarked on a detailed mapping project that showed the high BL incidence area to overlap with that of holoendemic malaria [1, 22, 23].

Burkitt's presentation at the Middlesex Hospital was the beginning of collaborative links with Epstein that included Burkitt sending BL biopsy material by a then recent direct jet route from Kampala to London. However, despite application of cell culture and EM expertise over the ensuing nearly 3 years, the Epstein laboratory could not detect virus in the

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Table 38.1 EBV-associated malignancies

Condition	Initial description of EBV association
African (endemic) Burkitt's lymphoma (eBL)	Epstein et al., 1964 [2]
Sporadic Burkitt's lymphoma (sBL) (subsets)	O'Conor et al., 1965 [7]
Nasopharyngeal carcinoma (NPC) (subsets)	Old et al., 1966 [8]
Non-Hodgkin's lymphoma (NHL) in immunocompromised patients (e.g. post-transplant lymphoproliferative disease, PTLD)	Crawford et al., 1980 [9]
Hodgkin's lymphoma (HL) (subsets)	Weiss et al., 1989 [10]
T/NK cell lymphoma (subsets)	Jones et al., 1988 [11]
Tumours with suspected EBV association	
Anaplastic gastric carcinoma	Imai et al., 1994 [12]
Salivary gland neoplasia	Raab-Traub et al., 1991 [13]
Smooth muscle tumours in immunosuppressed states	Lee et al., 1995 [14]

biopsy material or following its inoculation in vitro and in vivo. Equally, attempts to expand the biopsy material were unsuccessful. The situation changed on Friday 5 December 1963 when a biopsy arrived at the Middlesex Hospital after considerable delay due to the Kampala flight having been diverted to Manchester as a result of dense fog in London. The sample appeared cloudy, which was taken as indication of bacterial contamination, but Epstein decided to view the sample under light microscopy prior to the specimen being discarded. To his surprise, instead of bacteria, he observed free-floating, viable tumour cells that had detached themselves from the main biopsy and appeared to be growing as a cell suspension. Their appearance reminded him of the first successful in vitro cultures of murine lymphoid cells (using free-floating cell suspension rather than solid tumour fragment) that he had seen on a prior visit the same year to Yale University Medical School. As a result, Epstein decided that Friday in December of 1963 to attempt expanding BL biopsy material by suspension culture of the free-floating tumour cells of the newly arrived biopsy, the 26th one to arrive in his laboratory from Burkitt. And it worked – following in vitro culture in the London laboratory, this BL biopsy gave rise to the first BL-derived continuous cell line (still growing today) referred to then as 'Epstein-Barr 1' ('EB-1'; Yvonne Barr and Bert Achong had then joined the Epstein group as research assistants).

Once sufficient cellular material had been generated, Epstein undertook the first EM study of EB-1 on 24 February 1964, which revealed virus particles with the characteristic morphology of herpesviruses [2, 24, 25]. Subsequent collaboration from 1965 onward with Gertrude and Werner Henle in Philadelphia on EB-1 (and EB-2) demonstrated that the newly discovered herpesvirus was distinct from the then known human herpesviruses CMV, HSV and VZV [26] and became known in the Henle laboratory as the 'Epstein-Barr virus' (or 'EBV'). It was also in the Henle laboratory that the causative link between EBV and IM was established in 1967 when one of their research technicians manifest symptoms and signs of the disease (then of unknown aetiology) in August that year and her blood was shown by her colleagues in the Henle group to seroconvert to EBV seropositive status [5].

3 Biology

EBV is a large enveloped gamma-herpesvirus (around 120 nm in diameter) which contains a 172 kbp linear dsDNA genome within an icosahedral capsid consisting of 162 capsomeres [2, 27, 28]. Overall, the EBV genome has coding potential for about 70 proteins. The virus infects B cells in vitro and in vivo [29, 30]. In vitro, latent infection involves expression of latent viral genes without production of virus and is compatible with host cell growth, whereas expression of lytic genes in productive/lytic infection results in host cell death and release of infectious virus (virions).

The *Eco*RI and *Bam*HI genome restriction fragments have been cloned and restriction maps constructed [31–33]. The complete DNA sequence of the laboratory EBV B95-8 strain has been determined [34]. Open reading frames (ORF) are assigned a four-letter and number acronym based on the restriction fragment where transcription begins; for example, the BCRF1 ORF refers to the first [1] rightward ORF of the *Bam*HI C fragment [34].

3.1 In Vitro EBV Infection

EBV preferentially infects B lymphocytes in vitro through attachment of the major viral envelope glycoprotein gp350/220 to the B cell surface cluster designation (CD) 21 molecule (the EBV receptor), which is the complement receptor (CR) 2 that binds the C3d component of complement [35, 36]. Entry also involves binding to a cell surface co-receptor, the human leukocyte antigen (HLA) class 2 molecules, by a second virus envelope glycoprotein (gp), gp42, that complexes with virus-derived gp25 (gL) and gp85 (gH) mediating fusion of virus envelope with target cell membranes [37–41].

Whilst CD21 is the main receptor for EBV, deletion mutants lacking gp350 may still infect B cells suggesting alternative means of virus entry (e.g. through cell-to-cell contact) into B (and epithelial) cells [42, 43]. Although EBV has been shown to infect epithelial cells in vitro and in vivo [44–46], whether a CD21 homologue is normally present on epithelial cells (HLA class 2 molecules are absent), and the role of epithelial cells in EBV infection, is still unclear. Alternative (CD21-independent) modes of EBV entry into epithelial cells have been proposed since oropharyngeal epithelium in vivo may be one source of infectious virus in saliva. In an in vitro model of human polarised tongue and pharyngeal cells, EBV can enter epithelial cells by (1) direct cell-to-cell contact of apical cell membranes with EBV-infected lymphocytes, (2) interaction between the (lytic) virus envelope BMRF2 gp and β1 (or α5β1) integrins at basolateral membrane sites and (3) direct virus spread across lateral membranes [39, 46, 47]. In this model, virus egress occurred from apical and basolateral membranes. Recent evidence of EBV infection of polarised epithelial cells by transfer infection from memory B cells adds support to the model [48].

The EBV genome is divided into a unique short (US) and unique long (UL) sequence region by up to 12 tandemly reiterated 3 kbp internal direct repeats (IR1). Tandem repeat segments are contained in the unique regions with three of them (all in UL) referred to as IR2, 3 and 4. The linear genome is flanked at either end by reiterated 500 bp terminal direct repeats (TR). Following entry into target B cells, covalent linkage of the TR segments gives rise to a covalently closed circular (ccc) episome (or plasmid) in the nucleus of the latently infected cell, which is where the virus replicates [49, 50]. In each host cell, only 1 genome forms a ccc episome despite many becoming cell associated during B cell infection [51] indicating cellular or viral exclusion mechanisms [52], and redundant linear genomes are lost. Multiple identical copies persist in the latently infected cell as a result of amplification by cellular DNA polymerase [53]. The number of TR involved in formation of the ccc episome is characteristic of any circularisation event, and progeny cells retain the same episome thereby giving rise to a clonal virus population – a feature that has been used to assess clonality of the virus in tumours [54]. During latency, the episomes are replicated once every cell cycle in synchrony with host cell DNA and equal partitioning to progeny cells (for a review, see [55]).

In vitro, 1–10 % of the infected B cell pool is 'immortalised' by EBV giving rise to continually proliferating B lymphoblastoid cell lines (BLCL) [56, 57]. In contrast to B cells, EBV infection of epithelial cells in vitro is difficult and does not result in full latent gene expression or lytic infection. Therefore, BLCL serve as the main in vitro model of EBV infection, latency and oncogenesis. Similar to antigen-stimulated B lymphocytes, BLCL express the B cell activation markers CD23, CD30, CD39 and CD70 as well as the cell adhesion molecules (CAMs) lymphocyte function-associated antigen (LFA)-1 (or CD11a/18), LFA-3 (or CD58) and intercellular adhesion molecule (ICAM)-1 (or CD54) [55, 58]. In contrast, these markers are virtually absent on resting B lymphocytes.

BLCL express all EBV latent proteins which constitute only 10 of a possible 70 viral-encoded proteins [34]: six EBV nuclear antigens (EBNA-leader protein (LP), 1, 2, 3a, 3b and 3c) that constitute the EBNA complex, three latent membrane proteins (LMP1, 2a and 2b) and possibly one (or more) protein of the BARF0 open reading frame from the *Bam*HI A region of the virus genome (BART transcripts; for an overview of EBV latent transcripts, see Table 38.2 and [59]. Several EBV-encoded microRNAs (miRNA) have been described [60] that are clustered in two regions of the genome (BART and BHRF1) and are thought to function as host and viral gene regulators (as well as having anti-apoptotic effect), which, unlike proteins, do not elicit an immune response [61]. Additionally, two small non-polyadenylated (and, thus, non-coding) EBV-encoded RNAs (EBER1 and 2) are abundantly expressed during latency. Analysis of virus deletion mutants in the BLCL model has shown that of the latent virus proteins, only EBNA1, 2, 3a, 3c and LMP1 are essential for in vitro immortalisation, whereas EBNA-3b, LMP2a, 2b and EBERs are not (see Table 38.2). Whilst EBNA-LP deletion mutants have a reduced in vitro immortalising capacity in B cells compared with wild-type EBV, the role of BART transcripts in immortalisation remains unclear. The expression of the latent genes is tightly regulated by methylation of the gene promoters [62] in order to allow EBV to persist within the host without eradication by the host immune response. EBV DNA becomes CpG methylated slowly with time in latently infected B cells [63], and extensive DNA methylation is associated with transcription repression [64]. Such dinucleotide methylation is considered to explain BZLF1-mediated activation of latent cells to lytic cycle (see below; [65]).

Latent viral infection has been defined as latency 1, 2 or 3 based on EBV promoter usage and latent gene expression (see [59]). Latency 3 (EBNA expression driven by Wp/Cp in fragments *Bam*HI W and C, respectively; observed in BLCL and most PTLD) is characterised by transcription of all the EBNAs together with the three LMP proteins. In contrast, latency 1 (EBNA expression driven by Q promoter in fragment *Bam*HI Q; seen in EBV-associated BL and early passage BL cell lines) involves the transcription of EBNA1 only. Latency 2 (EBNA expression driven by Qp; detected in EBV-associated HL and NPC) entails EBNA1 expression together with a variable expression of the LMP proteins. EBERs and BARTs are expressed in all forms of latency [55]. However, since viral gene expression found in clinical samples does not always conform to the above latency types, this text uses an alternative scheme that refers to persistent

Table 38.2 EBV latent transcripts and their functions

ORF	Protein	Mol.wt (kDa)	Cellular site	Function	Required for immortalisation?
BKRF1	EBNA1	65–97	Nucleus	Episome maintenance; transcriptional transactivator	Yes
BWRF1	EBNA-LP	20–130	Nucleus	Promotes in vitro immortalisation; co-operates with EBNA2 in facilitating entry into cell cycle	Uncertain
BYRF1	EBNA2	75–105	Nucleus	Viral oncogene; transactivates cellular (e.g. CD21, CD23) and viral (e.g. LMP1 and 2) gene expression	Yes
BLRF3/BERF1	EBNA3a	130–195	Nucleus	Remains uncertain	Yes
BERF2a/b	EBNA3b	145–160	Nucleus	Regulation of cellular gene expression (e.g. CD40)	No
BERF3/4	EBNA3c	130–195	Nucleus	DNA binding protein; regulates viral (e.g. LMP1) and cellular (e.g. CD23) expression	Yes
BNLF1	LMP1	58–63	Membrane	Viral oncogene; acts as a constitutively expressed TNF receptor; upregulates cellular genes (e.g. *bcl*-2 and IL6) and activates cellular pathways (e.g. NF-κB); mimics CD40 function	Yes
BARF1/BNRF1	LMP2a	54	Membrane	Acts as a constitutively activated BCR homologue; prevents entry into lytic cycle	No
BNRF1	LMP2b	40	Membrane	Remains unclear	No
BCRF1	EBER1,2	–	Nucleus/cytoplasm	Confer resistance to apoptosis; induce IL10 production	No
BARF0	BARTs	–	Cytoplasm	Remains unclear	Not known

BCR B cell receptor, *IL* interleukin, *kDa* kilo ($\times 10^3$) Dalton, *Mol.wt* molecular weight, *NF* nuclear factor, *ORF* open reading frame, *TNF* tumour necrosis factor

infection as either 'unrestricted' or 'restricted'. The former expression pattern refers to a latency 3-type infection, whereas the latter refers to any other more restricted profile.

Lytic EBV infection involves productive viral DNA replication, expression of most virus genes, release of infectious virus and host cell death. The majority of EBV-encoded proteins are expressed during lytic infection although only a small number of BLCL cells enter lytic cycle at any given time. Since herpesviruses do not encode an RNA polymerase, EBV utilises host cell RNA polymerase II for transcription of its viral messenger RNA (mRNA). The immediate early (IE) viral proteins BRLF1 and BZLF1 initiate the lytic cycle, which is characterised by the sequential expression of early antigen (EA) complex, viral capsid antigen (VCA) complex and membrane antigen (MA) complex proteins. Each complex consists of several proteins (reviewed in [55]). The early (IE and EA) lytic proteins are operationally differentiated from the late (VCA and MA) lytic antigens by the persistent expression of the former, but not the latter, in the presence of viral DNA synthesis inhibitors. During lytic infection, EBV also expresses a human interleukin (IL) 10 homologue (viral IL10, or vIL10). Furthermore, EBNA1, LMP1, 2a, 2b and EBERs continue to be transcribed.

3.2 In Vivo EBV Infection

Following primary infection, EBV persists in vivo in a latent form at a fairly constant level in approximately 1–10 per 10^6 circulating B cells [66–68]. Around 90 % of seropositive virus carriers (cumulative results when assayed on several occasions) give rise to BLCL in vitro when (1) their peripheral blood leukocytes (PBLs) are cultured in the presence of the immunosuppressant cyclosporine-A (Cy-A), or (2) their throat washings are cocultured with susceptible EBV-negative cord blood mononuclear cells [69]. Circulating EBV-infected B cells are antigen-selected small, CD19+ve, CD23-ve, CD27+ve, CD80-ve, surface immunoglobulin (sIg) M+ve, sIgD-ve and resting memory B cells [56, 57, 66, 67, 70, 71].

Opinion is divided as to whether infection of epithelial cells occurs prior to infection of B cells, or whether infection of B cells occurs directly via the large surface area of the tonsillar crypts where a specialised discontinuous epithelium exists [72–74]. Early work found EBV-infected epithelial cells in IM [75, 76], a finding refuted by later studies [77–79], which may reflect differences in methodology. It is possible that a transient infection of epithelial cells occurs as a prelude to infection of B cells; this would occur prior to the onset of IM symptoms and could therefore be missed in studies involving tonsils excised in acute IM. In situ hybridisation (ISH) studies of tonsils removed during IM suggest that interindividual transmission of EBV occurred via the transfer of EBV-infected lymphocytes [77]. Recent evidence suggests, however, that EBV infection of epithelial cells may have a pivotal role in such transmission [80].

Based on coding sequence differences of EBNA2, 3a, 3b, 3c and EBERs, EBV is of two types referred to as 'type 1' and 'type 2' (or 'type A' and 'type B') [81]. The EBNA2 proteins derived from both types share only 56 % of

amino acids and have differing functional capabilities with type 2 being less capable of transforming B lymphocytes in vitro [82, 83]. Although both virus types have a worldwide distribution, Caucasian and Oriental populations are predominantly infected with type 1 virus, whereas type 2 virus is frequently found in African populations [84, 85]. Immunocompromised hosts may (more often than immunocompetent individuals) harbour both types simultaneously [86, 87], and infection with type 2 has been associated with high-risk sexual behaviour in HIV-infected and uninfected men [88]. In contrast, HIV-infected haemophiliac males have a prevalence of type 2 infection that is closer to that of the general population [89]. Amongst HIV-seropositive populations, type 2 EBV is over-represented in EBV-associated malignancies that arise in the context of HIV-induced immunosuppression [90].

EBV gene expression (transcriptional) patterns displayed by PBLs from healthy virus carriers have been analysed by reverse transcriptase polymerase chain reaction (RT-PCR) amplification of whole cell, or enriched B cell, population RNA extracts. Using such an approach, LMP2a transcription has been reported in vivo as has EBNA1, LMP2a and EBERs expression without detectable lytic BZLF1 transcripts [91, 92]. Similarly, EBNA1 and LMP2a expression have been described as well as EBNA1 and LMP1 only [93, 94]. In contrast, a further study detected lytic BZLF1 and BALF2 transcripts in 72 and 16 %, respectively, of enriched peripheral blood B cell extracts from virus carriers [95].

Taken together, the only consistently expressed latent virus gene in peripheral blood of healthy virus carriers is LMP2a ('latency programme'). Furthermore, cells showing full viral latent gene expression ('growth programme') have never been observed in peripheral blood in healthy virus carriers although such an expression profile has been detected during IM [92]. Thus, the LMP2a-only 'latency programme' has been proposed as the mechanism of in vivo latency with LMP2a providing the EBV-infected memory B cell with survival signals similar to those that maintain the long-term memory B cell pool [66]. In line with this is an observation that LMP2a inhibits the switch from latency to lytic infection in EBV-infected B cells in vitro [96]. According to current thinking, LMP2a and LMP1 provide infected B cells with B cell receptor (BCR: sIg) and CD40 co-stimulatory signals, respectively, and thereby mimic both survival signals required to maintain the infected B cell pool [97–104].

3.3 A Model of EBV Persistence

The EBV expression profile of tonsils from healthy EBV carriers has been examined in detail. The research group demonstrated latently infected naïve B cells in tonsils, and their presence was associated with viral replication [66]. Furthermore, in contrast to the studies above on peripheral blood, the same research group showed full latent viral gene expression (including EBNA2; 'growth programme') in purified naïve (sIgD+ve) tonsillar B cells [105]. These cells were activated CD80+ve (B7.1+ve) lymphocytes. Based on their findings, the research group suggested that infectious virus is restricted to regions of the tonsil that harbour only naïve B cells (e.g. the mantle zone). Latently infected memory B cells from the periphery could extravasate in the tonsillar marginal zones and reactivate EBV in response to signals in the mantle zone. The EBV-infected naïve B cells would either be killed by virus-specific T lymphocytes or differentiate within the tonsil to become resting memory B cells that would then leave the tonsil to circulate in peripheral blood whilst virus gene expression became restricted [102].

A model of EBV persistence in vivo has been proposed based on the observation that EBV persists in resting memory B lymphocytes that do not express any viral proteins [66, 106–111] and that this situation arises despite the virus's clear ability to drive proliferation of its host cells. Further support for the pivotal role of B cells in both primary and persistent infection comes from a study demonstrating that patients lacking Bruton's tyrosine kinase and, therefore, suffering from X-linked agammaglobulinaemia and the absence of functional B cells cannot be infected by EBV [112]. In the model of virus persistence, it is proposed that EBV promotes formation of long-lived virus-infected memory B cells using normal pathways of B cell response to antigen [113]. The oral lymphoepithelium of the tonsil (tonsillar crypts; part of Waldeyer's ring) is exposed to environmental antigens that are sampled by naïve B lymphocytes [73, 114]. When encountering specific antigen, naïve B lymphocytes are activated via their BCR and migrate as blast cells into tonsillar follicles to produce germinal centres where B cell maturation occurs [115]. Differentiation (involving antigen-specific T helper (Th) cells) entails rounds of proliferation associated with isotype switching and mutation of Ig genes followed by competitive selection for B cells that bind the antigen most avidly and their release as long-lived antigen-specific memory B cells. Non-selected B cells die by apoptosis. Paralleling this scenario, EBV expresses all of its growth-promoting latent antigens ('growth programme') following primary virus infection of naïve B cells in tonsillar epithelium [105]. Thus, the virus promotes B cell activation, blast formation, proliferation and formation of tonsillar follicles without specific antigen-mediated BCR triggering. In the tonsillar follicle, the transcription programme changes to the more restricted 'default programme' (EBNA1, LMP1 and 2 only; [107]). At this stage, LMP2 drives Ig isotype switching [116, 117], whilst LMP1 promotes Ig gene mutation and downregulates *bcl*-6 expression thereby retaining memory B cells in the germinal centre [116, 118, 119]. Therefore, the importance of the 'default programme' may be to offer

virus-infected B cells a selective advantage in the germinal centre [113]. For an overview of this model of EBV persistence, see [111].

Once they leave the germinal centre, latently infected B cells switch off virus protein expression ('latency programme') and are maintained as normal memory B cells [108]. EBV-infected memory B cells are thought to express virus proteins only rarely in the peripheral circulation [108]. Whilst the numbers of such virus-infected cells can form over half of the memory B cell pool during the acute stages of IM [70], a steady state of approximately 1–10 EBV-infected cells per 10^6 circulating memory B cells is detected within a year of IM reflecting a dynamic balance between cell proliferation and cell loss through lytic infection [108, 120]. However, the role of either EBV reinfection of B cells or direct virus infection of memory B cells, in maintenance of the virus-infected B cell pool is unclear [121]. Equally, short-term EBV persistence in the absence of a germinal centre reaction and a typical B cell memory pool has been demonstrated in X-linked hyper-IgM syndrome patients who (due to a mutation in the CD40 ligand gene) do not harbour such cells [122].

Virus reactivation in mucosal sites leads to virus transmission. As a result of antigen-mediated BCR triggering and homing of antibody-producing memory B cells to mucosal sites, an infected memory B cell activates EBV to lytic infection [114, 123, 124]. Studies in vitro and in vivo have demonstrated such a link between B cell differentiation and lytic infection [124, 125]. In the tonsil, this results in egress of infectious virus into saliva. The importance in transmission of reactivation of virus in genital sites is still unclear although virus is detected in samples from the male and female genitourinary tract suggesting the possibility of sexual transmission [126–128].

The above model of persistent infection centres on memory B cells as the site of EBV persistence, but oropharyngeal epithelium may support virus replication and amplify virus in the oropharynx and, by extension, play a central role in transmission [80, 112, 129, 130]. However, epithelial cells are not considered to support persistent EBV infection.

4 Laboratory Diagnosis

From an early stage, IM was known to be associated with an absolute lymphocytosis and atypical lymphocytes [131, 132] (Fig. 38.1). Recognised in 1932 by Paul and Bunnell, production of heterophile antibodies that cross-react with animal antigens, particularly agglutinins to sheep erythrocytes, was used for laboratory confirmation of IM [133]. In 1937, Davidsohn refined the Paul-Bunnell test with the use of guinea pig kidney antigen, which improved specificity, by removing Forssman antibodies (animal erythrocyte agglutinins in human serum not related to acute EBV infection) [134]. The approach forms the basis of the rapid heterophile ('monospot') test with subsequent amendments [133–135] (see also Public Health England (PHE) and US Centres for Disease Control and Prevention (CDC) websites). The heterophile test has a sensitivity of around 85 % and specificity of around 94 % for IM in a patient with symptoms of mononucleosis [136].

An absolute lymphocytosis is typical but not always present in IM, and many EBV-induced IM cases will have a normal total lymphocyte count; lymphopenia is rarely present, but atypical lymphocytes are usually present although the proportion of such cells will comprise ≥10 % of the total lymphocyte count in only around a fifth of IM cases [137]. However, the finding of ≥10 % atypical lymphocytes in peripheral blood of patients with mononucleosis has 75 % sensitivity and 92 % specificity for IM [138]. Mild thrombocytopenia is common but neutropenia less so [139, 140]. Elevations of one or more liver function tests, including bilirubin, alkaline phosphatase and aminotransferases, occur in around 80 % of IM cases, whilst clinical jaundice is apparent in up to 5 % of patients [141, 142]. Hyperbilirubinaemia may result from impaired hepatic clearance and/or increased destruction of red blood cells as a result of haemolysis. Jaundice is more common in older patients and is a frequent accompaniment of patients that succumb to rare fulminant infection [143, 144].

EBV-driven IM is confirmed in 20–25 % of patients where it is clinically suspected, and laboratory investigations are instigated with the remainder having 'IM-like' illnesses that are a heterogeneous group and may include drug reactions and/or infections by other herpesviruses (CMV, HHV-6, HSV), other viral agents (adenovirus, HIV, hepatitis viruses, influenza, parvovirus B19, rubella), bacteria (streptococci), parasites (toxoplasmosis) or other causes [145–147]. There is overlap in the clinical presentation and haematological features of acute EBV infection and these conditions, although the likelihood of any individual feature being present varies according to the diagnosis.

Common clinical features of patients with IM, CMV mononucleosis and acute toxoplasmosis comprise fever, sore throat, enlarged lymph nodes and splenomegaly [142]. However, CMV mononucleosis is primarily characterised by prolonged fever, lymphocytosis, atypical lymphocytes and abnormal liver function tests. Sore throat and cervical lymphadenopathy are not characteristic of CMV, but they do occur in a third and one-fifth of cases, respectively [142, 148, 149]. Similarly, acute toxoplasmosis is primarily characterised by the presence of lymphadenopathy and the absence of hepatomegaly, with sore throat, fever and splenomegaly occurring only occasionally.

Reminiscent of EBV-induced IM, other acute infections (e.g. CMV, HIV, HBV and toxoplasmosis) may present with atypical lymphocytes in the blood and severe perturbations

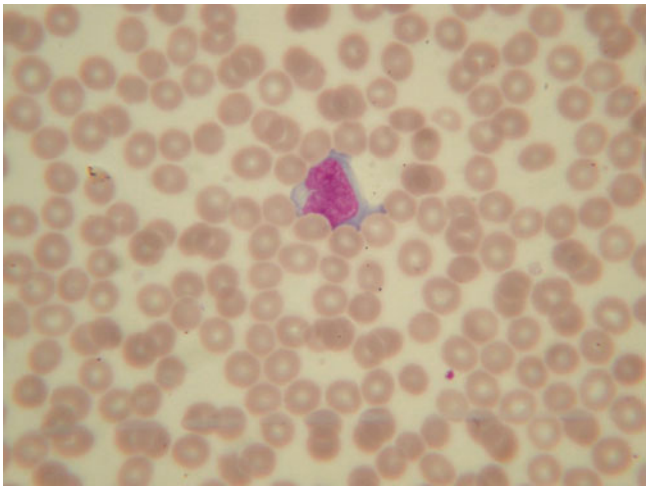

Fig. 38.1 Peripheral blood film in IM showing atypical lymphocyte (Photograph provided by Dr R Jones, St John's Hospital, Livingston, UK)

of lymphocyte subsets with inversion of the normal CD4:CD8 ratio [150–153].

4.1 Serology

Our knowledge of the host's immune response to primary EBV infection is modelled on IM (see Odumade et al. [154]). IgA, IgM and IgG antibodies to VCA are usually detected by the onset of IM together with IgG antibodies to EA and MA [155]. A range of (mostly IgM) heterophile and autoantibodies also appear transiently. During convalescence, IgG antibodies to EBNA1 arise, whilst IgA and IgM antibodies to VCA as well as IgG antibodies to EA (and heterophile antibodies) decline to undetectable levels [156]. There is a dichotomous antibody response to the nuclear antigens, with those against EBNA2 and 3 being formed early and those against EBNA1 being delayed. IM is rare in the first year of life – presumably as a result of protective maternal antibodies being transferred to the neonate in utero – and children uncommonly show typical symptoms of IM ([157]; for a review of IM, see [136]).

Current EBV serological tests assess host antibody responses to VCA, EA and/or EBNA by immunofluorescent (IFA) or enzyme immunosorbent assays (EIA). In the UK, test algorithms have been standardised by PHE (formerly, the Health Protection Agency, HPA) for use in diagnostic clinical laboratories as UK Standards for Microbiology Investigations (UK SMI; see the PHE website). For serological diagnosis of primary EBV infection, the SMI algorithm (endorsed by the UK Clinical Virology Network, or UKCVN: association of all UK clinical virology laboratories) is contained in UK SMI V26: 'Epstein-Barr Virus Serology' (issue 4, 2012). SMI V26 requires an initial test panel of either (1) VCA IgM and IgG followed by EBNA IgG if either test is reactive or (2) EBNA IgG on its own followed by the other two tests if a negative EBNA result is obtained. Furthermore, EBV IgG avidity testing may aid discerning between primary infection and past acquisition of virus [158, 159]. The approach highlights the role of EBNA IgG in determining past infection but recognises that such antibodies may also arise soon after acquisition of EBV.

For each assay platform, EBV serostatus can be assigned based on the results of VCA IgM, VCA (or VCA/EA) IgG and EBNA IgG as follows: 'no serological evidence of infection', 'early acute infection' (VCA IgM+ve), 'recent acute infection' (VCA IgM+ve, VCA IgG+ve) or 'past infection' (EBNA IgG+ve).

Historically, IFA has been considered the 'gold standard' for EBV serological assessments. However, IFA contains an element of observer subjectivity as well as falling prey at times to non-specific IF reactivity. As a result, IFA is rapidly being replaced in UK clinical virology laboratories with a more automated EIA approaches that do not require as much assay-specific expertise for interpretation as well as lending themselves to the high throughput and short turnaround times necessary in the hospital setting. Recent assessment of 3 EBV-specific IgM EIAs on such automated platforms found assay sensitivity to be 84–89 % [160], although sole reliance on EBV IgM may result in up to 8 % of IM patients being missed at first presentation [161].

The serological profile of primary EBV infection in organ graft recipients often differs from that seen in healthy immunocompetent individuals. Specifically, IgM antibody responses to VCA and/or heterophile antibodies are not always detected [162]. Furthermore, immunocompromised EBV carriers often have a 'reactivated' serological profile characterised by raised levels of IgG antibodies to VCA and EA but not to EBNA [163]. Also, in seropositive patients, suppressed EBV-specific cytotoxic T lymphocytes (CTL) activity is associated with increased oropharyngeal virus shedding and raised numbers of circulating EBV+ve B cells [164, 165].

4.2 Other Techniques

Propagation of EBV in vitro, from saliva or throat washings, as BLCL, is generally not offered in clinical laboratories. Tissue sections (e.g. biopsy material) can be probed for EBERs using ISH techniques or immunostained for various EBV proteins. These techniques and, in particular, nucleic acid amplification tests (NAATs; e.g. PCR) for EBV DNA have a role in determining active EBV infection in the immunocompetent host but are especially important in the diagnosis of EBV-associated disease in the immunocompromised host.

PCR analysis of EBV DNA viral load (VL) in the blood allows detection of EBV DNAemia which may be of use in

assessing active EBV infection during IM as well as in other EBV-associated diseases such as in pre-emptive management algorithms with a view to prevent progression to PTLD [166–169]. DNAemia is generally short lived following presentation of clinical symptoms of IM although EBV DNA assessments may aid early diagnosis of IM ([170]; for a review of the clinical utility of EBV PCR in the clinical setting, see [171]). In the UK, SMI guidance highlights the use of EBV PCR in the context of VCA IgM+ve, VCA IgG-ve and EBNA IgG-ve results in the setting of IM-like disease in order to exclude a false IgM result and address inconclusive serological results.

EBV PCR analysis is also recommended when assessing primary acquisition of virus, or reactivation of past infection, in immunocompetent and immunocompromised hosts. In the latter, serology may be unreliable (see above). Further guidance is available from the British Transplantation Society (BTS) [172] for EBV PCR testing post organ transplantation which does not recommend routine EBV DNAemia surveillance of adult transplant recipients by PCR with the exception of stem cell graft recipients. Conversely, routine surveillance is recommended to aid in pre-emptive identification of primary EBV infection in seronegative paediatric transplant recipients since that setting constitutes high risk of PTLD. Currently, routine EBV VL measurements aimed at monitoring response of PTLD to therapy are not recommended. There is as yet no clear consensus between UK clinical virology laboratories as to the clinical utility of EBV PCR in the diagnosis and management of EBV-associated disease (including IM). Thus, the use of EBV PCR varies somewhat between individual UK laboratories although rapid advances in NAAT technology will influence future use of the assays. Until recently, the lack of an international EBV standard for VL determinations compounded interpretation of PCR assay results and hindered comparison between clinical laboratories. The first such WHO international standard has now become available from the UK National Institute for Biological Standards and Control (NIBSC; see their website) which should aid such assessments. Equally, interpretation depends on type of samples analysed although plasma is generally accepted as being a suitable specimen for EBV PCR – but some centres prefer whole blood. Furthermore, it is clear that a single VL assessment cannot be used to predict the likelihood of PTLD since lesions may arise in patients with unremarkable VL [173, 174], and serial sampling is indicated. A rapid increase in VL in serial samples obtained following transplantation may indicate increased risk of PTLD development [167], and prompt reduction in immunosuppression may be indicated in such circumstances [175]. However, the role of EBV VL measurements in assessing treatment response to PTLD therapy, and in predicting risk of PTLD relapse, is still somewhat unclear since studies have not shown clear and consistent correlation between EBV VL and clinical course in solid organ transplantation [176, 177].

4.3 Challenges in Laboratory Diagnosis of IM

The typical findings of VCA IgM+ve, VCA IgG+ve and EBNA IgG-ve results together with a positive heterophile antibody ('monospot') test are not found in every case of EBV-induced IM. VCA IgM and IgG antibodies are usually present at the onset of symptoms/signs, but one or both may be delayed in arising [145, 146, 178]. Rarely, VCA IgM antibodies are not detected at outset of IM which may reflect low titre and screening dilution used [179]. Equally, high-titre IgM may give rise to a prozone effect although modern automated EIA platforms have in-built dilution steps to prevent such false-negative readings. Antibodies to EBNA are usually absent in the acute phase of IM (developing in convalescence), but may occasionally be detected during the first week of illness [146, 180]. Amongst patients with VCA IgM+ve IM, 85–96 % will have a positive heterophile antibody test using horse erythrocytes [145, 146, 179] although up to 25 % of IM patients may show negative heterophile test results during the first week of infection (up to 10 % during the second week; [136]). Similar to antibodies against VCA, heterophile antibodies may arise late in the course of IM [145, 146], and up to 75 % of children under the age of 12 years (particularly, under the age of 4 years) do not have detectable heterophile antibodies [181].

Laboratory investigations, rather than giving clarity, may add to diagnostic difficulty. In particular, false-positive heterophile antibody tests have been noted in health as well as in the context of other infections (notably HIV), connective tissue diseases and lymphoma [18], although, in general, primary viral infections other than EBV only rarely give reactive readings in the test [182]. False-positive VCA IgM+ve antibody readings have also been noted in the presence of rheumatoid factor [183] which is compounded further by the observation that rheumatoid factor (IgM or IgG) may be produced in the acute phase of IM [184]. Also, patients with EBV-induced IM may show reactivity in CMV IgM tests [185] which may be a result of cross-reactivity in EIA assays or a consequence of EBV being a polyclonal activator of B lymphocytes. In the immunocompromised setting, delayed, or atypical, serological profiles of primary infection, and virus reactivation, may occur [163, 186].

5 Pathogenesis and Immunity

Infection with EBV is a significant public health concern. Understanding of the host immune response, and strategies evolved by the virus to evade immune control, is important in devising vaccines, therapies and public health measures to reduce disease burden.

5.1 Persistent Infection

Stable levels of circulating IgG antibodies to VCA, MA and EBNA1 are detected in healthy virus carriers, and low levels of IgG antibodies to EA may also be present [155]. IgG antibodies against gp350/220 of the MA complex are retained and neutralising. Additionally, 21–30 % of virus carriers have IgA antibodies against gp350/220 in serum, and these antibodies are found in saliva of 12–19 % of virus carriers [187, 188].

Immune control of EBV infection is mediated primarily by CD8+ve HLA class 1-restricted EBV-specific CTLs against all the latent viral proteins (CTL precursor frequency ranges from 1:400 to 1:42,000 of the total T cell population) although the role of T cell responses to EBNA1 is still unclear ([189]; for an overview of immunodominant CTL epitopes and their HLA restriction, see [190]. In particular, CTL responses to EBNA3a, b and c antigens are immunodominant followed by LMP2 antigens [189, 191, 192], and cytolysis of the CD8+ve T cells is mediated by granule exocytosis mechanisms (perforin, granzyme). CD8+ve CTL specific for EBNA2, LP and LMP1 epitopes are less commonly detected. Analysis of phenotypic markers including IL7 receptor alpha and programmed death 1 (PD1) can aid delineate EBV epitope responses at different stages of infection [193, 194]. EBNA1 contains glycine-alaninine (gly-ala) repeats that interfere with proteosomal proteolysis necessary for HLA 1-restricted epitope presentation to CTLs [195, 196] and inhibit translation of EBNA1 [197]. Recent studies indicate that the protein may reduce its own translation by suboptimal codon usage within the gly-ala sequence [198] although transcriptional control may also be involved [199, 200]. Evidence suggests that EBNA1-specific CD8+ve CTLs can be generated in vitro [201–203]. Such EBNA1-specific CTLs could play a future role in adoptive immunotherapy against EBV-associated malignancies – all of which express EBNA1. Collectively, up to 5 % of circulating CD8+ve CTLs are committed to EBV immune control [204, 205].

In addition to immunosurveillance mediated by CD8+ve T cells, CD4+ve T cells also have a role to play, and EBNA1-reactive CD4+ve T cells have been detected in healthy seropositive individuals. In particular, such T cells have been shown to lyse EBNA1+ve cells [206, 207], and EBNA1 (and EBNA3c) is now considered highly immunogenic for CD4+ve T cells [208, 209]. In contrast to CD8+ve CTL, such CD4+ve T cells mediate their effector mechanisms primarily via interferon (IFN)-gamma secretion and Fas/Fas ligand (FasL) interactions rather than granule exocytosis [210]. The role of IFN-gamma in mediating CTL defences against EBV is further highlighted by studies of X-linked lymphoproliferative syndrome (XLP, or XLPS; see below), which results from a defect in the signalling adaptor SLAM-associated protein (SAP) gene that signals through signalling lymphocytic activation molecule (SLAM; CD150) and other Ig superfamily receptors including 2B4 (CD244). Using autologous BLCL as stimulators, EBV-specific T cell lines generated in vitro from XLP patients show markedly reduced IFN-gamma production and cytotoxic capacity compared to healthy controls [211].

During persistent infection, CTLs also control lytic infection and recognise specific IE (BZLF1, BRLF1) and EA (BMLF1, BMRF1, BALF2) antigens [212, 213].

Immunosuppression reduces EBV-specific CTL activity, and this can lead to EBV-driven proliferation of B cells that may, in the organ transplant setting, culminate in PTLD. Such lesions may be treated successfully by adoptive T cell immunotherapy [214, 215] which further underlines the role CTL surveillance plays in immune control of EBV.

5.2 Infectious Mononucleosis (IM)

IM is manifest by an absolute lymphocytosis including 'atypical' lymphocytes [131], and over 70 % of the lymphocytes have been shown to be antigen-driven mono- or oligoclonal HLA DR+ve, CD45RO+ve, CD8+ve, and CD38+ve CTLs when analysed using PCR amplification of, and monoclonal antibodies (mAb) to, the T cell receptor (TCR) Vβ-chain [216–219]; for serological responses, see Sect. 4. These cells express the CD45RO memory marker together with low expression of the anti-apoptotic gene *bcl*-2 that correlates with their apoptosis in vitro [220]. Evidence suggests that efficient resolution of IM symptoms is associated with a broad CTL response to a wide range of EBV-derived target latent and lytic epitopes [221]. To date, over 50 HLA class 1 and 2 epitopes have been characterised for EBV. Whilst EBV DNA VL in peripheral blood decreases rapidly during convalescence, salivary VL remains high (up to 6 months post-diagnosis), and IM patients remain highly infectious during this time [178, 222]. A role for superantigen (sAg)-driven expansion has not been demonstrated despite earlier reports [223, 224]. The transiently expanded T cell clones recognise primarily early (IE and EA) lytic (rather than latent) EBV antigens (e.g. BZLF1 and BRLF1) with up to 40 % of circulating CD8+ve CTLs being directed against a single virus epitope as evidenced by tetramer analysis, although the exact epitopes eliciting the CD8+ve CTL response varies with the HLA profile of the individual [213, 225, 226]. In contrast, only a minor component of the CTL response in IM is directed against the latent virus antigens. As the primary infection progresses, the vastly expanded CD8+ve T cells against lytic antigens reduce in number and may even become undetectable [204, 227–229].

Whilst CD4+ve T lymphocytes form a minor part of the observed lymphocytosis of IM, the cells display an activated phenotype and respond to lytic and latent (e.g. EBNA3a) viral epitopes [230–233]. In particular, CD4+ve T cell

responses have been described against the early lytic (BZLF1 and BMLF1) and latent EBNA1 and EBNA3a antigens in IM patients [231, 234]. However, such CD4+ve T cell-mediated responses decline rapidly during the disease and are at a low (or undetectable) level at 1 year after diagnosis of IM.

The massive CTL response observed in IM is thought to cause its characteristic symptoms and signs through excessive cytokine release such as raised Th1-type cytokine levels (e.g. IL2 and IFN-gamma [235, 236]). NK (CD56) cells form a component of the atypical lymphocytes observed in IM [219] and contribute to control of EBV [237, 238]. Further studies have demonstrated a correlation between the severity of IM symptoms and the level of activated T lymphocytes [233]. SLAM (CD150), SAP and 2B4 (CD244) were significantly upregulated on CD4+ve and CD8+ve T cells at time of IM diagnosis. At that time, NK cell levels were also significantly elevated suggesting a role for NK cells in immune control of primary EBV infection, and higher NK cell numbers at IM diagnosis were observed to correlate with significantly lower circulating EBV VL [239]. Also, human tonsillar NK cells can be induced to produce IFN-gamma in vitro that delays in vitro immortalisation of B cells [240] which further underlines possible role of NK cells in innate immune defences against EBV.

There is evidence to suggest deficiencies in CTL immune surveillance in patients suffering EBV-associated cancer [241, 242], but stronger links with EBV immune defences arise through work linking microsatellite markers close to the HLA-A locus to enhanced risk of EBV-associated HL [243], and these same markers have also been linked with increased risk of IM [244]. Similarly, markers linked with the HLA-A locus are strongly associated with risk of NPC [245, 246].

The role high-dose salivary and/or genital tract EBV exposure plays in IM is still unclear [247, 248]. Equally, it seems plausible that the release on a large scale of EBV-derived antigens from infected B cells under attack from CTL-mediated immunity may contribute to the signs and symptoms of IM [130].

5.3 Immune Evasion

Following acquisition, herpesviruses persist in the host for life and have evolved strategies to evade immune eradication, whilst sporadic reactivation from latency facilitates maintenance of the infected host cell pool and onward transmission. By providing all the necessary survival signals, EBV has evolved a strategy of persistence that entails mimicking antigen-mediated activation and maturation of its target B cell (see Sect. 3). To this end, it also shuts down expression of immunogenic viral proteins – apart from sporadic reactivation from latency that results in lytic infection with its ensuing replenishment of the virus-infected B cell pool and potential for onward transmission.

Similar to other herpesviruses, the EBV genome contains homologues of human genes which are thought to modulate the host's immune response. One such gene is BCRF1 whose product shows 84 % amino acid (and 71 % nucleotide) sequence homology to the human IL10 gene [249], the vIL10 homologue [250]. Like its human counterpart, vIL10 represses IL2 and IFN-gamma secretion (Th1 response) [249, 251] and promotes B cell growth and differentiation [252]; vIL10 is a lytic cycle protein that is non-essential for in vitro growth of latently infected B cells [253]. However, cells infected with deletion mutants were not able to inhibit IFN-gamma secretion by cocultured autologous mononuclear cells. IFN-gamma and NK cells provide non-specific immune defences against EBV infection [253–255], which vIL10 is postulated to inhibit. Furthermore, IL6 and IL10 are autocrine growth factors in vitro for BLCL [256, 257], and both growth factors are secreted by CD4+ve Th cells. IL10 has also been shown to be induced by LMP1 [258] produced by EBV+ve BL cell lines [259] and found at high levels in serum from humanised scid mice bearing PTLD-like tumours [260]. In such animals, a model of PTLD formation has been proposed that centres on CD4+ve T cell help for outgrowth of EBV-driven B cell lymphoproliferations followed by B cell-derived autocrine tumour growth involving B cell growth factors such as IL6 and IL10 [261–263]. Taken together, it is likely that EBV has a direct role in the induction and maintenance of autocrine growth factor production. Conversely, it has been suggested that IL10 gene promoter polymorphism correlates with susceptibility to primary EBV infection and that low IL10 production resulting from such mechanisms renders individuals more susceptible to primary EBV infection [264, 265].

EBV subverts the effects of class I and II IFNs by synthesising viral receptors which compete with the intended IFN receptor, generating interfering RNAs which inhibit IFN-directed protein synthesis and downregulating IFN-induced transcription [266–269]. The survival of EBV-infected cells is promoted by several anti-apoptotic mechanisms including the production of two homologues of the human gene *bcl*-2, BHRF1 and BALF1, and the upregulation of *bcl*-2 itself by the viral gene LMP1 [270–272]. The two genes appear to be required to prevent apoptosis at the outset of virus infection of B cells that will become latently infected but not necessarily during latency itself [273, 274]. Lytically infected cells secrete soluble gp42, a glycoprotein that binds HLA class 2 molecules, which may help EBV-infected cells to evade recognition by CD4+ve T cells [275]. Further studies have shown that the combined effect of the lytic viral proteins BNLF2A, BILF1 and BGLF5 is to lower the surface level of HLA class 1 molecules on infected cells. Whilst this may trigger NK cell recognition, the EBV micro-RNA BART2 reduces expression of the NK cell-activating ligand MICB that facilitates NK evasion [276]. Additionally, the IE protein BZLF1 has been shown to reduce expression of HLA class 2 molecules [277].

The immune evasive aspects of EBNA1 have been discussed already (see above). Clearly, the success of adoptive T cell immunotherapy for EBV-associated tumours arising following transplantation in the context of immunosuppression highlights the key role T cells have in mediating immune control of EBV.

6 Transmission

Studies of the epidemiology of IM have not shown any increased risk of IM amongst susceptible room-mates of cases [278, 279], whilst a retrospective study found a history of intimate oral contact with exchange of saliva in many IM patients [280, 281]. Transmission of EBV even in the context of a close personal relationship may be slow – a study of 45 EBV seronegative women, who were seen every 6 months in a British family planning clinic, reported a median time to seroconversion of 25 months (range 1–60 months) [282].

6.1 Oral Secretions

Saliva is thought to be the main route of EBV infection in children and adults. Cell-free virus, present in throat washings of seropositive individuals, may be detected by the immortalisation of cultured B lymphocytes obtained from an EBV seronegative donor. This technique, utilised in early studies, demonstrates the presence of infectious virus in the throat gargles of 14–25 % of healthy asymptomatic adults [283–285]. Longitudinal study of EBV-infected individuals showed that the excretion of cell-free virus in throat gargles was present on every occasion in 25 % of cases and intermittently detected in a further 66 %, with only 2/24 (8 %) individuals not having detectable EBV on any occasion [69]. In acute IM, 50 % of patients had cell-free virus in their throat gargles using the same technique [284]. The more sensitive technique of PCR has demonstrated EBV DNA in the 97–100 % of IM throat wash samples [178, 222] and in 48–62 % of samples from asymptomatic individuals [122, 285]. The salivary VL remains high for at least 6 months after diagnosis, and IM patients remain highly infectious during this time [178, 222]. Throat wash samples obtained from immunosuppressed patients show higher rates of B cell immortalisation of 24–100 % [283, 285].

6.2 Genital Secretions

Whilst saliva is thought to be the major route of EBV transmission, EBV has also been detected in genital secretions. Infectious cell-free virus was detected in cervical washings in 4/28 (14 %) of women [127], and additional studies have detected EBV DNA by PCR in 6–40 % of cervical samples [128, 286, 287]. Five to 48 % of male urethral specimens collected in sexually transmitted diseases clinics had EBV detected by PCR [126, 128]. EBV DNA has also been detected in 0.4–41 % semen specimens collected in infertility clinic [288, 289] and 3–3.5 % of sexually transmitted diseases clinic settings [128, 290]. In the sexually transmitted diseases clinic setting, males with concurrent HIV infection have higher rates of EBV detection in semen (41–56 %; [290, 291]).

Sporadic case reports record the temporal association of genital ulceration and IM, mostly in female patients, although not all had been sexually active [292, 293]. The VL in genital specimens appears lower than that in saliva, and sexual partners are more likely to share indistinguishable EBV strains in common than are individuals known to each other but not in a sexual relationship [128].

6.3 Breast Milk

Like CMV, EBV has been detected in breast milk. EBV DNA was present in 60 of 132 (46 %) of samples donated to a Canadian breast milk bank [294]. However, it is not clear whether breast milk can be a source of EBV infection in infants; the transplacental transmission of maternal antibodies to EBV in term newborn infants would appear to protect against early infection as evidenced by the abrupt onset of seroconversions after 8 months of age [295]. A seroepidemiological survey of bottle- and breast-fed Japanese children aged 12–23 months did not find any difference in age of EBV acquisition [296].

6.4 Intrauterine Infection

Primary EBV infection occurring during pregnancy is rare as most adult females are infected before pregnancy. A specific congenital syndrome relating to intrauterine EBV infection has not been described, although cases of congenital malformations associated temporally with primary infection in the mother during pregnancy have been reported [297, 298].

6.5 Tissue Transmission

EBV can be transmitted with grafted bone marrow or transplanted solid organs from seropositive donors to seronegative graft recipients [129, 299, 300]. In a study, two EBV seronegative heart/lung transplant recipients seroconverted shortly after transplantation and developed EBV-positive PTLD, and the lesions were shown by molecular analysis to harbour the donor EBV isolate. In contrast, EBV-positive PTLD from two seropositive heart/lung patients

were shown to contain the recipient original virus isolate. This indicates the significance of EBV transmission via the grafted organ as a risk factor for the development of PTLD in seronegative recipients [301].

EBV has also been shown to transmit with blood donations [302] prompting calls at the time for the blood from donors with a recent history of IM not to be given to EBV seronegative organ transplant recipients. However, the use of leukodepleted blood products has, to an extent, addressed the issue [303, 304].

7 Epidemiology

EBV achieves near universal infection in humans with over 90–95 % of the world population becoming seropositive [305–309]. The timing of primary infection shows marked geographical and socio-economic variation (see Fig. 38.2).

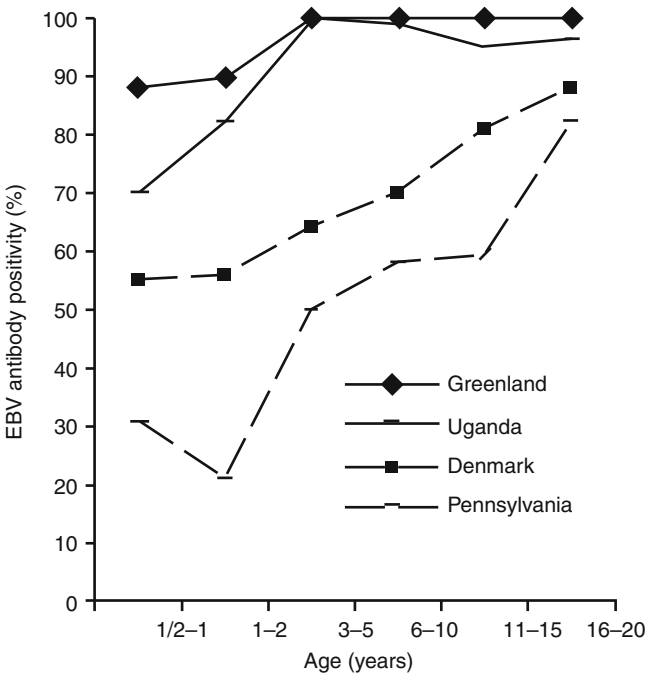

Fig. 38.2 Seroconversion by age in different countries (From Hjalgrim et al. [310])

7.1 Socio-economic and Geographical Factors

Seroepidemiological studies in Africa demonstrated very early EBV acquisition with 80–100 % of infants infected by their first birthday [311, 312]. Urban African children residing in more affluent homes showed slightly delayed EBV acquisition, in some cases until the third birthday [311]. Early serocoversion has also been demonstrated in other continents [313–315].

Studies in Europe and the USA have shown later acquisition of EBV, with only 39 % of children aged between 5 and 14 years having serological evidence of infection in a laboratory-based UK study [307] and 50 % acquisition by 2 years of age in a hospital-based study serving an area of low socio-economic status in Philadelphia [316]. Intermediate results have been obtained in Japan [309] and Brazil [317], with the Brazilian investigators noting later seroconversion in a neighbourhood of higher family income and maternal education.

A Danish study found an association between positive EBV serology and the occupation of the head of the household. Where the head of the household followed an 'unskilled' occupation or was unemployed, 54 % of children aged 0–6 years had antibodies as opposed to 34 % of children aged 0–6 years from a 'skilled' or 'professional' background [305]. Persistence of EBV seronegativity into teenage years has been associated with higher socio-economic status as assessed by the occupation of the head of the household [318], the income of the household [278] or residence in owner-occupied housing [318]. A study of over 1,000 children aged 6–7 years in primary schools in the UK, using a salivary antibody detection method, found significant associations between seropositivity and unemployment, or manual occupation, of the head of the household, sharing a bedroom or attending a school in an area with predominantly rented housing estates [319].

7.2 Serological Status by Age and Gender

IgG antibodies to EBV cross the placenta so almost all term newborn babies are seropositive. This wanes by 4 months of age with natural infection starting to occur around 6 months of age [295, 305, 320], and the rapidity of acquisition depends upon the affluence of the household and the community [311]. In affluent communities, where seroconversion is delayed, two peaks of acquisition may be seen; one in children under 5 years and a second in teenage years [306, 321]. A UK study, based on serological samples submitted for diagnostic purposes, showed 35 % of children aged 1–4 years and 54 % of 10–14-year-olds, and 72 % of 15–19-year-olds had evidence of past EBV infection [321]. In countries with late EBV infection, acquisition in adolescents aged 10–14 years occurs earlier in girls than boys [321] so that by age 19 significantly fewer males (64–73 %) than females (72–88 %) are seropositive [247, 306].

Studies from the UK and Japan suggest that age of primary EBV infection may be changing with virus acquisition now occurring later in childhood than previously [309, 321].

Table 38.3 Incidence of IM in general populations in the USA and UK

Author, year of publication	Location	Year	Criteria	Rate per 100,000
Henke et al., 1973 [327]	Rochester Minnesota	1950–1969	Reported to health department	181
			Strict based on laboratory and clinical features	99
Heath Jr et al., 1972 [325]	Atlanta, Georgia	1968	Collation of positive laboratory tests	45
Davidson, 1970 [326]	Grampian, UK	1969	Collation of positive laboratory tests	44
		1966–1968	Subgroup of two general practice populations above	100
Niederman and Evans, 1997 [328]	Connecticut	1983		71
		1985		61
White et al., 1998 [329]	England and Wales	1993–1995	Personal communication from Communicable Disease Surveillance Centre	69

7.3 Other Factors: Siblings and Day Care

The evidence for siblings and day care favouring EBV seroconversion is less consistent than age and socio-economic status. Some studies report no such association [278, 318], whilst others found an excess of seroconversions in those with siblings [306, 322]. A large study based in primary schools in the UK, using a salivary antibody detection method, also failed to find a significant association with numbers of children in the home [319]. Attendance of preschool children at nursery appears to promote seroconversion, although the studies to date are small and not all results achieved statistical significance [305, 308, 323].

7.4 Serological Status and Sexual Behaviour

In countries where EBV seroconversion is delayed into adolescence and young adulthood, several studies have noted an association between sexual activity and acquisition of EBV [88, 247, 324]; see also Sect. 6.

7.5 Epidemiology of IM

IM may result when primary EBV infection is delayed beyond infancy, predominantly occurring between the ages of 12 and 25 years [325].

The incidence of IM in a population may be estimated by the number of positive laboratory tests (heterophile antibody and/or EBV-specific IgM) or, where IM is notifiable, notifications to public health departments or by specific surveys. A limitation of population studies based on the finding of positive heterophile antibody tests is that they are likely to under-report IM, particularly in children, who are less likely to mount a heterophile antibody response before 4 years of age (see Sect. 4, [145, 146, 179, 181]). A UK study based on positive heterophile tests in 1970 found that the peak incidence of IM occurred between the ages of 15 and 19 years in both sexes, with incidence rising in females at a younger age than males [326]. A US study reported a similar incidence in the overall population of 45 cases per 100,000, a peak incidence in those aged 15–19 years, and noted that the peak incidence in females occurred 2 years prior to that observed in males ([325]; for an overview, see Table 38.3).

The incidence of IM in populations of young adults such as universities and military forces is much higher than in general populations as shown in Table 38.4. A recent report from the Israeli Defence Force gives an annual incidence of 880 per 100,000 between 2002 and 2009 [334]. A much earlier report from 19 US colleges and universities gave an overall incidence of 1,112 per 100,000 students in the 9-month academic year, with the rate from individual institutions ranging from 110 to 2,235 [335].

Akin to the seroepidemiological studies showing a relationship between affluence and delayed EBV acquisition, a study in London (UK), which compared the occupation of the head of the household of IM cases with that of the surrounding population, showed an excess of IM in those in higher socio-economic grouping [336].

Published information on hospitalisation rates for IM is scarce (see Table 38.5); however, in the UK, the incidence of hospital admissions for IM may be rising [337].

7.6 Epidemiology of IM in Student and Military Populations

IM is recognised as an important cause of ill-health in populations with large numbers of young people, including universities and the armed forces especially where a high proportion of new entrants are EBV seronegative. In the 1960s, IM was noted to be the third most common cause of days lost to hospitalisation in the US Air Force and fourth most common in the US Navy ([340]; for a summary of studies of IM in military recruits and students, see Table 38.4). Annual rates of IM in seronegative entrants range from zero to 13 %, but the proportion of EBV seroconversions that result in documented illness varies widely – from none to 74 % [4, 278, 279, 318, 331–333]. However, this may reflect the intensity of clinical follow-up.

Table 38.4 IM in military recruits and students

Author, Year of publication	Location	Sex	EBV seronegative at entry	Seroconversion rate per year	IM rate in seronegatives per year	Proportion of seroconversions symptomatic
Lehane, 1970 [330]	American Marine Recruits	Male	18 % (117/648)	7 % in training (calc) 2 % in Vietnam (calc)	Not stated Hospitalisations for IM in Vietnam 0.2 % per year	9 % (based on rate of hospitalisations for IM in Vietnam troops)
Niederman et al., 1970 [4]	Yale University	Male	65 % (97/150)	11.1 %	4.4 % per year	65 % 28/43
Niederman et al., 1970 [4]	Peace Corps Volunteers	Not stated	24 % (39/164)	27 % 8/30	No IM cases	No IM cases
Sawyer et al., 1971 [279]	Yale University	Male	49 % (175/355)	18 % per year (23/175, 13.1 % in 9 months)	13 % per year calc 9.7 per 100 in 9 months	74 % definite IM 100 % had some symptoms
Hirshaut et al., 1971 [331]	Cornell University	Not stated	25 % (200/800)	Not stated	4 % (15/200 in 2 years)	Not stated
University Health Physicians, 1971 [332]	6 UK Universities	Male and female	43 % (622/1,457)	21 % per year (60/496, 12 % in 7 months)	6 % per year (22/622, 3.5 % in 7 months)	37 % 22/60
Hallee et al., 1974 [278]	US Military Academy	Not stated presumed male	37 % (511/1,401)	21 % Overall 46 % in 4 years 12 % in first year 54/437	3 % (15/437) Definite + retrospective 6.2 %	28 % (15/54 definite IM cases) suspected in retrospect for additional 12, i.e. 50 %
Dan and Chang, 1990 [318]	Chinese University, Hong Kong	51 % male	7 % (71/1,039)	26 % 16/62	No IM cases	No IM cases
Crawford et al., 2006 [333]	Edinburgh University, UK	38 % male	25 % (510/2,006)	15 %	4 %	25 %

8 Host Response

8.1 Clinical Features of Acute Infection in Children and Adults

After an incubation period of between 4 and 7 weeks, the symptoms and signs of IM typified by fever, sore throat and lymphadenopathy may arise in up to 74 % of older children and adults [279, 281, 341–345]. In younger children, symptomatic seroconversion is much less frequently recognised, lymphadenopathy and pharyngitis is less prominent, and, when EBV seroconversion is detected, it may be associated with upper respiratory tract illness, otitis media, rash and/or pneumonia [316, 346].

In adults, IM may be subacute in presentation resulting in difficulty in pinpointing the exact date of onset [343]. Not all cases have pronounced sore throat or lymphadenopathy; in some, fever may predominate, whilst occasionally in others, the presentation may be with an unusual complication. Pharyngitis is evident in 85 %, and exudates may be seen in 22–58 % [342, 343, 346]; see Fig. 38.3. Lymphadenopathy, particularly affecting the posterior triangle of the neck, is present in most cases; axillary or groin nodes are detected in around half of cases [347]. Splenomegaly is clinically detectable in up to 17 %; an ultrasound study demonstrated splenic enlargement in all of 29 cases studied [348]. Hepatomegaly is clinically detectable in 10–37 % of cases, and in 50 % by ultrasound [341, 344, 348]. Other features include petechiae at the junction of the soft and hard palate and eyelid oedema [347]. Rashes, including macular erythema, petechiae and urticaria are reported in 3–16 % of cases; however, the concurrent administration of ampicillin results in a red rash in around 95 % [18, 341, 347, 349].

The acute features resolve over 3–4 weeks [350]. Thereafter, however, a fatigue state, lasting a median of 8 weeks, is common [329], and depression may follow occasionally [329, 351, 352]. Patients with IM also report prolonged hypersomnolence, impaired concentration and disruption of their usual work and recreational activities [161, 329, 353, 354].

A number of complications of IM are well recognised (see Table 38.6) – particularly hepatitis, jaundice, thrombocytopenia, haemolytic anaemia, splenic rupture and fatigue. Rarer complications include encephalitis, demyelination, arrhythmia, pancreatitis, gastrointestinal haemorrhage, interstitial nephritis and pulmonary infiltrates [342, 344]. When IM occurs beyond young adulthood, a more severe clinical presentation may be seen with protracted fever and severe hepatitis. Unusual complications may also be present, and

Table 38.5 Hospitalisation rate of IM

Author, year of publication	Location	Year	Rate per 100,000
Morris and Edmunds, 2002 [337]	England, UK	1989	2.6
		1998	4.8
Brabazon et al., 2008 [338]	Ireland	1997–2002	13.0
Ramagopalan et al., 2011 [339]	England, UK	1998–2005	4.2 Males 4.8 females 3.6

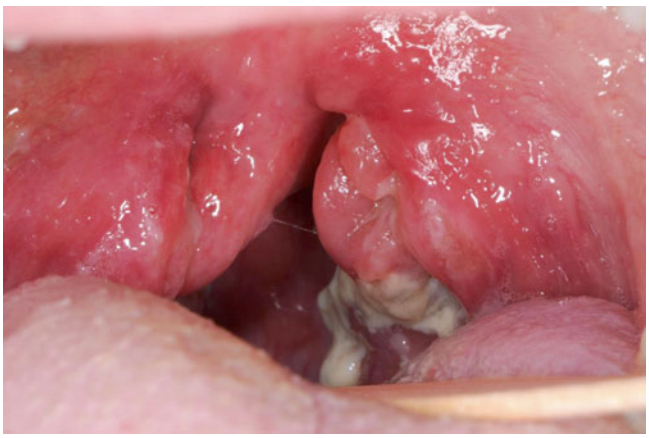

Fig. 38.3 Tonsillar swelling with exudate in acute IM (Photograph provided by Dr KF Macsween, Royal Infirmary of Edinburgh, UK)

the patient is likely to be investigated or treated for other conditions including haematological malignancy or hepatobiliary disease, as IM may not be considered at the outset of disease [143, 355, 356].

Death as a result of IM is very rare and may be the result of an underlying immunological defect, particularly XLP (see below). Other underlying defects resulting in severe disease are recognised, including defective NK cell function [237], perforin gene mutations [357] and selective IFN-gamma deficiency [358]. Even in those without immune defects, fatal outcomes may sometimes occur as a result of airway obstruction, neurological complications, hepatic failure, splenic rupture, myocarditis, cardiac arrhythmia, secondary bacterial infection or haemorrhage [144, 342, 344, 356, 359–361].

8.2 Fatigue After IM

Fatigue is reported by 77–100 % in the acute stages of IM and lasts longer than fatigue associated with other upper respiratory tract infections [154, 161, 329, 345, 352]. Studies in adults give a median duration of self-reported fatigue after IM of 4–16 weeks (interquartile range) [329], with 12–13 % of patients still fatigued at 6 months [345, 362–364]. The reasons for the prolonged fatigue are not understood, but risk factors for fatigue at 6 months include female sex, older age, negative beliefs about illness duration and severity of acute illness [352, 362–365]. Altered gene expression has been noted in a small study although it is not clear whether this reflects aetiology or is a consequence of altered behavioural patterns [366]. Most patients with IM recall being advised to rest by their general practitioner [367], yet the available evidence suggests that this impedes recovery [353, 368, 369]. A small study based in primary care found that an intervention of an individual treatment session which delivered ‘a personalised strategy of graded time-targeted activity and advice about a balanced lifestyle’ resulted in a significantly less fatigue at 6 months than in controls [370]. More studies are required in this area; however, the approach is in line with current practice in the management of chronic fatigue [371–374].

8.3 Treatment of IM

Treatment of IM is supportive, and no specific therapy is routinely recommended at present. The antiviral acyclovir reduces virus production in the throat during treatment; however, it does not alter the clinical symptoms or course of the illness, and its use is not recommended [375]. More recently, based on a small pilot study, it has been suggested that valacyclovir should be investigated for the treatment of acute IM; however, larger studies are required before this approach can be recommended [376]. Corticosteroids are not recommended for routine use in uncomplicated IM, but may be useful to reduce the need for surgical intervention when the degree of swelling in Waldeyer’s ring is compromising or threatening to compromise the airway [377–380]. Corticosteroids have also been used in the treatment of autoimmune haemolytic anaemia and severe thrombocytopenia [136, 344, 379]. Oral phenoxymethylpenicillin or intravenous benzylpenicillin may be given if superadded streptococcal infection is present; however, caution is advised as the risk of rash may be increased [136, 349]. Concurrent ampicillin or amoxicillin is contraindicated as almost all (around 95 %) patients will develop a generalised erythematous macular rash [136, 380, 381]. IM may be complicated by spontaneous or traumatic splenic rupture, and therefore it is advised that vigorous athletic participation should be deferred for at least 1 month, or until the patient is well and has no splenomegaly [382–384].

Table 38.6 Complications of IM

Organ/system	Complication
Respiratory	Respiratory tract obstruction, interstitial pneumonitis (rare)
Skin	Rashes 3–16 %; following the administration of ampicillin 86–98 %
Liver	Clinical jaundice 5 %, fulminant hepatitis (rare)
Haematological	Thrombocytopenia, haemolytic anaemia, neutropenia
Psychiatric	Depression, anxiety
Immunological	Secondary infection, streptococcal sore throat, sepsis in association with neutropenia, mesenteric adenitis, depressed cell-mediated immunity
Spleen	Splenic rupture 0.1–0.5 % spontaneous or after mild trauma (usually in males), splenic infarction
Neurological[a]	Encephalitis, acute cerebellar syndrome, aseptic meningitis, Guillain-Barré syndrome, cranial nerve palsy especially VII, transverse myelitis, seizures, mononeuritis, optic neuritis, cerebral haemorrhage
Gastrointestinal	Haemorrhage secondary to mucosal erosion, pancreatitis
Genito-urinary	Haematuria, interstitial nephritis, glomerulonephritis (rare), genital ulceration
Cardiac	Myocarditis, pericarditis, arrhythmia, electrocardiogram changes

Adapted from Macsween and Crawford [18]
[a]Pharyngitis and lymphadenopathy may be absent

8.4 X-Linked Lymphoproliferative Syndrome (XLP or XLPS)

In the early 1970s, deaths were reported from acute IM and/or malignant lymphoma of 6 male kindred within the same (Duncan) family [385, 386]. Similar disease was also reported in a 16-year-old boy whose 3 male cousins had succumbed to a similar disease [387] as well as in another three related young males [388]. The syndrome is due to a defective gene on the X chromosome [389] which results in the inability to control primary EBV infection – XLP, or XLPS – and is characterised by the clinical triad of susceptibility to severe primary EBV infection (IM), dysgammaglobulinaemia and lymphoma.

The typical clinical presentation of XLP is of a young male who is well prior to primary EBV infection but rapidly succumbs to fulminant IM following acquisition of EBV [390, 391]. Death may result from hepatic necrosis secondary to massive CTL infiltration and cytokine release, aplastic anaemia or pancytopenia or, in some cases, superimposed bacterial and/or fungal infection. A more chronic disease may also ensue which often progresses to a fatal B cell lymphoma, and death may occur by 40 years of age [392]. XLP is characterised histologically by massive solid organ lymphoproliferations of EBNA+ve B lymphoblastoid/plasmacytoid cells of polyclonal origin admixed with T, NK and phagocytic cells [393–397]. Haemophagocytosis is a frequent histological finding [393, 396]. Other phenotypes – including dysgammaglobulinaemia, lymphoproliferative disorders or autoimmune phenomena (including vasculitis, colitis or aplastic anaemia) – may arise before, or after, EBV infection [398].

Most XLP cases are caused by a defective gene located on the long arm of the X chromosome. SH2D1A (Src homology 2 (SH2) domain-containing gene 1A) that encodes for SAP [399–401]. SAP, a 128 amino acid protein with an SH2 domain, is an adaptor molecule expressed in T and NK (but not normal B) cells which binds to the cytoplasmic end of several cell surface proteins including SLAM, 2B4, CD84, Ly9 and NK-T-B antigen [402, 403]. Thus, SAP receives signals from the cell surface and modulates intracellular signalling through its association with the SLAM receptor family, such as SLAM and 2B4 [404], resulting in regulation of aspects of NK and T lymphocyte function including cytokine production, proliferation, cytotoxicity and antibody formation. If SAP is defective or absent, it is postulated that the SLAM family of receptors are unable to signal normally [398]. To date, over 70 mutations of the SAP gene have been identified [405–407] and most result in reduced or absent protein [398]; currently, there does not appear to be a correlation between mutation, phenotype and disease severity although the complex signalling interactions between SAP and its receptors are not fully understood [398, 408]. Recently, however, SAP has been shown to have pro-apoptotic, and EBNA1 anti-apoptotic, function that may provide an insight into XLP as well as EBV-associated tumourigenesis [409, 410]. Association of XLP with other genes – in particular, the X-linked inhibitor of apoptosis protein (XIAP) gene – and its subdivision into XLP 1 and 2 remains to be clarified.

XLP is usually fatal unless treated with either bone marrow transplantation [411] or, more recently, a humanised murine mAb against the B cell surface marker CD20, rituximab [412].

8.5 Chronic Active EBV (CAEBV)

Chronic active EBV (CAEBV) infection is a rare condition, distinct from chronic fatigue, which is typified, in a previously healthy person, by severe, chronic or recurrent IM-like symptoms following a well-documented primary EBV infection. An abnormal EBV antibody profile is present with

greatly increased antibody titres against VCA and EA – often accompanied by persisting IgM and IgA to VCA – together with the failure to mount antibodies against EBNA during the convalescent phase. In addition, there is a marked increase of EBV VL in tissues and peripheral blood [413] and evidence of major organ involvement – for example, interstitial pneumonia, bone marrow hypoplasia, splenomegaly, extensive lymphadenopathy, hepatitis, meningoencephalitis and uveitis [414–416]. EBV DNA testing aids diagnosis and assessment of prognosis in CAEBV [171].

CAEBV has a high morbidity and mortality from severe fever, hepatic failure, lymphoma, sepsis, cardiac failure or haemophagocytic syndrome. Diagnostic criteria have been proposed that include clinical features and virological and haematological parameters and, pre-fixing the acronym with 'severe', exclude patients with minor symptoms [415].

The exact pathogenesis is unclear although a perforin defect has been described that is not a constant phenomenon [417]. Interestingly, clonal expansion of EBV-infected T or NK cells takes place in preference to infection of the usual B cell target [357, 414, 418]. Severe cases are more common in Japan, China and Korea than elsewhere suggesting a possible genetic predisposition to CAEBV.

CAEBV is difficult to treat; bone marrow transplantation, and/or adoptive immunotherapy, may be helpful [419–421].

8.6 Multiple Sclerosis (MS)

EBV is one of several agents proposed as having an aetiological role in the pathogenesis of multiple sclerosis (MS). A Danish population-based study examined the subsequent occurrence of MS amongst over 25,000 patients diagnosed with IM. This study involved more than 550,000 person-years of follow-up and found a more than 2-fold increase in the risk of MS after IM [422].

8.7 Oral Hairy Leukoplakia

The corrugated white lesions on the lateral borders of the tongue occur in states of significant immunosuppression, including advanced HIV infection [423, 424], organ transplantation or, occasionally, systemic glucocorticoid therapy for other conditions [425–427]. As it is a productive (lytic) EBV infection [428], it may be controlled with oral acyclovir therapy [429].

9 Prevention and Control

Whilst EBV infects most of the human population and coexists without causing disease in the majority, it is an oncogenic virus that is an established factor/cofactor in the aetiology of many human cancers and (more recently) has been associated with the development of MS. In addition, primary EBV infection is a life-threatening condition for those with genetic defects, such as XLP (see above), and for those with profound immunodeficiency following organ transplantation. Furthermore, IM remains an important cause of morbidity in older children and young adults, occasionally causing very severe disease – especially in older age groups. Importantly, economic development may result in a rising incidence of IM due to delays in the age of primary acquisition of the virus.

9.1 Immunisation

The traditional approach to immunisation, using whole killed or live-attenuated virus, has been avoided by researchers in view of the known oncogenic potential of EBV and the difficulties in producing large quantities of virus [430]. Two main types of recombinant vaccine have been considered for prophylactic use prior to the occurrence of any disease. These include the use of the subunit glycoprotein gp350, which is known to induce neutralising antibody, and a peptide (epitope) vaccine – so-called polytope when multiple peptides are used – designed to induce cytotoxic cellular immunity [431]. Due to HLA diversity, however, it is estimated that 25 $CD8^+$ T cell epitopes would be needed to provide coverage for over 90 % of the population in Western countries [432].

The use of gp350 as a subunit vaccine has long been considered and was previously studied in the cotton-top tamarin (*Saguinus oedipus*) primate model. Following intraperitoneal and intramuscular injection of EBV, these small Colombian (New World) non-human primates develop large oligoclonal lymphomas, which could be prevented by prior injections with gp350 presented in artificial liposomes. However, only the animals with very high levels of virus neutralising activity in their serum were protected [433].

After a delay of over 25 years, phase 1 and phase 2 studies were undertaken in Belgium with a gp350 subunit vaccine, using glycoprotein expressed in Chinese hamster ovary cells [434, 435]. Fortunately, there is little sequence variation in this gene between the two EBV types [436]. After 3 vaccine doses, all subjects had produced anti-gp350 antibodies; however, one serious adverse event (reactive arthritis) occurred when gp350 with the adjuvant AS04 (a lipid and aluminium hydroxide adjuvant)* was given to an already EBV seropositive recipient, and there was also a tendency towards a higher frequency of local symptoms postvaccination in those who were already EBV positive. The subsequent part of the study and phase 2 placebo-controlled study were undertaken in EBV seronegative subjects [435, 437]. In the randomised double-blind placebo-controlled trial, 181 seronegative young adults in Belgium received either three

doses of gp350 with AS04 or three doses of a placebo-containing aluminium hydroxide alone. Asymptomatic primary infection was not reduced by vaccination with gp350; however, the number of cases of IM was reduced in the gp350 group (2 cases *versus* 8 in the placebo group, which was significant by intention to treat analysis; [437]). Unfortunately, this trial did not investigate VL or CTL responses in either group.

To date, trials to prevent IM using a polyepitope vaccine have not been conducted. A very small phase 1 study using a single EBV peptide epitope from EBNA3a mixed with tetanus toxoid and formulated in a water in oil squalene adjuvant 'Montanide ISA 720' has been conducted in ten HLA B*0801-positive subjects and 4 controls. Half of the epitope recipients, and 2/4 (50 %) of the placebo recipient, seroconverted to EBV. One case of IM occurred in each group. Although the study was too small to estimate vaccine efficacy, the authors concluded that the vaccine was safe, produced epitope specific T cell responses in 8/9 (89 %) recipients and was generally well tolerated [432, 438]. The decision to incorporate tetanus toxoid to provide CD4+ve T cell help was questionable as this probably led to increased local adverse reactions to the vaccine and excluded 45 % of potential volunteers due to pre-existing high levels of tetanus toxoid antibodies. The authors suggested that gp350 could be incorporated with the peptide vaccine as an alternative source of CD4+ve T cell help, although to date no trials combining the vaccine approaches have been published.

More recently, an EBV virus-like particle has been produced, and its use as a vaccine been proposed; however, this work is at an early stage, and further modification to remove additional EBV proteins and possibly RNA may be desirable before any in vivo work is undertaken [439].

Taken together, gp350 seems the most promising candidate for further trials at the present time, although epitope-based approaches may find a role as adjuvant therapies in EBV-associated malignancies [440].

*AS04 (adjuvant system 04) = 3-O-desacyl-4′-monophosphoryl lipid A (derived from Salmonella lipopolysaccharide) with aluminium salt [441]

9.2 Prevention of Disability After IM

Fatigue after IM – see above.

9.3 Other Methods of Control

EBV is occasionally transmitted by blood transfusion and organ transplantation – see Sect. 6.5.

10 Unresolved Problems

Although 50 years have elapsed since the discovery of EBV, many aspects of epidemiology, biology, pathogenesis, treatment and prevention remain incompletely understood or researched.

Many of the epidemiology studies were undertaken in the 1960s and 1970s, and there have been few recent studies to explore the effects of industrialisation and development in poorer parts of the world and the effects of changing behaviours (e.g. increased child care outwith the home), changing sexual behaviours and reduced household and family size. The events during the window period between virus entry and symptoms are largely unknown, as are the reasons leading to symptomatic rather than asymptomatic seroconversion. The proportion of infections that are symptomatic in children is not well studied, and the explanation for mainly asymptomatic acquisition in young children is not resolved. Whilst the main route of transmission is thought to be via saliva, the contribution from genital transmission in adolescents and adults is not clear.

There is no recommended specific treatment for IM despite the severity of the illness and its occurrence at often time critical periods of education, examinations, and early working life. This may become an increasing (and costly) problem as EBV epidemiology changes and primary infection is delayed into adolescence and young adulthood. IM is often followed by prolonged fatigue, hypersomnolence and disruption to usual work and other patterns of activity, and further studies to reduce or prevent this disability are required [161, 353, 370].

Work to produce an EBV vaccine has been limited, with to date only a single phase 2 study to prevent IM with the leading candidate gp350. The effect of vaccination with gp350 on VL and lymphocyte responses in natural EBV infection is so far unassessed, and any negative outcomes of immunisation such as increased severe IM are unquantified, or the effects of potentially altering the CTL repertoire are unknown [430]. It is not known whether EBV-related conditions such as BL, HL and MS might be prevented by immunisation.

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Useful Websites

Public Health England. www.gov.uk/government/organisations/public-health-england
UK Clinical Virology Network. www.clinicalvirology.org
UK National Institute for Biological Standards & Control. www.nibsc.ac.uk
UK Standards for Microbiology Investigations. www.hpa.org.uk/SMI
US Centres for Disease Control & Prevention. www.cdc.gov

Kaposi's Sarcoma-Associated Herpesvirus: Epidemiology, Biological Characteristics and Pathogenesis

39

Ronit Sarid and Maria Luisa Calabrò

1 Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), formally designated the human herpesvirus 8 (HHV-8), was first identified in a Kaposi's sarcoma lesion from a patient with AIDS [78]. KSHV is now recognized as the causative agent of all clinical and epidemiological types of Kaposi's sarcoma (KS) [286]. Based on adequate evidence supporting its etiologic association with KS, KSHV has been recognized by the International Agency for Research on Cancer (IARC) as one of seven known human tumor viruses (group 1) [44, 356]. KSHV is also causally associated with primary effusion lymphoma (PEL) [73] and a subset of multicentric Castleman's disease (MCD) [377]. In addition, it is related to large B-cell lymphoma arising in KSHV-positive MCD and to a germinotropic lymphoproliferative disorder. KSHV inflammatory cytokine syndrome (KICS) has been recently described as an additional condition related to KSHV [398].

The tumorigenic potential of KSHV is illustrated by its ability to alter the growth properties of cells in culture and to induce KS-cell-like morphology upon infection of endothelial cells. Viral-encoded genes are expressed in all KSHV-related disorders. At the molecular level, several KSHV gene products can induce cell proliferation, inhibit cell death, and may also generate genomic instability.

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2 Historical Background

In 1872 the Hungarian dermatologist Moritz Kaposi described five elderly male patients who presented an "idiopathic multiple pigmented sarcoma of the skin," which was characterized by multifocal nodules, localized mostly on the lower limbs but also on the mucous linings of certain organs and on the liver [45]. This disorder, later termed Kaposi's sarcoma (KS), is now classified into four epidemiological variants on the basis of clinical characteristics (localization and clinical course) and particular risk factors (age, gender, extent of immune suppression, and ethnogeographical origin) (Table 39.1).

The classic KS has a mostly benign course and is slowly progressing and not life threatening; it affects apparently immunocompetent elderly individuals, mostly men of Mediterranean or Eastern European origin [203]. The endemic or African KS, first described in the 1950s, is prevalent in parts of Central and Eastern Africa and affects adults, but also can develop in children, frequently as a disseminated lymphadenopathy with no cutaneous involvement [214]. The iatrogenic, or posttransplant KS, develops in immunosuppressed patients, mostly after organ transplantation [369]. Finally, the epidemic or AIDS-associated KS was initially described in 1981 in young men who have sex with men (MSM) infected by the human immunodeficiency virus type 1 (HIV-1) and subsequently became an AIDS-defining pathology and one of the most commonly observed malignancies in AIDS patients [200]. This variant is highly aggressive and is characterized by multiple, disseminated lesions with gastrointestinal and lymph node involvement. In Western countries, AIDS-KS develops mostly among HIV-infected MSM, whereas in Africa it has a similar incidence rate among males and females. In certain sub-Saharan African countries, KS is one of the most common cancers and is associated with significant morbidity and mortality [314, 406]. All four epidemiological forms of KS share similar histologic characteristics, though AIDS-KS tends to be highly aggressive whereas classic KS is relatively indolent.

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Table 39.1 Kaposi's sarcoma variants

Variant	First evidence	Population at risk	Age range	Gender ratio (M:F)	Cutaneous	Lymph nodes	Visceral	Clinical course	Reference
Classic KS	1872	Mediterranean area and Middle East origin	>50	3:1	Symmetrical, mainly localized lesions in lower limbs	Rare	Rare	Frequently slowly progressing and not life-threatening	[143, 203]
		Indigenous (Quechuas) and mestizo populations from Colombia and Peru	22–81 (Colombia)	8:1 (Colombia) 3:1 (Peru)	Confluent non-symmetrical nodules or ulcerated lesions in upper and lower limbs, associated with edema in 20–50 % of patients	20 % of patients	Rare	Locally aggressive	[284]
Endemic/African KS	1950s	African adults	25–40	10:1 (before the AIDS epidemic) 2:1	Localized or disseminated lesions in lower limbs	Rare	Rare	Indolent to locally aggressive; frequently observed prior to the AIDS epidemic	[5, 263]
		African adults	20–35	2:1	Disseminated lesions	Frequent	Frequent	Aggressive, more frequent after the AIDS epidemic, likely reflecting HIV infection	[263, 434]
		Prepubescent African children	11–13	5:1 (Tanzania)	Disseminated lesions	Rare	Rare	Locally aggressive, more frequent after the AIDS epidemic, likely reflecting HIV infection	[5, 6, 433]
		Young African children (around 3-year-old children)	<15	3:1	Rare	Diffused lymphadenopathy	Frequent	Rapidly fatal course in both HIV-infected and uninfected children	[215, 309, 433]
Iatrogenic KS	1969	Patients under immunosuppressive treatments or with autoimmune disorders	All	3:1	Frequent	Frequent	Frequent	Variable, ranging from chronic to rapidly progressing; may regress after modification of the immunosuppressive treatment	[132, 369]
Epidemic/AIDS-associated KS	1981	HIV-1-infected individuals, mainly MSM in developed countries	All	20:1	Disseminated lesions	Frequent	Frequent	Rapidly progressing before the introduction of HIV therapy; may regress after antiretroviral therapy	[29]

Epidemiological studies conducted on subjects affected by epidemic KS suggested an infectious etiology for this disorder. Indeed, KS incidence was higher among subjects infected via sexual contact as compared with other transmission groups, such as hemophilia patients and injection drug users (IDUs) [30]. A number of pathogens were initially proposed as potential KS agents, including human papillomavirus 16, hepatitis B virus, Cytomegalovirus (CMV), human herpesvirus type 6, *Mycoplasma penetrans*, and several others, yet a causative role could not be confirmed for any of these candidates. Herpesvirus-like particles had already been observed in KS neoplastic cells in 1972 [160], but those virions were subsequently ascribed to CMV infection [161]. Herpesvirus-like particles were also detected in KS biopsies by other researchers [407].

In 1994 Chang and colleagues identified DNA sequences of a previously unrecognized herpesvirus in a lesion from a patient affected by AIDS-KS [78]. These sequences were identified by using Representational Difference Analysis (RDA), a powerful technique available at the time to detect unique sequences through a comparison between DNA from pathological tissue and DNA of normal tissue from the same patient [246]. Sequence analysis revealed close homology of the newly discovered DNA sequences with two herpesviruses, Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS), which belong to the *Gammaherpesvirinae* subfamily. These sequences were subsequently found in KS lesions from patients affected by all variants of KS, supporting the involvement of this putative novel herpesvirus in KS. These herpesvirus-like sequences were subsequently detected in tumor cells from patients with a rare form of non-Hodgkin's lymphoma. This lymphoma presents primarily as lymphomatous effusion in serous body cavities, and it was thus named body cavity-based lymphoma, BCBL, or primary effusion lymphoma, PEL [73]. In light of a recognized clinical association of KS with multicentric Castleman's disease (MCD), a rare polyclonal lymphoid disorder characterized by lymphoid hyperplasia, the presence of KSHV sequences in MCD samples was also investigated, providing evidence for a possible link with a subset of MCD cases [377].

Cell lines established from effusion of PEL patients were found to stably maintain the virus, thus permitting the cloning and sequencing of its entire genome [351] and visualization of the viral particles by electron microscopy [340]. Nucleotide sequence analyses of the entire genome confirmed its initial classification as a gammaherpesvirus belonging to the genus of rhadinoviruses or gamma-2-herpesviruses.

Initial serological studies performed using PEL-derived cell lines as source of viral antigens confirmed the presence of antibodies against viral antigens in all patients affected by KS, while infection was shown to precede KS onset [152, 153, 218, 370]. Several subsequent serosurveys showed that KSHV seroprevalence globally mirrors the distribution of classic and endemic KS in the general population and is high in HIV-1 transmission groups in which epidemic KS is frequent. Taken together, these initial findings strongly supported the notion that KSHV is etiologically linked to the development of KS.

3 Methods of Epidemiological Analysis

Soon after the discovery of KSHV, most studies aiming to understand the epidemiology of KSHV infection were based on the detection of viral DNA in biopsy specimens and in peripheral blood mononuclear cells (PBMCs) through polymerase chain reaction (PCR) and by nested PCR. The initial search for KSHV sequences identified their presence in all epidemiological variants of KS, although the detection rates did not reach 100 %. Rates of PCR positivity were largely dependent on the sample type. DNA could be detected easily in fresh biopsies by standard PCR, but not all archival paraffin-embedded samples were found to be positive, largely because of the fixation conditions. In PBMCs, KSHV DNA was detected mainly by nested PCR in only 5–20 % of infected subjects, and the proportion was higher in KS patients [36, 413]. A highly sensitive and specific assay for detection of KSHV infection was based on nested PCR using two nonoverlapping primer sets for the open reading frame *(orf)25* and *orf26* genomic regions [41, 56]. Initial PCR analyses detected KSHV infection more frequently in PBMCs from HIV-infected patients prior to KS onset as compared with control HIV-1-infected subjects who did not develop KS [289]. This technique remains, however, highly prone to cross-contamination as well as contamination from previous amplification products and requires all procedures to avoid false-positive results. Since viral load in the systemic compartment of infected individuals, with or without KSHV-related pathologies, may be low or undetectable even by sensitive PCR techniques, the prevalence of infection cannot be reliably determined by qualitative or quantitative PCR.

The development of serological assays to detect antibodies to KSHV enabled more extensive studies and estimation of KSHV prevalence in the general population. KSHV-specific antibodies were detected initially by using PEL-derived cell lines as source of viral antigens. One of the most useful tests relies on an indirect immunofluorescence assay (IFA), performed on latently infected PEL-derived cell lines. Using these cells, sera from KSHV-infected subjects react with a viral nuclear antigen resulting in a characteristic speckled pattern of staining for the viral latency-associated nuclear antigen 1 (LANA-1), encoded by *orf73* (Latent IFA) [152, 153, 218]. PEL cell lines can also be chemically induced to increase the fraction of lytically infected cells and thus serve to measure antibodies against lytic viral antigens. In this case, sera from KSHV-infected individuals react with

several viral antigens and produce both cytoplasmic and nuclear fluorescence (Lytic IFA) [240]. In addition, various enzyme-linked immunosorbent assay (ELISA)-based diagnostic tools have been developed, using recombinant viral proteins, peptides, or whole virus lysates. The major antigenic proteins are LANA-1 and structural proteins of the viral capsid (encoded by *orf65*) and envelope (encoded by *orfK8.1*) [370, 379].

Combined serological analyses, using in-house or commercially available serological assays, have been proposed to estimate the seroprevalence of KSHV in different populations [233]. Serological tests may be performed by detecting antibodies to latent (employing latent IFA or ELISA) and lytic antigens (employing lytic IFA or ELISA using *orf65*- or *orf K8.1*-derived recombinant proteins) [333, 370]. These analyses should be followed by confirmatory immunoblots with a panel of latent and lytic antigens [370, 430]. The overall seroprevalence can be estimated as reactivity to at least one of the two analyzed antigens [55, 370]. These and other combinations of serological techniques were proved to be suitable for epidemiological studies, although the interpretation of some published prevalence data remains challenging. In fact, these techniques show only moderate concordance between each other and among different laboratories [319, 334, 379].

A standardized and universally accepted algorithm for KSHV diagnostics is not presently available. KSHV diagnostics should combine serological and molecular techniques to ideally delineate a broad virological profile. As asymptomatic KSHV-infected subjects may have antibodies to only latent or lytic antigens, and with very low titers, serological tests should include detection of antibodies to at least one latent and one lytic antigens. Antibody titration may also provide additional information. Higher antibody titers were associated with increased risk and faster progression to KS in HIV-1/KSHV-coinfected subjects [343]. Anti-lytic antibody titers may also reflect tumor burden and evolution in AIDS-KS patients [58, 70]. Lower CD4+ and CD8+ T-lymphocyte counts were found to influence antibody response to KSHV latent antigens in AIDS-KS patients [105, 175].

Concerning viral load assessment, initial studies indicated that detectable cell-associated KSHV viremia was correlated to a tenfold increased risk of KS in HIV/KSHV-coinfected individuals [129]. In untreated AIDS-KS patients, systemic KSHV load generally directly correlates with HIV-1 RNA levels in plasma. Introduction of highly active antiretroviral therapy (HAART) was shown to decrease systemic HIV and KSHV loads in patients, resulting in complete remission and durable responses. However, increasing KSHV loads in KS patients with partial or complete response to therapy, undetectable KSHV load before and after HAART, or lack of detection of KSHV DNA in the peripheral blood during KS progression were also reported [59, 71, 392, 419]. Therefore, viral load may be quantified in blood compartments (plasma and PBMCs) to determine the systemic levels of cell-free and cell-associated virus, but particular caution must be paid to data interpretation in HIV-1-infected subjects under HAART and in HIV-negative KS patients [184, 392]. In addition to peripheral blood, KSHV load analysis can be also carried out using saliva. Oral samples represent a site at which KSHV can be detected more frequently and at higher loads than in blood samples [270, 318], although detection may be intermittent [232], and factors controlling oral KSHV shedding must still be fully elucidated. Nevertheless, measurement of KSHV in saliva represents an estimate of the transmission potential of the subject. Viral load analysis should be thus performed in parallel with the search for anti-KSHV antibodies, although first-generation serological tests may not be fully adequate for diagnosis. Introduction of novel antigenic viral proteins, such as KSHV complement control protein (KCP) and vCYC [50, 307] in serological assays may be useful to better monitor the humoral responses of KSHV-infected subjects. In addition, innovative multiplexing techniques that combine the simultaneous detection of antibodies toward different latent and lytic viral proteins may, in the future, bypass the drawbacks of separate determinations by increasing sensitivity and by standardizing data interpretation.

4 Biological Characteristics

4.1 The Virus

KSHV is a lymphotropic gamma-2 herpesvirus (rhadinovirus) and is the only member of this group that infects humans. KSHV shares close homology with several primate and non-primate mammalian viruses, having the closest homology with retroperitoneal fibromatosis herpesviruses (RFHV) and rhesus rhadinovirus (RRV) [110, 347, 363]. Among human herpesviruses, KSHV is most closely related to the Epstein-Barr virus (EBV), a ubiquitous lymphotropic virus which is associated causally with several human cancers, including nasopharyngeal carcinoma and certain types of lymphomas.

The overall size of the KSHV virion is 120–150 nm. Four structural proteins encoded by the *orf25*, *orf26*, *orf62*, and *orf65* encompass the icosahedral capsid shell of KSHV which has a diameter of ~130 nm. Three of these proteins share significant homology with capsid proteins in alpha and beta-herpesvirus subfamilies, whereas ORF65 differs from its structural counterparts in the other subfamilies [397]. Cryo-electron microscopy (Cryo-EM) and Cryo-EM tomography studies suggest that KSHV capsomers are hexamers and pentamers of the major capsid protein encoded by *orf25*, with the small capsid protein, encoded by *orf65*, binding around the tips of both hexons and pentons [108]. An amorphous, but highly organized, proteinaceous tegument, wrapped with a lipid bilayer envelope containing surface viral glycoproteins, surrounds the capsid. Several cellular

and viral proteins, including ORF6, ORF7, ORF11, ORF21, ORF33, ORF45, ORF50, ORF52, ORF63, ORF64, ORF75, and heat shock protein 70 and 90, are incorporated within the KSHV tegument [27, 432]. These proteins appear to facilitate virion trafficking to the nucleus, decrease innate antiviral immune responses, inhibit cell death, promote the establishment of latency, and support viral egress during the late phases of productive infection [362].

The KSHV genome is a double-stranded linear DNA having a similar organization to that of other rhadinoviruses. The genome contains a central 140.5-kbp long unique region (LUR) encoding 87 open reading frames (ORFs) [351]. ORFs are generally consecutively designated according to their relative location on the genome from left to right and were annotated according to the corresponding herpesvirus saimiri (HVS) genes with which KSHV shares a relatively high homology. The LUR contains clusters of genes that are conserved among all herpesviridae members, including genes encoding structural and replication proteins. Genes that are either limited to rhadinoviruses or are unique to KSHV lie between the conserved herpesvirus gene blocks. These genes are separately numbered and have a K prefix, while unique genes identified following the primary designation have decimal K numbers. KSHV is notable for molecular piracy and contains several genes with similarity to cellular genes, which were probably pirated from the host during evolution. These genes encode proteins that function as immune modulators, signal transducers, as well as transcriptional, cell cycle and cell fate controllers [206]. Generally, unlike their cellular homologs, the viral counterparts escape cellular regulatory pathways and hence have altered activity. Furthermore, the expression of these genes is regulated via viral promoters that do not necessarily operate in accordance with the cellular promoters of the homologous genes. The LUR also encodes genes for untranslated RNAs. These include the PAN/nut-1 transcript, a nuclear polyadenylated yet mostly untranslated mRNA, several microRNAs and long antisense transcripts [76]. Of note, a systematic genome-wide survey of viral transcriptional and translational activity by using mRNA-sequencing and ribosome profiling uncovered strategies to expand the virus coding repertoire including extensive alternative splicing, mRNA editing, and the use of alternative translation initiation codons as well as numerous upstream and small ORFs [13]. The LUR is flanked by a variable number of GC-rich (85.5 %) direct terminal repeat (TR) units of 801-bp length. Each TR unit contains the latent origin of replication and two binding sites for the KSHV latency-associated nuclear antigen 1 (LANA-1) [199]. The overall length of the KSHV genome is approximately 165-kbp, though this size is not fixed, predominantly due to length variations of the TR region.

KSHV has several distinct subtypes which are thought to have diverged at least 100,000 years ago and track human migration through Africa [191]. Based on variation in the leftmost *orfK1* (VIP) gene, the first subtypes to be identified were A, B, C, and D, followed by subtype E. Additional variations were identified at the right end of the genome, in *orf K15* (TMP). *K15* genes fall into two alternative allelic subtypes, referred to as prototype (P) and minor (M), which have diverged by 70 % at the amino acid level. Variability in *orfK15* exists primarily among subgroups A and C of *orfK1*. In addition, small variations have been identified in 10 internal genomic loci. It is believed that the principal *K1* subtypes arose during the migration of modern humans out of East Africa first into sub-Saharan Africa (variant B), then into south Asia, Australia, and the Pacific islands (variants D), and then through the Middle East into Europe and north Asia (variants A and C), with very little subsequent mixing. A later expansion introduced the virus to the Americas (variant E) and Northern Europe [437]. Accordingly, subtypes A and C predominate in Europe, the USA, China, Southern Siberia, and Australia, whereas subtype B predominates in Africa. Subtype D is rare and has been found in individuals of Polynesian and Australian aboriginal descent and in Japan and Oceania. Subtype E has been reported to be hyperendemic among Amerindian populations of the Brazilian and Ecuadorian Amazon regions. Several novel subtypes including subtype F, which was identified in an Ugandan Bantu tribe; subtype Z, which is present in Zambia; and subtype N which has been identified in South Africa have been recently described. Viral genome analysis can be applied to track the origin and modes of virus infection and has been used to establish inter- and intrafamilial transmission of KSHV and genotypic differences in virus isolated from different body compartments of a single patient. Yet, it is unclear whether certain genotypes are associated with increased rates of disease progression or different virulence [254, 265].

4.2 Life Cycle of KSHV

Humans are the only known natural hosts for KSHV. In vivo, KSHV has a relatively broad cell tropism as suggested by the detection of its DNA and transcripts in various cell types, including CD19+ peripheral blood B cells, circulating endothelial cells, KS spindle cells, CD45+/CD68+ monocytes, keratinocytes, and oropharynx and prostatic glandular epithelial cells [74].

Multiple envelope proteins participate in the binding and entry of KSHV into cells. Like many other viruses, the cell surface glycosaminoglycan, heparan sulfate, serves as an attachment mediator for KSHV and functions to promote the binding and concentration of virions. Interaction between the KSHV envelope glycoproteins gB, gpK8.1A, ORF4, gH, and gH-gL and heparan sulfate has been reported [3]. The major KSHV envelope protein is gB, encoded by *orf8*, which mediates viral binding and entry through a specific interaction with the integrins α3β1, αVβ3, and αVβ5. Interaction between the dimeric glycoprotein H (gH) and glycoprotein L (gL)

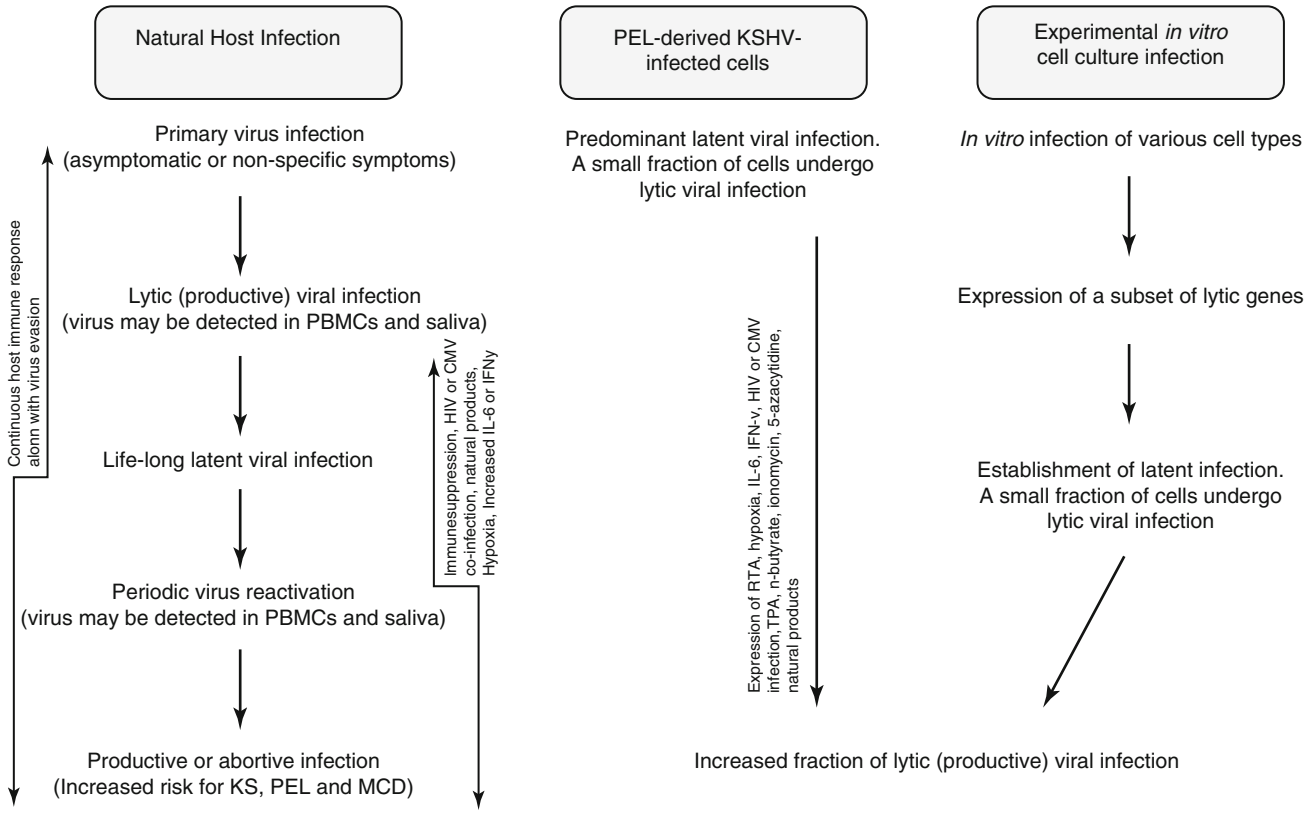


Fig. 39.1 Natural and in vitro KSHV infection. Primary host infection with KSHV involves a productive virus replication cycle, which precedes lifelong latent infection. Only a few viral proteins are expressed during KSHV latency, whereas extensive KSHV genome expression and viral DNA replication characterize the lytic phase of virus infection. Certain conditions, combined with reduced immune control, may lead to periodic virus reactivation, increasing the risk for disease onset. In PEL-derived cells and experimental cell culture infections, most cells undergo aberrant infection characterized by restricted expression of viral lytic proteins and virus latency in the majority of the infected cells. Spontaneous lytic virus reactivation in a small fraction of the cells may continuously take place while increased, but limited, virus reactivation is obtained following exposure to a variety of stimuli and treatment with chemical reagents

complex with the cellular ephrin receptor tyrosine kinase A2 (EphA2) also mediates entry and fusion of KSHV virions into different cell types including epithelial and endothelial [185]. The C-type lectin DC-SIGN/CD209 is another KSHV entry receptor used during infection of dendritic cells, macrophages, and activated B cells, yet its KSHV interacting glycoprotein(s) has not been defined [337]. Finally, the glutamate/cysteine exchange transporter protein xCT has been identified as a candidate entry receptor for KSHV [213]; however, the lack of evidence demonstrating a direct interaction between this protein and viral envelope glycoproteins suggests its involvement during other virus entry events. The interactions of KSHV glycoproteins with the cellular receptors initiate intracellular cascades that promote virus internalization by endocytosis or macropinocytosis, trafficking of viral capsids toward the nucleus, and later steps of infection.

Like all other members of the herpesvirus family, primary infection with KSHV results in the establishment of a lifelong infection. During lifetime infection, KSHV displays both latent and lytic (productive) phases, distinguished by their viral gene expression patterns (Fig. 39.1) [340, 357, 387, 428].

The latent phase is characterized by persistence of the viral genome as a highly ordered circular DNA (minichromosome/episome) which replicates synchronously with the host cell, expression of a limited subset of viral genes that ensure the maintenance of the viral genome while avoiding host recognition and promoting cell survival and proliferation, and lack of viral particle production. KSHV can establish latency in several cell types including CD19-positive B-lymphocytes, keratinocytes, and epithelial and endothelial cells. The host cell conditions and viral gene products required for avoiding lytic infection and initiating latency are currently unknown. Nevertheless, a limited number of viral lytic genes are transiently expressed prior to the establishment of latency and early after infection of endothelial and fibroblastic cell cultures, suggesting their ability to modify the intracellular environment to enable viral coexistence with the host cell during the initial time of infection and/or during the establishment of latency [223].

Extensive viral DNA replication and a well-controlled expression of a large array of viral genes characterize the lytic viral cycle, which may culminate in the assembly of

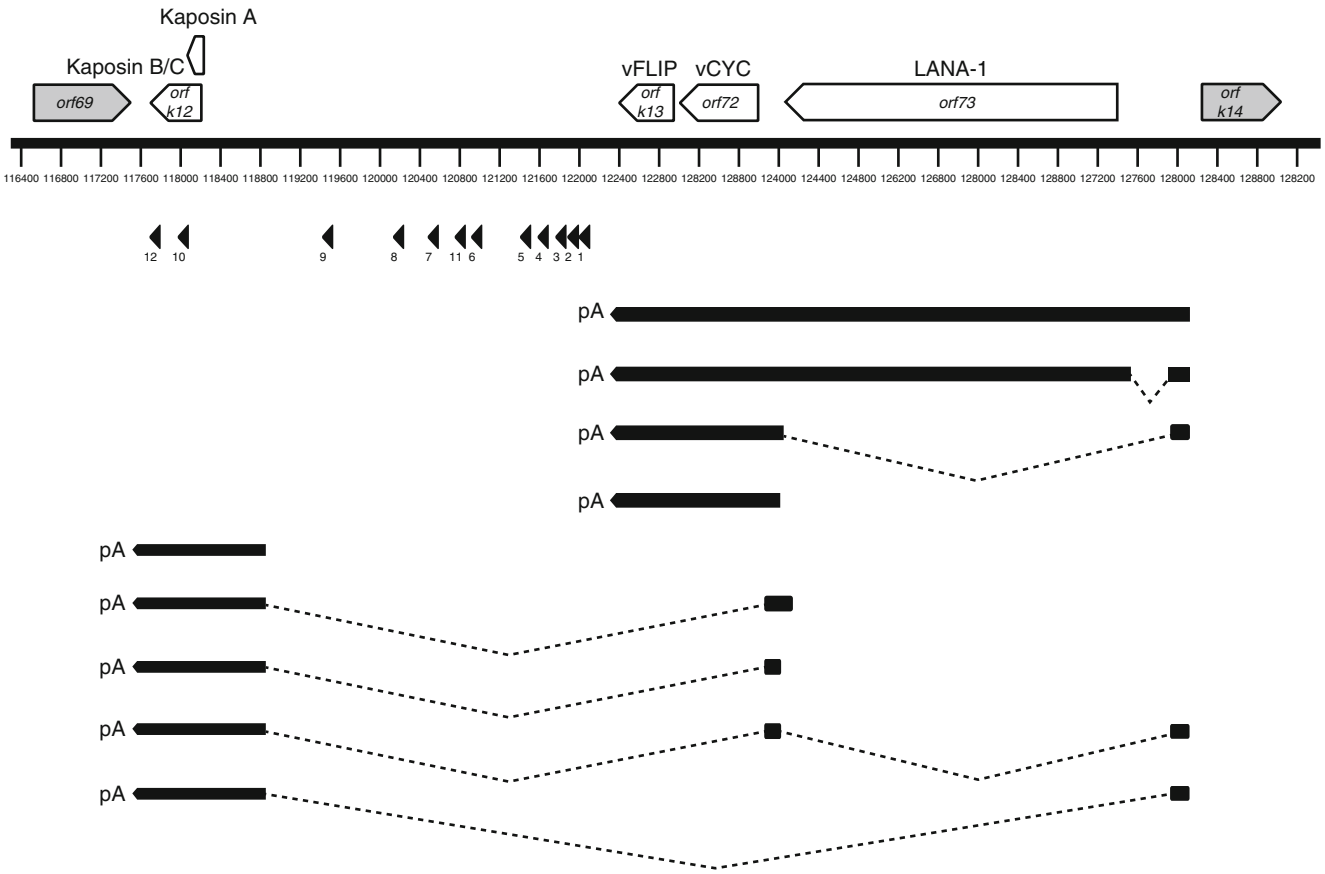


Fig. 39.2 Depiction of the major latent locus of the KSHV genome. The locus encodes four proteins, indicated by *open arrows*, as well as 12 pre-microRNAs (*black triangles*), and is flanked by lytic genes (*gray arrows*). Nucleotide positions are according to GenBank accession number AF148805.2. Latent transcripts are indicated by *black bars*, while introns are represented as *dashed lines* [53]

infectious mature virions that egress out of the cell while causing host cell death. A lytic cycle probably takes place during primary host infection, and it is crucial for virus dissemination within the host and between hosts. It is also likely to play an important role in preserving lifelong host infection and in the tumorigenesis induced by KSHV. Generally, the immediate-early lytic genes encode viral proteins that establish a permissive cellular environment and promote immune evasion; early genes encode proteins required for viral DNA replication and viral gene expression, whereas late genes include those encoding structural proteins necessary for assembly and maturation of the viral particles. Of note, certain viral genes can be expressed in a manner that does not follow the latent and lytic paradigm of the viral life cycle; hence, the expression of particular lytic viral genes can be inconsistent with their prescribed definition [77, 84, 300].

Latency is reversible, and certain conditions may periodically reactivate hidden latent virus to enter the lytic phase. Generally, reactivation occurs when the *orf50* viral gene promoter is activated, resulting in the expression of the replication and transcription activator (RTA) which acts as the principal switch for the viral lytic program [179]. Yet, an alternative apoptosis-initiated replication program, which produces virus with decreased infectivity and does not require RTA, may occur and have clinical significance [330]. Only some of the physiological factors involved in this switch are known, and the relationship between these factors is even less well understood.

4.3 Latent KSHV Genes

The major KSHV latent genes are clustered within a single genomic locus, located between *orfK12* and *orf73*, and are transcribed as polycistronic, differentially spliced, and alternatively polyadenylated mRNAs that are regulated through common promoters upstream to *orf73* and *orf72* [53, 114, 359]. These transcripts contain *orf73*, *orf72*, *orf71/K13*, and *Kaposin* and also serve as precursors for viral miRNAs. *orf K10.5*, encoding the viral interferon regulatory factor 3 (vIRF3), is exclusively transcribed in latently infected lymphoid cells to produce the latency-associated nuclear antigen 2 (LANA-2) [249, 346] (Fig. 39.2).

Below, we review some of the genes specifically expressed in the latent phase.

4.3.1 LANA-1

The major antigen expressed in all KSHV-infected cells is the latency-associated nuclear antigen 1 (LANA-1), encoded by *orf73* [336]. Upon infection, the expression of LANA-1 is promoted by the lytic immediate-early viral gene product, RTA, while LANA-1 subsequently represses the transcription of RTA and antagonizes its activity to promote the establishment and maintenance of latency [23, 229, 230, 400]. Thus, the tight control of LANA-1 and RTA expression and their mutual regulation appear to determine the fate of viral infection. LANA-1 is a multifunctional protein, which shares functional homology with EBNA1 encoded by EBV, and is required for the replication of the episomal viral DNA during cell division. It directly binds the latent origin of replication in the TR subunits and recruits multiple cellular proteins that act as components of the chromosomal replication machinery that also help in the efficient segregation of the viral episomes to daughter cells [22, 96, 199, 264, 313, 380]. In line, recombinant KSHV lacking LANA-1 fails to establish persistence and latency [421], while knockdown of LANA-1 reduces the copy number of viral episomes [164]. Similar to other oncoproteins encoded by DNA tumor viruses, LANA-1 interacts with various cellular and viral proteins and is capable of deregulating various cellular functions. LANA-1 binds and inactivates the retinoblastoma protein (pRB) [335], sequesters glycogen synthase kinase 3β (GSK-3β) in the nucleus and thereby stabilizes β-catenin [147], and inhibits transforming growth factor-β (TGFβ) signaling [111]. LANA-1 interferes with TP53-dependent transcriptional activation [145, 368] and forms a trimeric complex with TP53 and its E3 ubiquitin ligase HDM2 to promote cell survival [86, 355]. LANA-1 might impact angiogenesis, by targeting von Hippel Lindau (VHL) for degradation and by stabilizing hypoxia-inducible factor 1α [54]. LANA-1 alone does not transform cells in culture, but enhances cell activation events through the RAS/MAPK pathway and causes cells to become resistant to p16INK4-mediated cell cycle arrest [7, 335]. Furthermore, through interaction with the transcription factor Sp1, LANA-1 upregulates the transcription of telomerase reverse transcriptase (hTERT) and thus can extend the lifespan of cells [411]. When expressed in transgenic mice, LANA-1 causes increased B-cell hyperplasia and germinal center formation, as well as a B-cell lymphoma resembling MCD and plasmablastic lymphoma [133].

4.3.2 vCYC

vCYC is encoded by *orf72*, is expressed from a spliced latent transcript, and shares 54 % sequence similarity with cyclin D2 [53, 114, 359]. Like its cellular homolog, vCYC associates with specific cyclin-dependent kinases (CDKs), in particular CDK6, and phosphorylates pRB family members to overcome cell cycle arrest. Yet, the vCYC/CDK complex has more promiscuous substrate specificity, which is manifested by the phosphorylation of a broader range of cellular targets including histone H1, CDC6, Cdc25A, $p27^{KIP}$, $p21^{CIP}$, Bcl-2, human caldesmon (hCALD1), and nucleophosmin [79, 97, 163, 243, 306]. Unlike its cellular counterparts, vCYC/CDK imitates constitutively activated kinase complexes and is resistant to inhibition by the CDK inhibitors p16INK4, $p21^{CIP1}$, and $p27^{KIP1}$; likewise, it does not require CDK-activating kinase (CAK) for activation [212, 388]. In vitro expression of vCYC initiates S-phase but also results in cytokinesis defects and generation of polyploid cells that survive as an aneuploid population only in the absence of TP53 [401]. This phenomenon is probably related to the ability of vCYC to interact with nucleophosmin, which leads to the decreased formation of TP53-MDM2 complexes, and promotes TP53 accumulation [226]. In line with these findings, inactivation of the TP53 pathway has been shown to be strictly required for lymphomagenesis in transgenic mice expressing vCYC constitutively in the lymphoid compartment [402].

4.3.3 vFLIP

orf71/K13 is translated from an internal ribosome entry site located within the vCYC coding region and encodes vFLIP [174, 248], a homolog of the cellular FLICE (caspase 8)-inhibitory protein (FLIP) [394]. It blocks apoptotic pathways and protects cells from CD95/FAS-mediated apoptosis by inhibiting activation of caspase 3, caspase 8, and caspase 9 [116]. vFLIP also suppresses autophagy by preventing ATG3 from binding and processing LC3 [236]. Through interaction with TRAF2 and the inhibitor of κB kinase-γ (IKKγ), vFLIP constitutively activates nuclear factor-κB (NF-κB) signaling [137]. This in turn induces the expression of an array of cellular cytokines, chemokines, and antiapoptotic proteins implicated in the pathogenesis of KSHV. NF-κB prevents lytic reactivation, and hence, vFLIP may play a role in maintaining latency [102]. Furthermore, NF-κB activation by vFLIP appears to be responsible for the formation of spindle cells upon infection of endothelial cells [171]. vFLIP has been recently found to upregulate the host hsa-miR-146a which results in downregulation of CXCR4. Thus, it was suggested that vFLIP may promote premature release of KSHV-infected endothelial progenitors into the circulation [331]. vFLIP transgenic mice demonstrate an increased incidence of lymphoma, which develops after a long period of latency, while a reduced latent period is evident in mice co-expressing vFLIP and Myc [2]. Knockdown of vFLIP or inhibition of the NF-κB signaling in PEL cells induces apoptosis, further supporting the importance of this protein for lymphomagenesis [176].

4.3.4 Kaposin

Kaposin transcripts are abundantly expressed throughout all stages of KSHV infection. Increased levels of the Kaposin transcripts are evident during lytic virus reactivation as a result of the activation of its strong promoter [81]. A complex translational program involving initiation at non-AUG codons generates three protein isoforms of Kaposin – A, B, and C [352]. miRNA-K10, one of several microRNAs produced by KSHV, is derived from the middle of Kaposin A. Kaposin A has transforming potential in rodent fibroblasts and in athymic nude mice; however, this activity could be due to either its protein product, miRNA-K10, or both [294, 395]. Kaposin B activates the p38/MK2 signaling and blocks the degradation of particular short-lived transcripts that contain AU-rich elements (AREs) in their 3′-untranslated region [274]. As several cytokine mRNAs contain AREs, Kaposin B expression enhances production of proinflammatory cytokines creating a cellular environment more compatible with transformation. Stabilization of the mRNA encoding PROX1, a master regulator of lymphocytic endothelial cell (LEC) differentiation, is also modulated by Kaposin B [423]. Of note, editing of the Kaposin transcripts by the host RNA adenosine deaminase 1 controls certain functions of its products [150].

4.3.5 miRNAs

KSHV encodes 12 pre-miRNAs that produce 25 mature miRNAs through the cellular pathway of miRNA biogenesis [244, 324]. These miRNA precursors are clustered within the latency-associated locus in a uniform transcriptional orientation. Thus, all viral pre-miRNAs, produced through the usage of alternative polyadenylation signals, share common promoters with the above latent transcripts and hence are transcribed during latency [53]. These transcripts are also detected during lytic infection, and the expression levels of those controlled by the Kaposin promoter increase upon induction of lytic virus replication. RNA editing of pre-miR-K12-10 further increases the range of mRNA targets of this pre-miRNA. One of these miRNAs, miRNA-K12-11, is an ortholog of the human hsa-miRNA-155 which has the same seed sequence and is involved in immunity, hematopoiesis, and oncogenesis [167, 373]. Since miRNA-155 is known to function during late stages of B-cell differentiation, miRNA-K12-11 could contribute to the plasmablastic phenotype of PEL cells or may protect these cells from death. KSHV miRNAs are thought to be involved in various functions including control of lytic reactivation and viral latency maintenance, immune regulation, cell differentiation, and survival. The exact function of each of the viral miRNAs is just beginning to be revealed. Confirmed cellular targets include the NF-κB inhibitor, IκBα [238]; the stress-induced natural killer (NK) cell ligand, MICB [297]; the antiangiogenic molecule, Thrombospondin 1 [353]; and the cellular transcription factor, MAF, which promotes reprogramming of endothelial cells [188]. Furthermore, circulating viral miRNAs and intercellular transport of miRNAs via exocytosis has been demonstrated, suggesting possible modulation of the microenvironment through the transport of KSHV-encoded miRNAs to uninfected cells [88, 275].

4.3.6 LANA-2

LANA-2, also termed vIRF-3, is a viral homolog of interferon regulatory factors and is a latent protein that is constitutively expressed in PEL cells and KSHV-infected MCD cells, but not in KS lesions [249, 316, 346]. Experiments involving ectopic expression of LANA-2 revealed complex regulation of the interferon response by LANA-2. It activates the interferon promoter through its interaction with the cellular IRF-3 and IRF-7 proteins, but on the other hand may suppress interferon-mediated induction [249, 250]. Yet, LANA-2 inactivates TP53 and reduces the activation of the interferon-stimulated gene product, protein kinase R (PKR); thus, LANA-2 may function to evade the cellular antiviral response [131, 346]. Expression of LANA-2 appears to be required for the survival of PEL cells, as knockdown of LANA-2 in these cells results in reduced cell proliferation and increased caspase 3/7 activity [417].

4.4 KSHV Cultivation

One of the barriers to the study of KSHV has been the lack of an in vitro system that supports lytic virus replication and produces high viral titers. Cultures from KS lesions lose KSHV DNA and can only be maintained for a limited number of passages [28, 100, 299]. Therefore, they are of almost no use in the study of KSHV [385]. Several cell lines, retaining stable multiple copies of viral genome per cell, have been established from patients with PEL. Under standard growth conditions, PEL cells are predominantly latently infected by KSHV, while only a small, but steady, fraction expresses lytic viral proteins and produces new viral particles. An increased, but limited, lytic virus replication can be induced in these cells by various stimuli, including Rta overexpression [26, 300, 412], coinfection with another virus [399, 405], hypoxic conditions [101], interleukin 6 (IL-6) [80, 84, 375], gamma interferon [40, 80, 277], and chemical agents, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) [288, 340], n-butyrate [280], ionomycin [436], 5-azacytidine [85], H_2O_2 [422], certain plant extracts [416], and cytotoxic chemotherapeutic agents [330]. The mechanisms and signal-transduction pathways that are activated by these manipulations are not fully understood; nevertheless, it appears that most involve the activation of the lytic replication and

transcription activator (RTA) which serves as an important trigger of the lytic phase [179]. By using PEL cells, KSHV genes have been classified as latent or lytic, and mechanisms that control the switch to lytic replication were initially revealed. These cells, however, do not support serial transfer of infection and therefore could not help elucidate mechanisms involving primary infection and establishment of latency. In addition, since PEL cells are of lymphoid origin, they cannot be used to study the consequence of viral infection on endothelial cells representing the predominant cells comprising KS lesions.

An in vitro model that allows transmission of KSHV in cell culture is valuable, since it would permit investigation of the early events of infection and the use of mutant recombinant viruses in which individual KSHV genes have been disrupted. Furthermore, such a system would allow the application of recombinant viruses containing marker genes, such as the green fluorescent protein (GFP), which can be used to track the infectious process. In vitro, KSHV has been shown to infect various human cells including primary endothelial cells of various differentiation states and lineages (vascular, lymphatic, endothelial, precursor cells), epithelial cells, fibroblasts, keratinocytes, monocytes, dendritic cells, and B cells. KSHV also infects monkey and baby hamster kidney cells, and mouse fibroblast cells [26], yet almost no virions can be generated in mouse cells [18]. These infections are nonproductive and in most cases, in a manner similar to PEL cells, result in the establishment of a latent infection that can be partially shifted into a lytic one. Infected endothelial cells develop an elongated spindle shape resembling that of KS spindle cells and acquire features of a transformed phenotype [89]. Infection of primary endothelial cells with KSHV induces the formation of spindle cells that express markers of the lymphatic endothelium. Interestingly, transcriptome analysis revealed that infection shifts the gene expression profile of vascular endothelial cells toward lymphatic endothelial cells (LEC) [63, 197, 410], suggesting that infection of blood or circulating endothelial cells by KSHV could drive them to differentiate into lymphatic endothelium. Yet, KSHV-infected primary endothelial cells cannot be maintained in long-term culture [89, 227]. By using LEC spheroids, it was shown that the viral proteins vFLIP and vGPCR cooperate in the activation of Notch pathway, leading to KSHV transcriptional reprogramming of LEC to mesenchymal cells via an endothelial-to-mesenchymal (EndMT) transition. Therefore, KSHV infection of LEC induces EndMT, leading to an increased invasiveness and survival of infected endothelial cells [83, 155]. Unlike infection with EBV, infection of primary B cells by KSHV does not produce immortalized cells in culture.

Several recombinant KSHV genomes have been generated and serve as important tools to investigate various aspects of this virus. rKSHV.219 has been isolated from the JSC-1 PEL cell line and expresses red fluorescent protein (RFP) from the strong KSHV early lytic promoter PAN, green fluorescent protein (GFP) from the EF-1α promoter, and a puromycin selection marker from the RSV promoter [403]. This recombinant virus allows detection of infected cells and discrimination between lytic and latently infected cells that can be accurately quantified by fluorescence microscopy or FACS analysis. The bacterial artificial chromosome (BAC) system serves as an alternative powerful tool for obtaining viral mutants that can be applied for examining the function of viral genes in the context of the full-length genome. BAC sequences, containing antibiotic resistance markers as well as a GFP marker, have been inserted into the entire KSHV genome without disruption of any known gene and thus allow the application of this powerful system for the study of KSHV [49, 429].

4.5 Animal Models

Host-virus interactions and virus pathogenesis are preferably investigated by using animal models that follow natural infection, including virus spread within the host, establishment of long-term infection, development of an immune response, and onset of disease. Given the close phylogenetic relationship between KSHV and RRV, experimental infections with RRV can serve as useful surrogate model for KSHV infection [115]. Indeed, coinfection of macaques with simian immunodeficiency virus and RRV produces in certain animals hypergammaglobulinemia, extranodal B-cell lymphoma, and proliferative mesenchymal disease, referred to as retroperitoneal or subcutaneous fibromatosis [311, 420]. Attempts to transmit KSHV into rhesus macaques failed to induce seroconversion or pathological alterations, suggesting that this primate host is unlikely to provide a useful model [341]. Persistent infection of KSHV has been established following experimental inoculation of common marmosets, a New World primate. Sustained serological responses, latent infection of PBMCs, and infrequent development of lesions with histopathological features similar to KS characterized this infection, which has been suggested to serve as an important animal model of KSHV infection [82].

Several tumor graft models of PEL and KS have been established. For example, injection of PEL cells to SCID/NOD mice leads to the development of ascites, diffuse infiltration of organs, and subcutaneous tumor formation that varies according to the injection route and PEL cells used [42, 326]. Injection of KSHV into normal human skin engrafted on SCID mice induces KS-like lesion formation containing spindle-shaped cells latently infected with KSHV coupled with substantial induction of angiogenesis [140]. Similarly, KSHV-infected normal mouse bone marrow endothelial cells form angiogenic KS-like tumors that express KS

signature genes in nude mice, with no evidence of productive KSHV replication. The lesions produced in this murine model require the presence of the viral genome [295]. Nevertheless, results obtained with graft models have limited application to the human disease, and spreading of KSHV infection to murine tissues has not been observed [42, 326]. However, intravenous injection of NOD/SCID mice with purified KSHV results in latent and lytic viral gene expression in murine spleens and long-term infection of specific leukocyte populations [317]. Taken together, current experimental systems employing murine models enable the study of immune responses and evaluation of potential therapies. Yet, most models do not take into account the dynamics of host-virus interactions that occur during natural infection and disease onset.

4.6 Transforming Capacity

The potential transforming capacity of KSHV has been shown in several systems. KSHV infection of endothelial cells leads to reduced growth factor dependence and to morphological alterations, higher survival, loss of contact inhibition, and growth factor and anchorage independence [89, 139, 290, 408]. Furthermore, transfection of a KSHV genome into murine endothelial progenitor cells generates KS-like lesions when transplanted into mice [295]. KSHV is required for continued survival of PEL cells in culture, and knockdown of several viral gene products, in particular that of vFLIP, LANA-1, and LANA-2, induces apoptotic death [164, 177, 417], supporting the requirement of the expression of viral genes for the survival of these cells.

Individual KSHV proteins exhibit transforming capacity in experimental systems and in transgenic mice. These include ORFK1/VIP, ORF74/vGPCR, ORFK9/vIRF-1, ORFK12/Kaposin A, and ORF73/LANA-1, which have been reported to have transforming capacity in classical transformation assays. Others, including ORF72/vCYC, ORF73/LANA-1, and KbZIP, have been shown to alter cell cycle control, while ORF71/vFLIP, ORFK2/vIL-6, ORF K7/IAP, and ORFK10.5/vIRF-3 were shown to increase cell survival. Other proteins that could promote cell transformation include the cellular homologs vBcl-2, vIRFs, and vCCLs. The expression of individual viral genes in transgenic animals provides information about the function of the viral proteins. However, these experiments involve expression of the gene out of the natural framework of the global viral effects; hence, these data must be carefully interpreted.

Based on the notion that productive virus infection culminates in cell death, most efforts to identify KSHV genes responsible for its tumorigenic potential focused primarily on the few viral genes that are expressed during latent infection. However, the KSHV latency program is not sufficient to initiate tumorigenesis, and many viral proteins that are expressed during the lytic phase harbor an oncogenic potential. Findings suggesting that oncogenesis is not directly linked to latency introduced the new concept in virus oncology of "paracrine tumorigenesis," in which viral latent and lytic proteins act in concert to promote the onset and the progression of the tumor. In fact, most KSHV-infected cells present in KS lesions and PEL tumors are latently infected, yet some of the cells express genes associated with lytic infection. These cells may release viruses to sustain infection but may also produce paracrine signaling molecules that modulate inflammatory and angiogenic processes within lesions allowing aberrant cell division and immune evasion.

Among the lytic genes, multiple lines of evidence were provided suggesting a role of the viral G protein-coupled receptor (vGPCR) in KSHV-induced transformation. Expression of vGPCR generates constitutive signaling and enhances proliferation [15, 20]. vGPCR can activate various key intracellular molecules, including mitogen-activated protein kinase, p38, c-Jun, and Akt, which in turn may control the expression and activity of numerous growth-promoting proteins. Because vGPCR induces the secretion of angiogenic growth factors, it was suggested to play a role both in direct autocrine and indirect paracrine maintenance of the transformed state. In line with the paracrine model, vGPCR generates KS-like lesions in transgenic mice, even when only expressed in a proportion of cells [196, 285]. Furthermore, xenografts of KSHV-transfected murine endothelial cell lines were found to require the signaling activities of vGPCR for tumorigenicity [169, 295].

The viral IL-6 homolog (vIL-6) is another cytokine that could act through a paracrine/autocrine mechanism. vIL-6 is expressed in vivo in a subpopulation of PEL cells and in a large number of KSHV-infected B cells in MCD lymphoid follicles [287, 316]. It induces proliferation, angiogenesis, and hematopoiesis and serves as an autocrine factor in PEL cell lines [12, 195]. Blocking the interaction of vIL-6 with the IL-6 receptor complex inhibits proliferation of PEL cells [222]. It also induces vascular endothelial growth factor (VEGF), which has been implicated in the pathogenesis of PEL and KS [11].

5 Descriptive Epidemiology

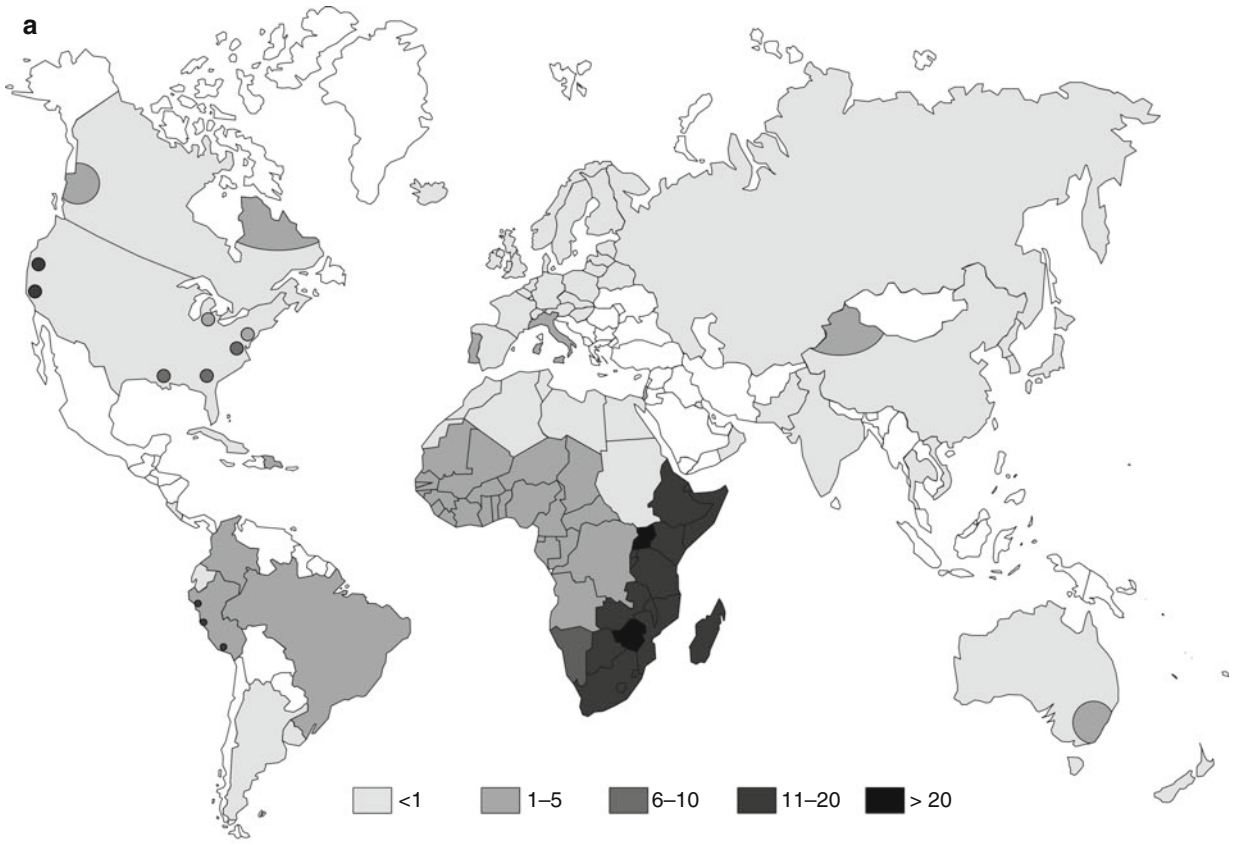
Before the AIDS epidemic, KS was viewed as a tumor affecting elderly individuals, predominantly men, of Mediterranean or Eastern European origin. This was based on crude rates from Israel [231] and population-based incidence data from the USA [202, 349]. A pronounced gradient of classic KS incidence from northern to southern Europe was reported [143, 172]. Similar variations were observed in Scandinavian

countries, with the lowest incidence rates in Denmark and the highest in Sweden; Italy exhibits similar gradients, with an increasing incidence from north to south and higher rates in towns along the Po Valley [157, 192]. In sub-Saharan Africa, incidence rates of endemic KS showed great variability between different areas and different native populations prior to the AIDS epidemic [305]. Immune suppression is associated with higher rates of disease, as 500- to 1,000-fold higher incidence rates of KS have been recorded in immunosuppressed renal transplant patients versus those seen in control populations [189, 258]. With the advent of the AIDS epidemic, KS begun to appear in young MSM; it was found to be 20,000 times more common in HIV-1-infected individuals than in the general population and 300 times more frequent than in other immunosuppressed groups, such as renal transplant recipients [30]. KS incidence was found to be higher among HIV-1-infected young homosexual or bisexual men than among other HIV-1 transmission groups, suggesting an infectious etiology of the tumor, and that the putative infectious agent was transmitted through sexual contact [30]. In several African countries, KS incidence in children and adults, mainly women, increased concomitantly with HIV-1 spread, and KS was found to represent up to 40 % of all adult malignancies and 10 % of all childhood cancers in certain regions of sub-Saharan Africa [406]. In this region, 22,000 KS cases in males and 12,000 cases in females were diagnosed in 2008, and the corresponding estimated age-standardized incidence rates were 8.1 and 3.6 per 100,000, respectively. The majority of these cases were observed in countries of Eastern Africa, including Ethiopia, Rwanda, Uganda, Zambia, and Zimbabwe [207]. The main epidemiological and clinical features of the four KS variants (classic, endemic, iatrogenic, and epidemic) are summarized in Table 39.1, while Fig. 39.3 illustrates worldwide incidence rates of KS and seroprevalence rates of KSHV.

5.1 Classic Kaposi's Sarcoma

Classic KS is a rare disease diagnosed primarily in elderly men (>50 years, with a 3:1 male:female gender ratio) of Mediterranean/Southern European, with relatively high incidence rates in Italy, Greece, Turkey, and Israel [143, 203]. Incidence rates are 10 times higher in these countries than in other European countries and in the USA [33, 172]. The disease is typically characterized by single or multiple pigmented skin lesions that primarily appear in the lower limbs and may spread to the arms, face, and torso over a period of years or decades. This disorder is mostly indolent, not life threatening, and only very rarely involves visceral mucosa. In Mediterranean countries KSHV seroprevalence increases with age, but KS remains a rare disease. For instance, in Italy only 0.03 and 0.01–0.02 % of KSHV-infected men and women above age 50, respectively, develop KS annually, indicating that cofactors influence the natural history of infection and contribute to tumor onset. Data on incidence rates over age 50 prior to the AIDS epidemic indicated that KS was more common in Italy (1.05/100,000 men, 0.27/100,000 women), especially in Southern Italy (Sicily and Sardinia) (3/100,000 men, 0.54/100,00 women), than in England and Wales (0.14/100,000 men and women), the USA (0.34/100,000 men, 0.08/100,000 for women), and Sweden (0.4/100,000 men, 0.31/100,000 women) [157, 172, 349]. The incidence rates of classic KS in two southern regions of Italy and in Israel are the highest registered in developed countries (2.07/100,000 men, 0.75/100,000 women) [181]. A higher risk of classic KS was reported for Israeli Jewish residents born in Iraq and North African and Eastern European countries as compared with Jewish immigrants from Western European countries and the USA and compared with the Israeli born population [181, 201]. Indeed, the Maghreb region of Northwest Africa, including

Fig. 39.3 Global incidence of Kaposi's sarcoma (KS) and seroprevalence of KSHV infection. (**a**) Age-standardized incidence rates (ASR) of KS (in males per 100,000) are reported. Incidences in Africa were taken from the Globocan database 2008 and [207]. The majority of KS cases diagnosed in 2008 occurred in the countries of Eastern Africa (including Uganda, Zambia, and Zimbabwe) with a corresponding ASR of 14.9. The countries of Southern Africa (including Botswana, Namibia, and South Africa) had the highest rates (11.5 in males per 100,000) after Eastern Africa. In sub-Saharan Africa, ASR was 8.1, followed by Middle Africa (4.1) and Western Africa (1.9). ASR were obtained also from the International Agency for Research on Cancer (Cancer Incidence in Five Continents). In the USA, the highest incidences were measured among Hispanics (6.6–13), non-Hispanic Caucasians (8.4–16.7), and African-Americans (8–14.5) in Los Angeles and San Francisco Bay areas (1993–1997). Slightly lower rates were observed in Georgia, New Orleans, New York, and Washington, whereas incidence rates were generally low in other US areas, mainly mirroring classic KS incidence in the general population. Classic KS was found to be frequent also in Israel (2.8), in Southern Italy (3), in the Xinjiang region of China, in Colombia, in the coastal region of Peru (>20), and in New South Wales in Australia (2.5). Low rates were reported all over Canada, except for British Columbia (2) and Quebec (2.1) (years 1993–1997). (**b**) Seroprevalence rates of KSHV infection among the general population, mainly blood donors, are reported as the percentage of seropositive subjects. KSHV infection is highly prevalent among Amerindians living in the Amazon regions, in countries of the Eastern Africa, in Peru, and among some Melanesian populations. KSHV seroprevalence is intermediate in the Mediterranean area, in some Middle Eastern countries, in other parts of South America, and in some ethnic groups and regions of China, with the highest rates reported in the Xinjiang region. KSHV is less prevalent in Northern Europe, North America, and most of Asia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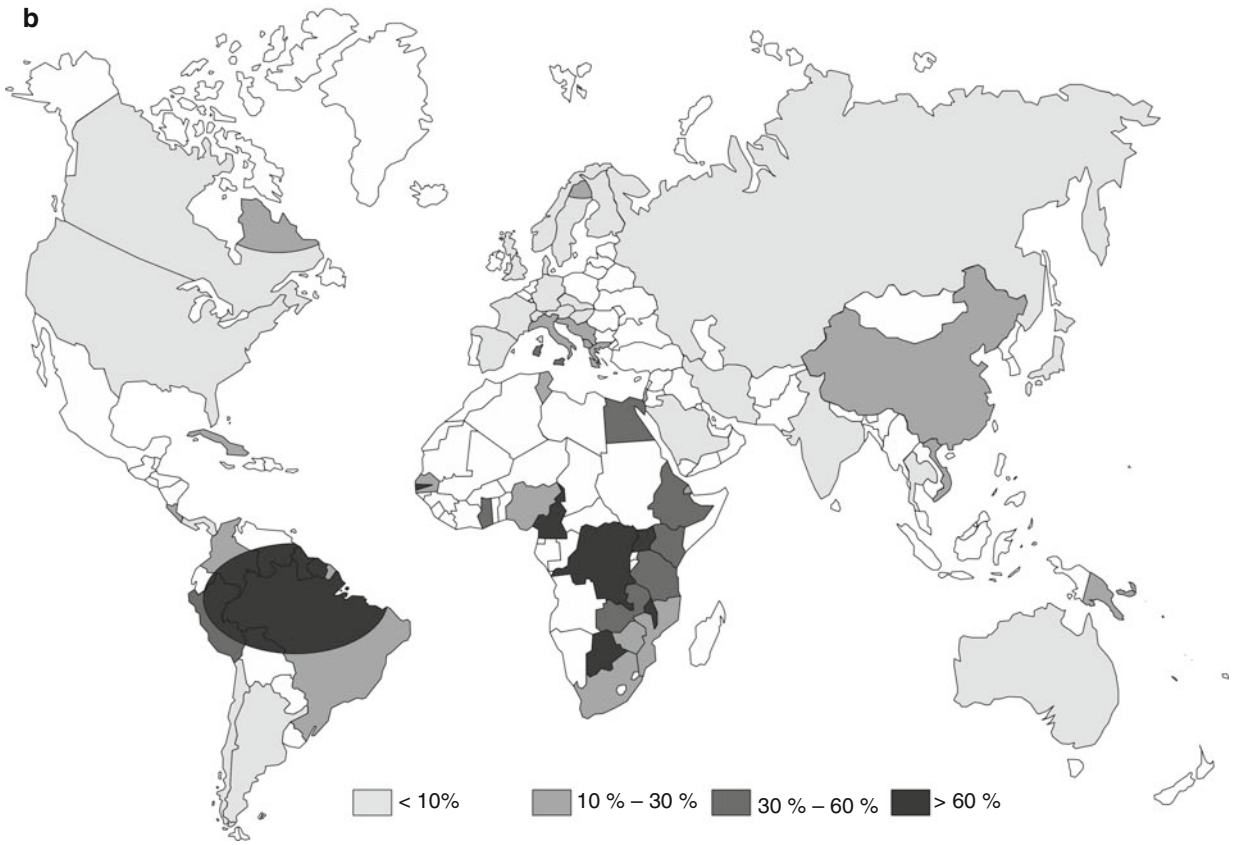
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Morocco, Tunisia, and Algeria, is a geographical area where KS is frequent [291].

Classic KS is a prevalent pathology also in South America, and three large studies were done in Argentina, Colombia, and Peru. Reports from Colombia and Peru observed KS cases mainly in indigenous populations (Quechuas) and in mestizos, while cases from Argentina presented more frequently in European descendents. An overall incidence of 2.54/10,000 attended patients (1946–2004) was reported in a study conducted in Peru, and some clustering was observed in the coastal region [283, 284]. An interesting clinical feature observed among certain classic KS cases from Colombia and Peru is an unusual presentation with confluent exophytic, nonsymmetrical nodules or eroded or ulcerated lesions. Low socioeconomic status and delayed consultation very likely represent the main cause of the numerous and extensive lesions in these patients. A few cases of classic KS were also reported in the Inuit population of Northern Quebec [21, 348], suggesting that KSHV may circulate in this population.

5.2 Endemic Kaposi's Sarcoma

Endemic or African KS is highly prevalent in sub-Saharan Africa. This form affects mainly young adults (median age 35 years, 2:1 male to female gender ratio) and children (median age 3 years), in contrast to the other three epidemiological forms [406]. Endemic KS has complex features and wide heterogeneity in terms of clinical presentation and affected population. At least four sub-variants have been described; one has a relatively indolent clinical course, with localized lesions similar to that of classic KS, but presenting at a younger age. The other three forms are more aggressive and quite similar to epidemic KS in their clinical progression; one has a prevalent disseminated cutaneous localization and may also affect prepubescent children, the other is characterized by extended mucocutaneous and visceral involvement, while the last one presents mainly in children with a median age of 3 years and shows a rapid diffusion to lymph nodes and internal organs, frequently without cutaneous involvement. The onset of AIDS led to an increase in prevalence rates of KS in both adults and pediatric populations in African countries where KS was endemic and complicated the study of the epidemiological and clinical features of endemic KS. However, the wide variety of clinical presentations in African KS was also present prior to the spread of HIV-1 [263]. Localized nodular KS was the most common presentation before the AIDS epidemic, in addition to a locally invasive form, which had a worse prognosis. KS with a disseminated mucocutaneous involvement, frequently associated with lymphadenopathy, and/or visceral diffusion was rare before AIDS, though it is very common at present. Visceral KS is highly aggressive and consistently fatal whether it occurs in association with HIV-1 infection or not. The lymphadenopathic type was the most common presentation in children, but rare in adults. Starting from the AIDS epidemic, this presentation became common in young adults, mainly women, but continues to have a rapidly fatal course in children whether associated with HIV infection or not.

During the AIDS epidemic, many of the epidemiological features of African KS changed. KS presented in adulthood at a younger age (30–39 years of age in men, 20–29 years of age in women), and the rising incidence of KS in women led to a decline of the gender ratio from 10:1 in the 1970s to 2:1 in the mid-1990s. Women from South Africa experienced an increase in KS incidence, changing the gender ratio from 7:1 in 1998 to 2:1 in 1996 [372, 433]. In Uganda, prior to the spread of HIV-1 (1954–1970), KS represented 6.5 % of all male cancers and no KS cases in women were registered. After the beginning of the AIDS epidemic (period analyzed: 1989–1991), the prevalence of KS among all cancer patients was 48.6 % in men and 17.9 % in women, and the incidence increased from 2.6 to 30.1/100,000 in men and from 0 to 11/100,000 in women [406]. In parallel with HIV-1 spread, the incidence of pediatric KS rose up to 40 % in Uganda, and an increase in oral and facial-cutaneous KS was reported among prepubescent children in central African countries. In Zambia, analysis of the clinical course of KS before puberty (age 3–15 years) revealed a more frequent primary lymphadenopathy in younger children [215]. In African countries where AIDS did not dramatically increase KS incidence, clinical features of this tumor were nevertheless profoundly changed. In pediatric populations of Tanzania, KS incidence rates and gender ratio (about 5:1) did not vary, but the disease acquired a more aggressive clinical course and a change in the anatomical distribution of KS lesions, reflecting an association with HIV-1 infection [6].

5.3 Iatrogenic Kaposi's Sarcoma

Iatrogenic or posttransplant KS occurs in patients undergoing immunosuppressive therapy mainly after organ transplantation but also in association with other pathological conditions and autoimmune disorders. This variant presents two peculiar features. First, the course of these KS lesions can be modulated, as documented by KS regression after withdrawal, reduction, or replacement of immunosuppressive agents [322], thus highlighting the significance of immunosuppression as a risk factor for KS development. Second, the presentation and clinical course is variable. Patients may show localized or disseminated skin lesions, and visceral involvement may be frequent in the more

aggressive cases, with a clinical course ranging from a chronic to a rapidly progressing disease.

Incidence of this form follows the ethnogeographical differences characteristic of KSHV seroprevalence and also depends on the type of organ received. Posttransplant KS affects 0.4 % of posttransplant recipients in the USA and Europe, but about 4–5 % of patients in Saudi Arabia, where KS represents 87.5 % of all posttransplant neoplasia [134, 321, 332]. This form is more frequent in renal recipients compared to other solid-organ or bone marrow recipients. Among kidney transplant recipients, reported KS incidences are 0.2–0.6 % in Spain, Germany, the USA, and Canada; 0.4–3.3 % in France, Italy, Israel, and Turkey; and 4–5.3 % in South Africa and Saudi Arabia [258]. An Italian study measured a seroconversion rate that was higher among liver recipients (21.4 %) compared to renal recipients (8.6 %), but the renal recipients showed a higher risk of developing KS [9]. The degree of immunosuppression represents a critical factor for KS occurrence in posttransplant patients. In fact, regimens including cyclosporine A were found to increase the risk of KS [320]. Regression of posttransplant KS was achieved by substituting the immunosuppressive regimen including mycophenolate mofetil and cyclosporin A with sirolimus [98]. Sirolimus was also shown to promote the development of KSHV-specific T-cell immunity and the recovery of a functional T-cell repertoire, able to contribute to the regression of posttransplant KS [25].

In countries with intermediate and high seroprevalence, the majority of posttransplant KS cases develop in transplant patients with a preexisting KSHV infection, indicating that KS occurrence is mainly linked to KSHV reactivation due to the immunosuppressive regimen. A French study showed that 8 % of renal transplant recipients were seropositive before transplantation, and 28 % of them developed KS within 3 years after surgery, whereas none of the patients seronegative before transplantation developed KS [141]. In areas with lower seroprevalence, posttransplant KS is primarily due to primary infection through the donated organ [339].

5.4 Epidemic Kaposi's Sarcoma

Epidemic or AIDS-associated KS develops in HIV-1-infected individuals. According to the Centers for Disease Control and Prevention (CDC) guidelines, KS is considered an AIDS-defining disorder. Although KS develops in all groups at risk for HIV-1 infection, it is more common among men who have sex with men (MSM) and bisexual men than among transfusion recipients, women and children, hemophiliacs, and heterosexual IDUs [30]. Similar to classic KS, KSHV seroprevalence reflects AIDS-KS incidence in the different groups at risk for HIV-1 infection [365, 413]. The clinical course of AIDS-KS is characterized by a wide spectrum of disease manifestations ranging from the incidental finding of asymptomatic skin nodules to explosive growth of ulcerative plaques associated with edema and visceral involvement, resulting in significant morbidity and mortality [242].

While the frequency of AIDS cases presenting with KS was 40 % in the early 1980s in MSM, this figure decreased subsequently to 13–25 %, due to the introduction of initial antiretroviral therapies (ART) and safer sexual practices that reduced new HIV-1 infections [30, 260]. KSHV seroprevalence was 24.6 % in MSM in San Francisco in 1978, while HIV-1 infection was present in only about 2 %, indicating that KSHV infection was present in the homosexual population prior to the AIDS epidemic [312]. After the introduction of HAART, the incidence and severity of KS declined consistently [148, 235], mainly in association with improved immune function directly related to this therapy. A large study including HIV-1-infected subjects from North America, Europe, and Australia showed a decrease in incidence rates of KS from 15.2 in 1992 to 1996, to 4.9 in 1997 through 1999 [60]. KS incidence rates in San Francisco over a period of 25 years were 0.5/100,000/year in 1973, prior to the AIDS epidemic, increased to 31–33.3/100,000/year from 1987 to 1991, and then declined to 2.8/100,000 in 1998, in the post-HAART era [124].

Several studies have shown a direct correlation between the course of HIV-1 infection and the onset and progression of KS. Untreated patients presenting with progressive AIDS-KS typically exhibit low CD4+ T-lymphocyte counts (below 150–200 cells/ul) and high HIV-1 loads (above 10,000 RNA copies/ml). In the majority of patients, time to KS regression after the initiation of HAART depends upon tumor burden at baseline. Complete or partial response may occur within 6–24 months and is frequently accompanied by a significant increase in CD4+ T-lymphocyte count and suppression of HIV-1 viremia, two markers of good clinical efficacy of HAART, as well as a decline in systemic KSHV load. HAART-induced reconstitution of immune function may drive KS regression [70, 235, 383]. During 52 weeks of HAART, some AIDS-KS patients showed a significant decrease in HIV-1 and KSHV loads, which was associated with significant increases in CD4+ T-lymphocyte counts, and restored anti-KSHV cytotoxic activity [418]. Moreover, HAART-driven immune restoration was also associated with improved NK-cell-mediated immunity directed against KSHV-latently infected cells [371]. It was proposed that HAART-induced KS remission may be also mediated by direct antiangiogenic and antitumor effects on KS neoplastic cells exerted by some protease inhibitors (PI). Systemic treatment with PI, mainly saquinavir and indinavir, was shown to control the development and to induce regression of angioproliferative KS-like lesions in murine models [396].

KS regression and HIV suppression may also be achieved using non-PI-based HAART regimens [261]. Moreover, persistent or de novo KS was also reported in clusters of AIDS patients with high CD4+ T-lymphocyte counts and low or undetectable HIV-1 viremia [224, 266], indicating that some patients may experience effective HIV-1 suppression and restoration of immune function not accompanied by KS remission. Other factors possibly associated with the lack of partial or complete response of KS, such as age, duration of HIV-1 infection, tumor burden, and KSHV pattern of expression, need to be analyzed in-depth.

In a subset of HIV-1-infected subjects, KS may initially worsen following the initiation of HAART, as a result of an inappropriate immune reconstitution and a pathological inflammatory response to either previously treated or subclinical infections. This process is known as immune reconstitution inflammatory syndrome (IRIS). The risk of IRIS was found to be mainly associated with low CD4+ T-lymphocytes at baseline [293], although other factors, such as detectable plasma KSHV DNA, were proposed to predict IRIS-associated KS [241].

5.5 Epidemiology of KSHV Infection

Unlike most other human herpesviruses, the infection with KSHV is not ubiquitous. Prevalence of KSHV infection in the general population mainly reflects the local incidence of classic or endemic KS and depends on the geographical area and analyzed population. Factors that influence the epidemiology of KSHV infection in a given population mirror the predominant modalities of KSHV transmission and the prevalence of risk factors for KS [358]. Older age, Mediterranean or Eastern European origin, HIV-1 infection, homosexual, and high-risk sexual behaviors are predictors of higher seroprevalence. A higher risk for KSHV infection is associated with having a seropositive mother or siblings in endemic countries [183, 327]. Seroprevalence is almost equally distributed between men and women, and a gradual increase with increasing age in both endemic and non-endemic populations has been frequently observed [55, 240]. However, the age-related increase in seroprevalence begins during childhood in endemic countries, and after puberty in countries with lower seroprevalence [240, 267], indicating a difference in the prevalent modality of transmission among these populations.

Globally, KSHV infection is highly prevalent in sub-Saharan Africa and among some Amerindian and Melanesian populations and diffuse in the Mediterranean area, in some Middle Eastern countries, and in parts of South America. KSHV is less prevalent in Northern Europe, North America, and most of Asia. Variation of KSHV seropositivity across small-area regions within countries also exists, and the KSHV-KS geographical correlation does not apply everywhere. In fact, high KSHV seroprevalence matched with relatively low KS incidence has been reported in Gambia [14], in some Amerindian populations [34], and among Ethiopian immigrants to Israel [170]. This incongruence may reflect biases, but it could also indicate the presence of yet unidentified cofactors that protect these populations from KS development.

Seroprevalence rates in the general population, mainly based on blood donor surveys, but also on other specific categories, are reported below and illustrated in Fig. 39.3. It has to be kept in mind that data are taken from serosurveys that may use different serological assays and criteria to estimate the overall seroprevalence in the analyzed populations.

The highest KSHV seroprevalence rates worldwide have been reported in Native American populations (up to 93 % in adults) in South America, mainly among Amerindians living in the Amazon regions from Brazil and Ecuador (77 %) and in the transitional area between the Amazon forest and the savannah of central Brazil (50–83 %). KS incidence in these populations appears to be low, although difficulties in diagnosis and records of diseases in subjects of remote villages may underestimate the actual prevalence of KSHV-related malignancies [34, 105, 216, 284, 302]. Amerindians from Bolivia, Paraguay, and French Guiana showed an intermediate seroprevalence (18–45 %), and those from Southern Brazil the lowest rates (0–14 %). Amerindian and non-Amerindian populations living in the same geographical area showed diverse KSHV seroprevalences, suggesting differences in the prevalent transmission routes between the two populations. In the Amazon region, KSHV seroprevalence was 65 % in children and increased to 93 % in adults (≥35 years) among Mapuera Amerindians, whereas it was 10 % in children and 50 % in adults among Mapuera non-Amerindians [302].

KSHV antibodies were found in lower proportions of the general population, mainly blood donors, from Chile (3 %), Jamaica (3.6 %), Argentina (4–8 %), Southeast Brazil (3–7 %), Costa Rica (10 %), Honduras (11.3 %), French Guiana (11.8 %), Colombia (13 %), and Cuba (17 %) [67, 104, 135, 136, 284, 323, 376]. In contrast, a seroprevalence of 56 % was found in Peruvian blood donors [283]. A large multicenter survey of Brazilian blood donors from three state capitals, representing a population composed of descendents of three ethnic groups (Caucasian, African, and Indigenous populations), reported an overall seroprevalence of 25 % [301].

KSHV seroprevalence is much lower in North America. Large studies in the USA showed a seroprevalence of 0.5–7 % among the general population [130, 153, 219, 319]. The prevalence in Canada has been documented by studies on transplant recipients, showing 0–2 % seropositivity before transplantation [364]. Cases of classic KS reported in Inuit

people living in northern Quebec may indicate that KSHV is more diffuse in this population [348], although seroprevalence data are not yet available.

In Africa, KSHV prevalence is generally high, but is unevenly distributed. Overall, adults living in sub-Saharan countries show infection rates that reflect the cumulative incidence of endemic KS [93, 106]. Uniform criteria to estimate seroprevalence were adopted in a reanalysis of five seroepidemiological studies conducted in sub-Saharan Africa. Among adults (>20 years of age), seropositivity was 23.5 % in Nigeria, 40.2 % in Kampala and Uganda, 45.4 % in Tanzania, and reached 70.6 % in the rural West Nile, in Uganda. Among children, seroprevalence rates ranged from 18.1 % in Kampala to 33.8 % in Tanzania and increased steeply with age in all populations [325]. Consistently, other studies showed that about 55 % of the general population in Uganda had KSHV antibodies [38, 367], and higher rates were reported in rural territories [38, 52, 118]. Seroprevalence estimates in Tanzania ranged from 30 to 89 %, depending on the studied population and the geographical area, with a mean prevalence of about 50 % in blood donors [118, 269, 278]. KSHV seroprevalence was reported to be 43 % in Kenya [19], 54–67 % in Malawi [109], 58 % in Zambia [308], and 82 % in the Democratic Republic of Congo [128].

In West Africa, KSHV seropositivity was found to be 60 % in the adult population of Cameroon [328, 344] and 32.3 % in Ghana [304]. Early epidemiological surveys found a higher seroprevalence (75 %) in Gambia [14], not matched by the low KS incidence in this African region, whereas a seroprevalence of 14.3 % was reported among pregnant women in Senegal [156]. In Northern Africa, seroprevalence estimates are available for Tunisia (14 % in blood donors) [187] and Egypt (24–42 %) [272, 366]. In North-Eastern Africa, seroprevalence in Eritrea was found to be 5–8 % among women and 26 % among members of the Rashaida tribe [125]. A seroprevalence of 37 % was reported among HIV-1-uninfected pregnant women in Ethiopia [239], a rate similar to that found among Ethiopian Jewish immigrant to Israel [170].

In Southern Africa, a seroprevalence of 21.4 % was found in Mozambique among individuals attending public health centers [68], and a similar rate (20.3 %) was reported in Zimbabwe [118]. Seroprevalence rates ranging from 17.6 to 30 % were measured in South Africa [118, 253], whereas higher estimates (87 % in Sans, an indigenous group, and 76 % in Bantus) were found in Botswana [128].

A marked variation of KSHV seroprevalence/KS incidence is also present in Europe. In general, KSHV infection is diffuse in Mediterranean countries, such as Italy, and lower in Northern Europe. Italy showed an increasing gradient of KSHV prevalence from north (7–13 %) to south (22–35 %), as well as some hot spots in the Po Valley (16–27 %), mirroring classic KS incidence at the microgeographical level [55, 69, 354, 390, 414]. KSHV seroprevalence similar to that reported among blood donors from Central/Southern Italy was found in subjects from Albania (20–29 %) and from the Kosovo region of former Yugoslavia (18 %) [87, 168].

Lower KSHV seroprevalence rates were reported in the UK (5 %) [370], France (1–4 %) [121, 256], Germany (3 %) [292], Switzerland (5 %) [338], and in a cohort of IDUs in the Netherlands (1–3 %) [342]. Slightly higher rates were measured in Spain (4–8 %) [104, 149] and Greece (6–14 %) [370, 425].

The prevalence of KSHV in Nordic countries showed pronounced variation among and within countries, again reflecting classic KS incidence [192], as KSHV seroprevalence was found to be 16 % in Northern Sweden [393], but only about 4 % in young adults attending an STD clinic before the AIDS epidemic in Denmark [193].

A low seroprevalence was found in the general population of Russia (0–5 %) [180], Czech Republic (0.3–2.4 %) [386], and Hungary (2 %) [209], whereas higher rates (25 %) were reported in an adult population of Southern Siberia [65].

Prevalence in Asia varies from low to moderately high. A systemic review and meta-analysis based on the available literature showed that KSHV seroprevalence in China is 11.3 % in the general population, but it varies in different regions, being higher (18–23 %) in Xinjiang, an area in which incidence rates of classic KS are high [113, 427]. KSHV seroprevalence (0.2–1.4 %) and KS incidence were found to be low among the general population in Japan [146]. Of note, a higher KSHV prevalence has been reported in patients with adult T-cell leukemia/lymphoma, suggesting an increased risk of KS in these patients [282]. A large study reported a prevalence of 5 % for the Republic of Korea, 8–10 % for Thailand, and 11–16 % for Vietnam [104]. In India, a prevalence of 4 % has been reported among healthy individuals [1].

In Middle Eastern countries, similar seroprevalence rates were reported in Saudi Arabia (2 % in healthy controls, 18 % in renal transplant recipients) [4] and in Iran (2 % in blood donors, 17 % in hemodialysis patients, 25 % in renal transplant patients) [205]. In Israel, KSHV seroprevalence among the different Jewish groups was found to correlate with the incidence of KS. KSHV seroprevalence rates ranged from 3 to 10 % among Israeli Jewish populations, and higher figures were reported among Ethiopian immigrants (26–39 %) [99, 170, 183, 204].

Some indigenous Melanesian adult populations were shown to harbor KSHV infection. Seropositivity rates ranged from 21 % in Port Moresby to 30 % in rural Bensbach in Papua New Guinea [345], and native Melanesians from New Caledonia, located in the southwest Pacific, and Vanikoro island, belonging to the Solomon Archipelago, showed an overall seroprevalence of about 24 % [66]. A seroprevalence of 11.2 % was reported among HIV-negative homosexuals from Sidney, Australia [173].

6 Mechanisms and Routes of Transmission

Modalities of circulation of KSHV infection in the general population depend on the geographical area and on the relative seroprevalence in the analyzed group. KSHV is transmitted mainly via nonsexual routes during childhood in areas with intermediate and high seroprevalence and primarily via sexual contact during adulthood in countries with low seroprevalence. Saliva is an important reservoir of infectious virus [404]. This biological fluid has a highly infectious potential, as viral particles isolated from saliva can establish productive infection in B cells regardless of KSHV load [154]. Therefore, saliva represents the foremost vehicle of KSHV in the context of both sexual and nonsexual routes. Oral transmission of KSHV may also involve the IgMλ subset of tonsillar B-lymphocytes, which, once latently infected, would increase their proliferation rate and differentiate toward a plasmablast phenotype highly reminiscent of MCD [190].

6.1 Horizontal Transmission

In areas with intermediate and high seroprevalence, horizontal, primarily intrafamilial transmission is the main modality of KSHV spread. Infection is acquired early in childhood, from members in and outside households, and infection rates increase with age [51, 55, 159, 183, 267, 269, 327]. A high KSHV load in maternal saliva was associated with transmission to the child [107, 269, 391]. Other risk factors for infection before puberty include HIV infection [253, 281] and environmental factors [271]. It has been also postulated that horizontal transmission may take place through exposure to food premasticated by the mother [269, 327], although a more recent population-based study in a rural community in Uganda failed to prove this hypothesis [52]. The habitual use of maternal saliva as first-aid medication of insect bites has also been suggested to play a role in promoting mother-to-child transmission of KSHV in endemic countries [91].

6.2 Vertical Transmission

Vertical mother-to-child transmission, not involving saliva exchanges, may also occur in areas with intermediate and high KSHV seroprevalence. Initial studies on vertical transmission showed that KSHV seroreactivity in newborns is mainly due to transplacental passage of maternal antibodies [57, 159]. However, rare cases of in utero or intrapartum KSHV transmission were documented in newborns by the detection of KSHV DNA in a newborn's blood at birth [46, 245, 255, 273] and by the detection in an infant of primary infection with the mother's *orfK1* subtype [245]. African women also showed higher KSHV detection rates in cervicovaginal secretions compared to women from non-endemic or subendemic areas [56, 228, 415], indicating that KSHV load in the female genital tract might influence vertical transmission. Like other herpesviruses, KSHV may be reactivated during pregnancy, as demonstrated in HIV-1-infected women [245]. KSHV can also infect trophoblasts and placental endothelial cells in vitro and in vivo, providing evidence that transplacental transmission may, though rarely, occur in endemic countries [112].

6.3 Transmission Through Sexual Contact

In areas where KS is sporadic, KSHV infection is acquired in adulthood and is mainly associated with sexual activity [39, 260]. KSHV infection is common among MSM, and KSHV seroprevalence is higher in those infected by HIV-1. Epidemiological surveys indicated that the risk of KSHV acquisition in MSM is associated with the number of lifetime sexual partners, a history of other sexually transmitted diseases, HIV-1 infection, and specific sex practices [39, 165, 173, 218, 260, 374]. Prevalence of KSHV infection and incidence of AIDS-KS are higher among HIV-1-positive MSM or bisexual men compared with other HIV-1 transmission groups, and this finding suggests that KSHV could be a sexually transmitted agent. Several studies validated the association of KSHV seropositivity with the number of sexual partners, thus confirming the relevance of sexual transmission among HIV-infected MSM [259, 262].

In endemic countries, KSHV seroprevalence increases with age among adults as well, suggesting that transmission is ongoing in adulthood, very likely including both sexual and nonsexual routes [19, 51, 234, 367].

KSHV loads in cervicovaginal, seminal, and prostatic secretions are much lower than in saliva [56, 103, 318, 415], and the infectious potential of these biological fluids has not been fully elucidated, to date. It is conceivable that saliva also plays a predominant role in KSHV dissemination in the setting of sexual transmission [259, 262].

6.4 Parenteral Transmission

Parenteral transmission of KSHV appears to be highly inefficient [75, 127, 162, 310]. Some reports indicated prolonged use of injecting drugs, with extensive needle sharing, as a risk factor for KSHV infection [17, 61, 138, 166].

Infection through the administration of blood or blood products is also infrequent. Several variables, including lack of viremia in healthy donors and pretreatment and storage of blood, may limit KSHV spread through this route [126]. Nevertheless, some studies indicated increased risk and

KSHV transmission by blood transfusions in endemic countries [117, 194, 268].

6.5 Transmission Through Organ Donation

Posttransplant KS has been reported in solid-organ transplant patients, mainly recipients of kidney grafts. In endemic countries, posttransplant KS is primarily due to reactivation of a preexisting KSHV infection in the recipient. However, reported cases of KSHV seroconversion in pediatric and adult recipients after organ transplantation indicate that KSHV transmission through organ donation may also occur [92, 251, 252, 315, 360]. Furthermore, it was shown that KSHV-infected neoplastic cells in the posttransplant KS lesions from some renal transplant recipients may harbor either genetic or antigenic markers of their matched donors, suggesting that KS progenitor cells may be transmitted through solid grafts [24]. It has suggested that transfusions during surgery could also play a role in reported cases of primary infections after organ transplantation in countries with intermediate and high KSHV seroprevalence.

7 Pathogenesis

7.1 Primary Infection with KSHV

The characteristics of primary or acute infection with KSHV have been incompletely defined, though like all other infections with herpesviruses, it results in a long-term lifelong latent infection. A febrile illness that was sometimes associated with maculopapular skin rash and active KSHV viremia has been associated with KSHV primary infection in immunocompetent children [8, 215, 361]. Primary KSHV infection in immunocompetent adults is associated with mild diarrhea, fatigue, localized rash, and lymphadenopathy [409]. Fever and cutaneous rash, coinciding with KSHV viremia, have been reported as part of primary infection in immunosuppressed patients after transplantation [252], while a severe, sometimes fatal, outcome (e.g., bone marrow or multiorgan failure) of primary infection was described in rare cases in immunocompromised patients [257, 360].

Generally, the development of KSHV-related disorders in KSHV-infected individuals occurs after long-term infection and is fairly rare, suggesting the involvement of cofactors. Along with the major clinical entities (KS, PEL, and MCD) that are etiologically associated with KSHV, additional, less common, clinical manifestations related to KSHV infection are also described below. The involvement of KSHV with other disease conditions has been widely investigated, and its association with a broad spectrum of diseases has been ruled out [90]. Of note, several cases exhibiting the concomitant occurrence of two or three of the KSHV-related syndromes have been reported in the literature, thus supporting their common causative agent.

7.2 Kaposi's Sarcoma

The most frequent KSHV-associated malignancy is Kaposi's sarcoma (KS). KS is an unusual multifocal angioproliferative disease presenting various-sized lesions composed of multiple cell types. KS lesions contain spindle-shaped endothelial cells and abnormal and leaky vessels with extravasation of red blood cells and hemosiderin deposits. Inflammatory lymphocytic infiltrates including monocytes, T cells, B cells, histiocytes, and plasma cells are also present in these lesions, in particular during early development. KS may develop in various organs but lesions are mostly seen on the skin. Cutaneous presentation usually has a benign course, whereas visceral dissemination, involving the lungs, liver, lymph nodes, and the gastrointestinal tract, can be aggressive. Clinically, lesions have been classified as patch, plaque, nodule, or tumor stages; though a given patient may simultaneously present different types of lesions, while certain lesions never progress and others initially present at an advanced stage. Of note, although KS was originally defined as a malignant tumor, this classification is still a matter of debate, and the current model suggests that KS represents a consequence of a multistep proliferative, inflammatory, and angiogenic process involving both viral and cellular factors, which could give rise to a clonal metastatic neoplasm under certain conditions [151, 279]. This view is supported by studies showing that KS lesions display several patterns of clonality (mono-, oligo-, and polyclonal), suggesting that KS begins as a polyclonal hyperplasia with potential for subsequent evolution to a monoclonal tumor.

KS lesions harbor KSHV mostly as a latent genome, while a small fraction of the cells undergo lytic infection, as demonstrated by the expression of early and late transcripts and proteins [381]. Cells undergoing lytic infection produce viral and cellular cytokines and chemokines that probably support the growth of latently infected cells allowing sustained growth of the lesions.

The predominant cell type in advanced lesions is the elongated-shaped spindle cells that proliferate and promote disease progression. The cellular origin of these cells remains a subject of debate. Most spindle cells express markers of lymphatic endothelial cells (LECs), including CD31, CD34, PROX1, lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1), vascular endothelial growth factor receptor 3 (VEGFR3), and podoplanin [122, 210]. Yet, some spindle cells also express markers of dendritic cells, macrophages, or smooth muscle cells [350]. Interestingly, in vitro infection of blood vessel endothelial cells (BECs) induces LEC markers,

whereas infection of LEC induces transcriptional reprogramming toward BEC-like characteristics [197, 410]. The finding of endothelial cell reprogramming by KSHV provides insights into the pathology of KS, and suggests that KSHV infects circulating endothelial precursor cells, and reprograms their transcriptional profile toward a unique lymphatic lineage [279].

KSHV is a prerequisite etiologic agent for all variants of KS. This notion is based on consistent and reproducible evidence obtained by several research groups worldwide. Specifically: (1) almost all KS lesions test positive for KSHV DNA, yet KSHV is absent from the adjacent tissue. (2) Infection with KSHV precedes the onset of KS. Detection of antibodies directed against KSHV antigens in the blood of untreated HIV-infected subjects is strongly predictive of their future development of KS. Furthermore, elevated antibody titer, both for lytic and latent viral antigens, is associated with increasing risk of KS. (3) Elevated levels of viral DNA in PBMCs are detected among KS patients and in individuals subsequently progressing to KS, compared with healthy subjects. (4) A general correlation is well established between KSHV prevalence and population risk for developing KS. (5) KSHV is present in critical cellular components of KS, namely, the spindle cells. (6) KSHV encodes several potentially oncogenic proteins, and it is closely related to other mammalian tumorigenic viruses.

Infection with KSHV is not sufficient to cause KS. Most carriers of KSHV never develop KS, suggesting that other host-related factors along with lifestyle and environmental cofactors are likely to affect the risk of KS development. The most important cofactor predisposing KSHV-infected individuals to KS is immunosuppression, which could be iatrogenic but is most commonly due to HIV infection. The introduction of effective antiretroviral therapy led to a sharp decrease in KS incidence among HIV carriers, yet the risk of KS remains relatively high in HIV-infected individuals [144]. This illustrates the significance of the immune system in the development of KS but also reflects specific interactions between HIV and KSHV proteins that yield a synergistic effect which stimulates KSHV replication. Age is an established cofactor for the development of KS. Except in areas endemic for KSHV, KS in immunocompetent individuals usually develops after 50 years of age. Whether the early age of KSHV acquisition in Africa is a contributing factor to KS development in children in this area is unknown. In addition, the reasons for the tendency of KS to selectively affect men are unknown. Additional possible cofactors for the endemic disease include malaria, schistosome and other parasitic infections, and contact or ingestion of specific plants that might increase lytic reactivation of KSHV [416]. Genetic determinants, possibly those that reflect ethnicity, such as the human leukocyte antigen (HLA) tissue type may be influential. Selected alleles of the interleukin-6 promoter and of the low-affinity Fcγ receptors IIIA for IgG are associated with an increased lifetime risk of the development of KS in men infected with HIV. Among KSHV-seropositive Italians, the risk of classic KS is associated with diplotypes of IL-8RB and the promoter region of IL-13 [47]. Underlying immune activation, reflected by elevated levels of neopterin or β-2 microglobulin, was also found to be associated with the disease.

Environmental factors probably play an important role, and certain were evaluated. For example, KS is associated with exposure to volcanic soil, and this disease seems to be more common in highland areas located at altitudes >2,000 ft, although high incidence rates have been found at a broad range of altitudes and temperatures. Chronic exposure of the skin to aluminosilicate or ferrous particulates, leading to lymphatic obstruction and local inflammation, has been also suggested to increase the risk of KS emergence [435].

Lifestyle factors have been demonstrated to influence KS. Cigarette smoking is inversely associated with classic KS risk, and HIV-infected MSM in the USA who also smoke were found to have a reduced risk of AIDS-associated KS. However, in other studies, tobacco use was not associated with reduced risk of classic, AIDS-associated, or endemic KS. Crack cocaine also has been inversely associated with KS. Infrequent bathing was demonstrated to be associated with an increased risk of classic KS. A history of several diseases, including asthma and allergies, was associated with an elevated risk of KS. At present, it seems that the virulence of specific strains of KSHV is not likely to play an important role in its ability to induce KS. However, the effect of viral factors remains to be further explored.

Familial clustering of KSHV infection has been reported [10, 99]. However, only few cases of familial clustering of KS have been documented [182, 211]. The rarity of familial clustering of KS may be because individuals infected with KSHV have a low lifelong risk of developing the disease; yet, familial cases suggest that virological, environmental, and genetically determined susceptibility factors, independently or in combination, may determine the development and pathogenesis of KS.

7.3 Primary Effusion Lymphoma

The etiologic association between lymphoma and KSHV is mainly based on cases of primary effusion lymphoma (PEL) that were primarily observed in HIV-infected patients, and on a small number of cases that have been described in post-transplant immunosuppressed patients, and in elderly individuals who are apparently immune competent except for functional immune senescence [62, 73, 298]. PEL is a rapidly fatal distinct type of non-Hodgkin B-cell lymphoma (also referred to as body cavity-based lymphoma). In elderly subjects, PEL may have a less aggressive clinical course and less

frequent EBV coinfection. It typically presents as lymphomatous effusion in the pleural, peritoneal, or pericardial body cavities without an associated tumor mass. Rarely, lymphomatous cells, presenting similar molecular and immunophenotypic characteristics, occur as solid tumors with or without evidence of effusions and are considered as an extracavitary/solid variant of PEL [426]. This presentation sometimes precedes or follows a typical case of PEL and may involve the skin, gastrointestinal tract, lung, central nervous system, and lymph nodes. Recurrent and aggressive polyclonal effusions containing KSHV-infected atypical lymphomononuclear cells may also develop in body cavities of patients with concomitant KSHV-associated disorders (KS and/or MCD), not exclusively in association with HIV infection [16, 43].

The tumor cells in PEL present diverse morphologic features ranging from large immunoblasts or plasmablasts to anaplastic morphology. Binucleated or multinucleated cells resembling Reed-Sternberg cells are found as well. Indicative of their B-cell origin, PEL cells display clonal immunoglobulin (Ig) gene rearrangements, yet they lack pan-B-cell markers including CD19, CD20, CD79a, and surface and cytoplasmic Igs. Expression of plasma cell markers, such as CD30 and CD138/syndecan, and hypermutation of Ig genes as well as frequent somatic hypermutation of the noncoding region of the *BCL6* gene suggest that PEL cells originate from postgerminal center B cells. Unlike several other lymphoid disorders, PEL cells lack c-*myc* rearrangement and do not exhibit dysregulated c-Myc expression [208, 221].

PEL cells harbor dozens of copies of episomal KSHV genome per cell and are generally latently infected. Accordingly, these cells express LANA-1, vCYC, vFLIP, and LANA-2 proteins as well as Kaposin and microRNAs, while viral interleukin 6 (vIL-6) is expressed by a variable proportion of the tumor cells. The involvement of KSHV gene products in the pathogenesis of PEL is supported by the requirement for latent KSHV gene expression for the survival of PEL cells [164, 176]. Given the strong association between PEL and KSHV infection, immunohistochemical analyses, in particular using antibodies to the major KSHV antigen, LANA-1, serve as a helpful diagnostic procedure. This approach allows the distinction between PEL and secondary lymphomatous effusion and also serves to diagnose extracavity PELs that otherwise could be incorrectly classified.

PELs are frequently coinfected with EBV; however, there is no difference in the clinical presentation, tumorigenicity in mice, and response to treatment between EBV-positive and EBV-negative PELs. In PELs, EBV presents a restricted latency I gene expression pattern, involving the expression of EBNA1 protein, and the small noncoding RNAs EBERs and microRNAs [198, 389]. The rarity of PEL suggests that KSHV infection represents only one of the several events involving the cascade that leads to the development of PEL. EBV infection has been included among cofactors associated with the establishment of PEL, yet its limited viral gene expression suggests that the major driving force of PEL is KSHV infection.

7.4 Multicentric Castleman's Disease (MCD) and KSHV Inflammatory Cytokine Syndrome (KICS)

Multicentric Castleman's disease (MCD) is a rare polyclonal lymphoproliferative disorder which manifests as a generalized lymphadenopathy with systemic inflammatory symptoms of fever, cachexia, weight loss, hypoalbuminemia, cytopenia, and hyponatremia [72]. Like PEL, MCD occurs at increased frequency in AIDS patients and in this setting is almost always associated with KSHV infection. MCD also may occur in HIV-negative individuals, yet only 40–50 % of these cases are KSHV positive.

In MCD, the cells harboring KSHV occur predominantly in the mantle zone of B-cell follicles. These cells are monotypic plasmablasts that almost invariably express high levels of cytoplasmic IgM with an exclusive λ light chain restriction. Microscopic foci of clonal plasmablastic lymphoma may eventually occur within MCD-affected tissue [119].

KSHV-infected MCD is associated with an abundant viral lytic infection, suggesting the involvement of viral lytic proteins in the pathogenesis of this disease. A major role in the pathogenesis of MCD has been attributed to the lytic gene product vIL-6 that is expressed in scattered plasmablasts surrounding the lymphoid follicles and acts as a stimulator of B-cell proliferation and VEGF secretion. This notion is based on the association between high levels of IL-6 and worse prognosis of MCD. Treatment of three MCD patients with ganciclovir, an inhibitor of herpesvirus lytic replication, has been reported to ameliorate MCD [64], while treatment with cidofovir, an inhibitor of herpesvirus DNA polymerase, was not effective [31, 94].

An IL-6-related systemic inflammatory syndrome has been recently reported in patients with HIV and KS, but without a pathologic diagnosis of MCD. This clinical condition, termed KSHV inflammatory cytokine syndrome (KICS), resembles KSHV-MCD but lacks prominent lymphadenopathy and pathological nodal changes [329, 398]. Like MCD patients, elevated loads of KSHV and high vIL-6 and IL-10 levels characterize KICS patients.

7.5 Other KSHV-Associated Lymphomas

KSHV is associated with large B-cell lymphoma arising mostly in HIV-positive patients with KSHV-associated MCD [62]. The lymphoma cells present features of MCD plasmablasts, including IgMλ expression and absence of EBV

infection. Unlike PEL, large B-cell lymphomas arising in KSHV-infected MCD lack somatic hypermutation of Ig genes; hence, they are thought to originate from pre-plasma cells that circumvented the germinal center [123].

Germinotropic lymphoproliferative disorder, predominantly involving germinal centers of lymph nodes and characterized by plasmablasts that are EBV and KSHV coinfected and a favorable response to chemotherapy or radiotherapy, has also been associated with KSHV [120].

8 Immunity

Acquired and genetic deficiencies in cellular immunity are strongly associated with increased risk of KSHV-associated disorders. Decrease in the number of CD4 cells is associated with AIDS-KS [35] and classic KS [48]. Furthermore, lower KSHV-specific CD4 cell counts are detected among HIV-infected KS patients as compared with HIV- and KSHV-coinfected individuals without KS [178]. Similarly, increases in both absolute CD4 count and KSHV-specific T-cell responses are coupled with response to therapy, hence illustrating an interaction between the clinical state and cellular immunity [25, 37].

Defects in the humoral activity may also play a role in the pathogenesis of KSHV. Reduction of B-lymphocyte counts correlates with the development and progression of KS [382, 384]. Lower levels of KSHV neutralizing antibodies have been recorded in the serum of patients with clinical KS, as compared with KSHV-infected individuals with no evidence of KS [220].

At the same time, KSHV is an active regulator of the host immune response, encoding multiple proteins that inhibit innate and adaptive host immunity [95, 237]. Two modulators of immune recognition, MIR1 and MIR2, are encoded by KSHV *orfK3* and *orfK5*, respectively, and strongly impair MHC-I presentation. Four homologs of cellular IRF, encoded in a single cluster, which probably originated through gene duplication, regulate the antiviral interferon response. Two other proteins that are not members of the IRF family, ORF45 and RTA, abrogate induction of interferon response by IRF-7 [424, 431]. Furthermore, vIL-6 impairs the formation of STAT1-STAT2-IRF-9 complexes that ordinarily promote expression of interferon-stimulated genes. Three viral homologs of CC chemokines, vCCL-1/MIP-I, vCCL-2/vMIP-II, and vCCL-3/vMIP-III, encoded by *orfK6*, *orfK4*, and *orfK4.1*, respectively, function as negative regulators of inflammatory and immune responses. A homolog of the cellular CD200/OX2, encoded by *orfK14*, suppresses TNF-α production by macrophages and represses inflammation. Another viral gene which has a cellular counterpart is the KSHV complement control protein (KCP), encoded by *orf4*, and functions to limit and disrupt the progression of the complement activation cascade. In addition, KSHV encodes a microRNA which represses the expression of the stress-induced NK cell ligand, MICB, allowing recognition escape and consequent elimination by natural killer (NK) cells [297]. However, most of these genes are expressed during lytic infection and thus probably enable virus evasion from the host response and promote greater virus production.

9 Control and Prevention

Prevention of primary KSHV infection could reduce the incidence of KSHV-related disorders. Yet, no successful attempts to control virus transmission have been described. The reason for this is because most infections are asymptomatic, while salivary exchange, which probably represents the predominant route of virus transmission, cannot be feasibly controlled. Nevertheless, improvement of hygiene could reduce the spread of the virus. Cost-effectiveness of a KSHV vaccine has not been thoroughly evaluated and is hard to predict, but at present, vaccine development appears to be unwarranted.

KSHV transmission via organ donation is of great clinical concern, since transplant recipients undergo immune suppression. Threefold higher risk of KS has been reported among KSHV-seropositive recipients of solid organs as compared with seronegative recipients, with no significant differences in the clinical outcome in both groups [142]. This suggests that screening of potential transplant recipients and donors may be required for clinical monitoring, yet the presence of antibodies in either recipient or donor should not exclude transplantation. Preemptive antiviral therapy could offer an approach for the prevention of KS among transplanted patients who are KSHV positive or receiving an organ from a donor infected with the virus. Hence, routine screening for KSHV should be considered prior to transplantation, at least in countries with intermediate and high KSHV seroprevalence.

Lytic KSHV infection can be inhibited in vitro by antiherpesvirus drugs, including ganciclovir, foscarnet, and cidofovir, but not acyclovir [217, 276]. The ability of these drugs to reduce the risk of KS in AIDS patients and to induce regression of KS has been evaluated by several investigators, providing inconsistent results. A recent study reported no detectable effect of oral valganciclovir therapy on tumor growth, KSHV antigen positivity, or gene expression [225], and together with negative findings reported for cidofovir [247] provide little support for the use of these drugs alone for KS treatment. HAART decreases circulating HIV and hence reduces the incidence of KS and can induce regression of AIDS-KS, yet KS remains a clinical issue, and only 50 % of cases completely resolve [303]. Adoptive immunotherapy, involving infusion of specific cytotoxic T cells, is already in

use for the treatment of patients with EBV-infected lymphomas and is expected to be effective toward KSHV-associated diseases as well. Finally, the accumulated knowledge about the biology of KS and KSHV is expected to be translated into new therapeutic approaches targeting KSHV infection and paracrine mechanisms.

The prognosis of PEL patients is poor even with standard chemotherapy regimens. Several nonchemotherapy approaches have been tested including HAART alone or antiviral treatments with promising results [32, 186, 378], while therapies that target specific viral oncogenes may provide benefits in the future [32]. A monoclonal antibody targeting CD20, rituximab, has been evaluated in the treatment of KSHV-positive MCD, yet was associated with slight exacerbation of KS in some patients [158].

10 Unresolved Problems

Research on KSHV during the recent years has led to better understanding of its pathogenesis and its related disorders. The functions of several proteins and microRNAs encoded by KSHV have been studied extensively, yet various aspects are still poorly understood. Much of what we know so far has yet to be translated into clinical practice. Furthermore, understanding the involvement and regulation of host genes, including microRNAs, during infection may shed light on cellular pathways that could be targeted and thus could offer new treatment approaches.

The site of KSHV latency has not been resolved yet. Some of the signaling cascades that induce virus reactivation have been identified, but we cannot yet combine them into a model with pathophysiological relevance. Furthermore, the events and conditions that determine long-term asymptomatic infection, cell proliferation, angiogenesis, and transformation among infected individuals have not resolved yet. Since the fate of infection is determined by several signaling networks, involving multiple viral and cellular gene products, this issue is complex. Furthermore, the interactions of KSHV with other microbial agents, such as malaria, that could affect its replicative phase remain to be elucidated. Specific immunological factors and events controlling disease onset are not fully understood. Newly identified animal models as well as in vitro systems to study the biology of KSHV employing recombinant viruses are expected to largely increase our understanding on molecular mechanisms involved in KSHV pathogenesis.

A standardized algorithm for KSHV diagnostics in subjects at risk for KSHV-driven malignancies, such as post-transplant patients and HIV-1-infected individuals, is not yet available, and uniform criteria to estimate seroprevalence in a given population have not yet been defined. Research and diagnostic laboratories should work in concert to develop and test new serological assays and search for a consensus on the diagnostic flowchart related to detection and follow-up of KSHV infection. Moreover, there is the need to provide laboratories in developing countries with simple and reliable tests for KSHV detection, which would help pathologists to confirm KS diagnosis in suspected and/or borderline tumor conditions in adults and in children. Newly defined parameters, such as circulating exosome-associated viral or cellular miRNAs, may in the future provide more reliable predictive and prognostic markers and guide physicians to plan and tailor therapeutic strategies.

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Human Herpesviruses: Malignant Lymphoma

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1 Introduction

The human gammaherpesviruses, Epstein-Barr virus (EBV, or HHV4) and Kaposi sarcoma-associated herpesvirus (KSHV, or HHV8), are associated with lymphoma. EBV was first discovered in the 1960s in association with Burkitt lymphomas arising in sub-Saharan Africa, while the discovery of KSHV in 1994 was associated with Kaposi sarcoma tumors and driven by the AIDS epidemic. While EBV infection is ubiquitous, KSHV is restricted to certain populations. EBV-associated lymphoproliferative diseases are likewise more common than KSHV-associated lymphoproliferative disease. These two herpesviruses nonetheless share many characteristics. Particularly relevant to lymphoma and lymphoproliferative disease is the ability of these two viruses to infect and establish a reservoir of infection in lymphocytes. Whereas some herpesviruses establish latency in nondividing terminally differentiated cells such as neurons, the gammaherpesviruses establish latency in lymphocytes. The gammaherpesviruses have evolved mechanisms for persisting as extrachromosomal genomes in dividing cells. These cells may be driven to proliferate or be protected from cell death pathways by viral gene expression. The result may be a self-limited lymphoproliferation that establishes or maintains a reservoir of latently infected cells or, under certain circumstances, a malignant lymphoproliferation.

Although the focus of the chapter is EBV- and KSHV-associated lymphomas, the development of lymphoma is a rare outcome of viral infection. Some lymphoma entities are nearly universally virus associated, such as African Burkitt lymphoma (BL), nasal-type extranodal NK-/T-cell lymphoma, AIDS-associated primary central nervous system lymphoma, and AIDS primary effusion lymphoma (PEL); others such as diffuse large B-cell lymphoma (DLBCL) and Hodgkin lymphoma (HL) are variably associated with the virus. Consistent association with the virus does not indicate that herpesvirus infection is sufficient to cause these lymphomas. As the names of some of these lymphomas hint, geographic, anatomic, or immune factors contribute to the pathogenesis of these diseases. In this chapter, we review aspects of the biology of the viruses and aspects of the relevant lymphoma entities and discuss approaches to diagnosis, treatment, and prevention that are virus specific.

2 Aspects of Viral Biology and Viral Gene Expression

EBV is a ubiquitous gammaherpesvirus that infects over 90 % of the world's population by adulthood [1, 2]. By contrast, KSHV infection is unusual in most parts of the world. KSHV seroprevalence is greatest in regions of Africa, Mediterranean populations, and among men who have sex with men in North America and Europe [3, 4]. Both viruses are primarily transmitted through saliva, and both infect B lymphocytes and establish a lifelong reservoir. In healthy adults, the EBV reservoir is in resting memory B cells. The KSHV B-cell reservoir is less completely characterized, and there may be reservoirs in other tissues.

EBV and KSHV genomes are present in cells as closed circular plasmids in the nucleus. Viral DNA may be replicated either as part of the process that leads to the production of virions or in tandem with cellular DNA in cycling cells. For virion production, linear double-stranded viral DNA is synthesized in a process that relies on viral DNA polymerase and a variety of other viral enzymes. This process is referred to as lytic replication, and the viral gene expression that

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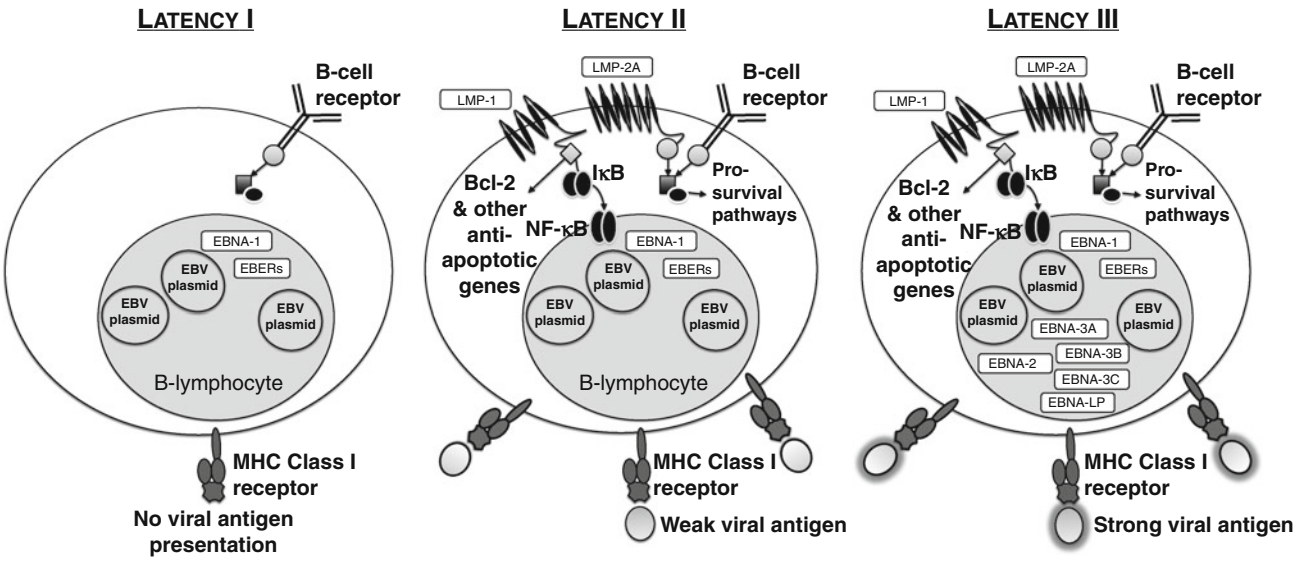

- Burkitt lymphoma
  - African
  - HIV-associated
  - Sporadic
- Plasmablastic lymphoma
- Primary effusion lymphoma

- Hodgkin lymphoma
  - Classical
  - HIV-associated
- Some PTLDs
- Most NK/T-cell lymphomas

- Most PTLDs
- AIDS-associated primary CNS lymphoma
- Some DLBCL associated with chronic inflammation

Fig. 40.1 Latent EBV gene expression patterns and the lymphomas associated with each are shown. Pro-survival and antiapoptotic pathways are targeted by EBV latent membrane protein-1 (*LMP-1*) and LMP-2A. Viral antigen presentation by major histocompatibility complex (*MHC*) class I receptors varies by latency pattern. In all cases, the EBV genome is present as a nuclear plasmid and replicates in tandem with cell division using cellular machinery

makes it possible is the lytic program. Most of the open reading frames encoded by the virus are expressed in the lytic program. For viral plasmid replication, which occurs during latency in tandem with cellular DNA replication during normal cell division, only a single viral protein is required. For EBV, this is the EB nuclear antigen 1 (EBNA-1). It serves to activate an origin of replication in the viral genome, allowing cellular enzymes to replicate the viral DNA plasmids. The KSHV latency nuclear antigen (LANA-1) serves a similar function for plasmid replication.

Several different EBV gene expression programs may accompany latency replication. These expression programs have been most completely defined in tumors. A schematic of these latency viral gene expression programs and associated lymphoma types is shown in Fig. 40.1 [5]. Expression of viral proteins may be limited to EBNA-1 (latency 1) or may include other latency proteins. Expression of the full spectrum of latency proteins drives host cell proliferation and has been referred to as the growth program or latency 3.

EBV infection of resting B cells in vitro leads to proliferation and ultimately to immortalized cell lines, referred to as lymphoblastoid cell lines (LCL). LCL grow as EBV-driven human B-cell tumors in immunodeficient mice [6]. Viral DNA replication is almost exclusively in a latency program, and viral gene expression is that of the growth program (latency 3). Among the viral proteins expressed in the growth program are viral proteins that activate and modulate key cellular pathways including the notch and nuclear factor kappa B (NF-κB), drive cell proliferation, and confer resistance to apoptosis [7–9]. Some of these proteins mimic normal B-cell signaling and activation, perhaps allowing B lymphocytes that harbor EBV plasmids but fail to express functional, high-affinity immunoglobulins to escape regulatory checkpoints and survive [2, 10, 11]. KSHV infection of B cells does not lead to immortalization, and establishment of the B-cell reservoir that harbors latent KSHV genomes is less well understood.

Following primary EBV infection, there is a brisk cytotoxic T-lymphocyte (CTL) response to viral antigens, particularly lytic and growth program antigens. This cellular immune response limits the production of virions and virus-driven B-cell proliferation. The EBV plasmids in memory B

lymphocytes, which are long-lived cells that divide at low frequency, do not express the viral antigens targeted by this cellular immune response and thus elude immune surveillance. The periodic activation of viral lytic gene expression necessary for virion production likely accounts for intermittent viral shedding that occurs throughout the lifetime of the host. Inadequate or waning cellular immunity to viral antigens associated with the growth program may allow virus-driven lymphoproliferation.

Both EBV and KSHV encode mimics of host cytokines and chemokine receptors. EBV encodes a viral homologue of IL-10 homologue, among others [12–14]. IL-10-mediated inhibition of interferon-gamma production may impact on the function of NK cells and cytotoxic T cells, lessening their reactivity against virus-infected B lymphocytes [15]. Viral IL-10 may also limit the presentation of viral antigens by MHC I molecules, again impairing the responsiveness of CTLs to EBV infection. LCL do not require IL-10 to maintain latent infection, but IL-10 appears to be important for the initial steps in immortalization of these cell lines [16]. KSHV encodes a viral homologue of IL-6 as well as a G-protein receptor homologue. These cytokines function during lytic replication, perhaps to dampen immune responses and allow latent infection to be established. There is also evidence that Toll-like receptor (TLR) stimulation is important for EBV-infected B-lymphocyte proliferation. It has been postulated that pathogens bearing TLR ligands, such as malaria, may serve this function and potentially perpetuate latent EBV infection. Other coinfections may also contribute to the increased risk for EBV-associated lymphomas seen in immunosuppressed patients by acting in similar ways.

Both viruses also encode viral homologues of the anti-apoptotic protein bcl-2 [17, 18]. The EBV proteins BHRF1 and BALF1 act as homologues of Bcl-2 (B-cell lymphoma 2), blocking apoptotic signals and allowing dysregulated or mutated lymphocytes to persist. The function of EBERs is only beginning to be elucidated, but there is evidence that they may upregulate Bcl-2 expression; facilitate the maintenance of malignant molecular events, such as the c-myc translocation in BL; and prevent interferon-α-mediated apoptosis [19]. In some cases, viral gene expression may hinder p53 tumor suppressor protein activity [20]. In particular, EBV BALF1 can block the FAS-mediated cell death pathway [18].

3 Overview of EBV- and KSHV-Associated Lymphoma

EBV is associated with B-, T-, and NK-cell lineage lymphoma or lymphoproliferative disease. KSHV is only known to be associated with B-cell lineage lymphoma or lymphoproliferative disease. Some B-lineage tumors are nearly always EBV positive, including African BL, DLBCL associated with chronic inflammation, AIDS-associated primary DLBCL of the central nervous system, and post-transplantation lymphoproliferative disorder (PTLD). Other B-cell lymphomas are associated with the EBV, but not universally so. These include sporadic and HIV-associated BL, classical HL – particularly mixed cellularity and lymphocyte-depleted subtypes – and systemic HIV-/AIDS-related lymphomas. EBV-associated hematologic diseases of T-cell or NK-cell lineages include extranodal NK-/T-cell lymphoma (ENKTL), aggressive NK-cell leukemia, peripheral T-cell lymphoma not otherwise specified, angioimmunoblastic T-cell lymphoma (AITL), and EBV-positive T-cell lymphoproliferative disorders of childhood [21–24]. While the nasal type of ENKTL is always EBV(+), the same lymphoma presenting at other anatomic sites is often but not always EBV(+). AITL is a T-cell lymphoma in which the tumor cells are EBV negative but are amid a background of EBV-positive B lymphocytes. Lymphomatoid granulomatosis, a lymphoproliferative disorder, is also consistently associated with EBV [25].

KSHV is associated with B-lineage lymphomas and lymphoproliferative disorders. Primary effusion lymphoma (PEL) is always associated with KSHV and is often associated with EBV. Germinotropic lymphoproliferative disorder is always associated with both KSHV and EBV coinfection of tumor cells. Multicentric Castleman disease (MCD) is a lymphoproliferative disorder often associated with KSHV, particularly in HIV-infected individuals, but is EBV negative. Table 40.1 lists various types of lymphoma and comments on consistency of the viral association and other cofactors.

Although the list of EBV- and KSHV-associated lymphomas includes many varieties of lymphoma, it is important to recognize that there are many histologic subtypes that seem never to be associated with either virus. Thus, among common B-cell lymphomas, follicular lymphomas are not EBV or KSHV associated; and among common T-cell lymphomas, lymphoblastic lymphoma is never EBV associated. The determinants of the association remain poorly understood, but the cell of origin; the histologic subtype; and host factors including age, gender, HIV status, comorbid conditions such as chronic inflammation, ethnicity, HLA, and other genetic polymorphisms all seem to be important.

4 EBV Lymphoma and Lymphoproliferative Diseases

4.1 Burkitt Lymphoma (BL)

Denis Burkitt called attention to what is now known as endemic Burkitt lymphoma (BL) and highlighted its

Table 40.1 EBV- and KSHV-associated diseases

Disease	EBV association	Associated factors
Burkitt lymphoma		
Endemic	~100 %	*Plasmodium falciparum*, male predominance
Sporadic	15–30 %	
Immunodeficiency associated	25–40 %	HIV
Hodgkin lymphoma		
Classical	30–40 %	Hispanic ethnicity, heightened risk for ~10 years following primary EBV infection, male predominance
HIV associated	90 %	CD4 counts typically >200 cells/μL – HAART therapy does not decrease risk
Nodular, lymphocyte-predominant subtype	~0 %	
PTLD		
B-cell origin	90 %	Use of immunosuppressive agents or transplant techniques that selectively deplete T cells
T-cell origin	20–30 %	
NK-cell origin	70–90 %	
DLBCL		
AIDS-associated primary CNS lymphoma	100 %	Advanced HIV/AIDS with very low CD4 count (<50 cells/μL)
Plasmablastic lymphoma	70–80 %	HIV, advanced age, congenital immunodeficiencies, male predominance
HIV associated	40 %	
DLBCL associated with chronic inflammation	>80 %	Long-standing foreign material or seromas, Asian ethnicity, male predominance
EBV(+) DLBCL of the elderly	100 %	possible immunosenescence with aging
Without immunodeficiency	<5 %	
Lymphomatoid granulomatosis	100 %	Immunodeficiency
Extranodal NK-/T-cell lymphoma	100 %	Asian ethnicity, CAEBV with associated hemophagocytosis
Aggressive NK-cell leukemia	>95 %	Mosquito bite allergy, Far East Asian ethnicity
Angioimmunoblastic T-cell lymphoma	90 %	EBV present in B cells of the tumor microenvironment, but not within malignant T cells

Disease	KSHV association	Associated factors
Primary effusion lymphoma	100 %	EBV – 80 %, advanced HIV/AIDS, transplant patients Mediterranean individuals, male predominance
KSHV-positive multicentric Castleman disease	100 %	EBV – 0 %, HIV/AIDS, older age, no previous HAART exposure, CD4 nadir >200 cells/μL, male predominance
Germinotropic lymphoproliferative disorder	100 %	EBV – 100 %

Abbreviations: *HIV* human immunodeficiency virus, *HAART* highly active antiretroviral therapy, *PTLD* posttransplantation lymphoproliferative disorder, *NK cell* natural killer cell, *DLBCL* diffuse large B-cell lymphoma, *AIDS* acquired immunodeficiency syndrome, *CNS* central nervous system, *CAEBV* chronic active EBV

occurrence in equatorial Africa in 1958 [26, 27]. The epidemiology hinted at an infectious etiology. Epstein and Barr succeeded in culturing tumor cell lines in which herpesviruses were observed by electron microscopy [28]. Serologic studies in African children solidified the viral association [29, 30]. Demonstration that the virus could immortalize B lymphocytes in vitro lent biological plausibility to the association as did evidence of experimental induction of EBV lymphoma in nonhuman primates [31, 32]. Thus, EBV was the first virus associated with a human cancer.

Endemic BL or African BL has several distinctive features: it arises in young children in areas where malaria is holoendemic, including equatorial Africa (Fig. 40.2, adapted from Burkitt and Wright [33]) and Papua New Guinea. It characteristically presents as a rapidly enlarging jaw or abdominal mass, and it is nearly 100 % associated with EBV. It is 3–5 times more common in boys than girls [34].

BL is also recognized outside these regions. The age distribution and the clinical presentation of sporadic BL are distinct. BL outside endemic regions is relatively rare, accounting for only about 1 % of adult-onset lymphomas and 30 % of lymphoma in children in the Western world [34, 35]. Whereas endemic BL occurs with an incidence of 50 per million, the incidence of BL in non-endemic areas is only 2 per million [34, 35]. It only very rarely presents as a jaw

Fig. 40.2 The distribution of endemic Burkitt lymphoma, depicted as *circles*, is contained within the malaria belt of Africa (outlined and shaded in *gray*)

mass. The EBV association is variable but is higher in other equatorial areas, such as parts of Brazil, Columbia, and Malaysia [36]. In the United States, it has been estimated that 20 % of sporadic BL cases are EBV positive. BL also occurs with increased frequency in HIV-infected patients (see below).

Histologically, BL appears as a relatively homogeneous proliferation of medium-sized lymphocytes with scant, intensely basophilic cytoplasm filled with lipid vacuoles. The mitotic index is high and apoptotic cells, often engulfed by macrophages, give the classic "starry sky" appearance. BL tumor cells express CD10 and Bcl-6, both germinal center markers, as well as CD20, CD19, and CD79a [37]. Essential for diagnosis is a Ki67 of greater than 99 % and demonstration of a c-myc translocation [37, 38]. The chromosomal translocation occurs between the c-myc gene on chromosome 8 and one of the B-lymphocyte immunoglobulin genes (on chromosomes 2, 14, or 22). The characteristic chromosomal abnormalities are common to both endemic and non-endemic BL (although differences in the precise chromosomal breakpoints have been noted between the endemic and sporadic variants of BL) [39, 40].

Whether EBV-associated BL occurs in the setting of malaria endemic areas, HIV infection, or sporadically, the pattern of viral gene expression appears to be the same. EBNA-1 is the only viral protein expressed. Regulatory RNAs are also expressed. Investigation of the EBV-positive BL cell lines such as the Akata cell line and an isogenic derivative cell line which lacks EBV genomes demonstrates that loss of the viral genome corresponds to slower proliferation, increased sensitivity to apoptotic stimuli, and loss of tumorigenicity in immunocompromised mice. The precise roles that EBNA-1 and viral RNAs play in determining these characteristics have yet to be determined.

The geographic distribution of endemic BL is that of holoendemic malaria. The prevalence of active malarial parasitemia in young children in these regions is approximately 60 % and the risk of transmission is yearlong [41]. Primary EBV infection usually occurs before 1 year of age [42]. Holoendemic malaria may alter the risk of primary EBV infection in very young children [43]. Malaria may alter the character of the EBV-infected B-cell population and predispose to the chromosomal translocations that are associated with BL. The details of the interplay between viral infection, malaria, and associated immune dysfunction or other environmental factors remain a subject of speculation. However, attention has been called to the observation that the *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1) on the surface of malaria-infected erythrocytes activates memory B lymphocytes and induces lytic EBV replication [44, 45]. This might provide an explanation for the observation that EBV DNA is detected at higher levels in blood from children in areas with holoendemic malaria in comparison with blood from children in other areas [42, 46]. Malaria infection may also blunt the interferon-γ-mediated EBV-specific cytotoxic T-cell responses, activate Toll-like receptors (TLR), and increase the frequency of immunoglobulin double-strand breaks and translocations, with potential mechanisms of pathogenesis depicted schematically in Fig. 40.3 [41, 47–50]. EBV gene expression may permit genetically Unstable B lymphocytes to escape apoptotic signals [51, 52].

4.2 Hodgkin Lymphoma (HL)

EBV can be detected in tumor cells of HL in a variable fraction of cases [53–55]. When present in tumor cells, the virus is usually present at all sites involved by tumor, both at presentation and at relapse [56]. Approximately 9,000 new cases of HL are diagnosed annually in the United States, representing around 12 % of all lymphoma [57]. Approximately one third of HL tumors in the United States and Europe are EBV associated, making HL the most common EBV-associated lymphoma in the Western world [58, 59]. In Latin America and Africa, HL are more frequently EBV positive [60–62].

Factors associated with EBV positivity include mixed cellularity subtype, history of symptomatic infectious mononucleosis, male gender, Hispanic ethnicity, low

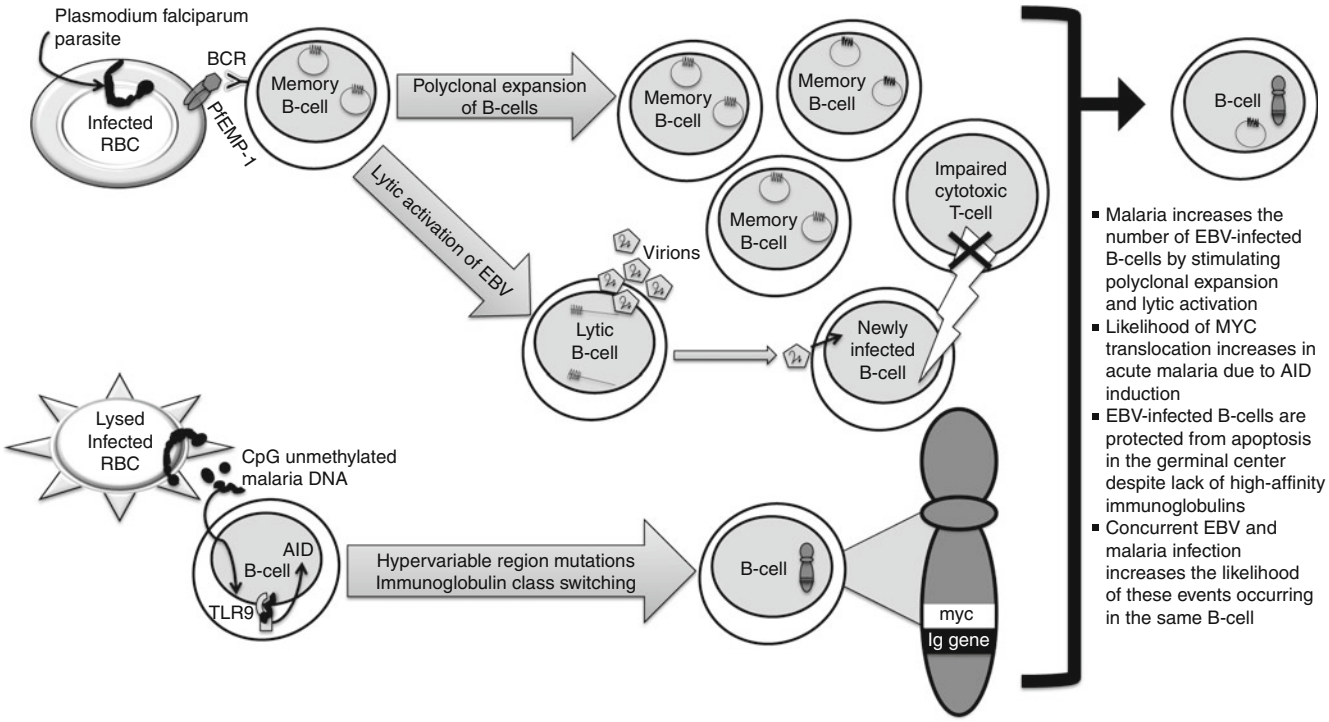

Fig. 40.3 Potential mechanisms by which *Plasmodium falciparum* (malaria) infection interacts with EBV to promote the development of endemic Burkitt lymphoma. The cysteine-rich interdomain region 1α of the *Plasmodium falciparum* erythrocyte membrane protein 1 (*PfEMP1*) interacts with B cells via the B-cell receptor (*BCR*). Infected red blood cells (*RBC*s) release CpG-unmethylated malaria DNA, which may stimulate Toll-like receptor 9 (*TLR9*) and induce activation-induced cytidine deaminase (*AID*) gene expression, thereby increasing the likelihood of a MYC translocation during immunoglobulin class switching and hypervariable region mutation. Impaired function of EBV-specific cytotoxic T cells may also play a role in endemic Burkitt lymphoma pathogenesis

socioeconomic status, and very young or very old age [58, 60, 63, 64]. Other at-risk populations for EBV-positive HL include HIV-positive patients and recipients of solid organ or hematopoietic stem cell transplants, with EBV positivity seen in 90 % of the HL tumors in these immunosuppressed populations [65–67]. The risk of developing EBV-positive HL after symptomatic primary EBV infection is approximately threefold over baseline, peaking at 2 years postinfection and remaining elevated for about 10 years after infection [64]. There is also evidence that the serologic response to viral infection differs in patients who develop EBV-associated HL [68].

In industrialized countries, HL follows a bimodal age distribution, occurring more commonly in young adults and the elderly. In economically developing countries, there is a higher incidence of HL in young children. Variation in the incidence of HL with regard to age seems to depend also on factors such as social class, hygiene, and housing density. Children who grow up in economically developed countries with fewer infectious exposures and low housing density may have a higher risk of HL in young adulthood [69]. In the United States, day care attendance is associated with lower risk of HL, although not specific to EBV-positive HL. One hypothesis to explain these results is that immune stimulation following infectious exposures is protective [70].

The EBV gene expression pattern in HL is latency 2, including latent membrane protein 1 (LMP-1), LMP-2A, and EBNA-1 expression and lacking EBNA-2 expression [71]. LMP-1 and LMP-2A may allow B cells lacking functional immunoglobulin genes rearrangements to circumvent apoptotic signals. The cellular gene expression profile in HL may reflect the effects of EBV gene expression in tumors. HL tumors expressing the tyrosine kinase Lyn cytoplasmic protein, a Src family protein tyrosine kinase, are more commonly EBV positive [72]. LMP-2A, a viral gene whose expression interferes with normal B-cell receptor signaling, appears to exert its effects by signaling through the Lyn protein [10]. In contrast to BL, there are no tumor-derived cell lines that retain EBV genomes and show this pattern of gene expression.

HL tumor cells (Reed-Sternberg cells) are infrequent amid a mix of infiltrating macrophages, lymphocytes, and eosinophils. A skew toward Th1 activity in the microenvironment of EBV-positive tumors has been described, although the significance of this remains undefined [73].

EBV-positive HL tumors have also been found to have increased expression of the chemokine CCL20, which increases the migration of regulatory T cells in HL tumors [74]. Several reports have described increased numbers of CD68(+) or CD163(+) tumor-infiltrating macrophages (TAMs) in EBV-associated HL, but not EBV-negative HL the same histologic subtypes [75–80].

4.3 Posttransplantation Lymphoproliferative Disorder (PTLD)

PTLD was first recognized when EBV-associated lymphomas were noticed with increased frequency in the posttransplant period. Over the years, PTLD lacking EBV has also been recognized. PTLD occurs weeks to decades after solid organ transplantation (SOT) or hematopoietic stem cell transplantation (HSCT) [81–83]. In the first year after transplant, most cases of PTLD are EBV positive, while cases occurring many years after transplantation are rarely EBV positive [84–89]. Advances in immunosuppressive regimens and approaches have lowered the incidence of EBV-PTLD, especially those that typically arise within the first year of transplantation. The percentage of transplant recipients varies from less than 1 % in some series to greater than 25 % in others. The organ transplanted, the EBV serostatus of the recipient, the age of the recipient, the immunosuppressive regimen, the occurrence of organ rejection or graft-versus-host disease, and the treatment for these complications are all important determinants. Immunosuppression to prevent or treat graft rejection, and in HSCT recipients to prevent or treat graft-versus-host disease (GVHD) leads to an inadequate cellular immune response to the proliferation of EBV-infected lymphocytes [90–92]. EBV-PTLDs most commonly exhibit a type 3 latency pattern. Such tumors never arise in an immunologically intact host.

Clinically, PTLDs are a heterogeneous group of lymphoproliferative disorders. They are often extranodal and are often associated with systemic symptoms. In SOT recipients, they very often involve the transplanted organ. Pathologically, most are of B-cell lineage but T-cell PTLD is also well recognized in this setting. PTLD has been classified into four major categories by the World Health Organization: early lesions, polymorphic PTLD, monomorphic PTLD (subclassified by cell lineage and then by lymphoma), and classical HL-type PTLD [93]. Early lesions are always EBV positive and tend to occur in the weeks to months following transplantation [91]. Polymorphic lesions contain a mixture of lymphocytes, immunoblasts, and plasma cells. Unlike early lesions, the lymph node architecture is not preserved, and cellular atypia, high proliferation, and necrosis will often be present. The involved B lymphocytes are EBV positive and have both clonal immunoglobulin rearrangements and clonal EBV genomes [89]. Monomorphic PTLDs are lymphomas that are morphologically similar to aggressive non-Hodgkin lymphomas (NHLs). However, these lesions are classified as PTLDs based on a history of transplantation, even if remote [89]. Monomorphic lesions are less often EBV positive and typically harbor more cellular mutations and cytogenetic abnormalities than polymorphic or early lesions [84, 85]. NK-cell and T-cell PTLDs can arise, typically years after transplantation [94, 95]. Most NK-cell PTLDs are EBV positive [95, 96]. Classical HL-type PTLD tumors are nearly always EBV positive, despite their tendency to arise years after transplantation [89]. Primary CNS PTLD is rare (less than 1 % of PTLD cases), yet they are much more common than primary CNS lymphoma in the general population, and in contrast to the general population, over 90 % are EBV positive [97, 98].

In SOT, PTLD typically arise in the lymphocytes of the recipient, and thus the EBV status of the donor is important, with higher incidence of EBV-PTLD seen in EBV-seronegative transplant recipients receiving organs from EBV-seropositive donors [99–101]. Not unexpectedly, EBV-PTLD is more common in the pediatric SOT population as compared to the adult SOT population, as this patient group has often not yet been exposed to primary EBV infection.

In contrast to SOT, the lymphocytes that evolve into EBV-PTLD in HSCT patients are typically donor derived [99]. The most vulnerable period for HSCT patients to develop an EBV-PTLD is in the first several months following transplantation, before reconstitution of an EBV-specific CTL+ response, with the majority of cases arising within the first year post-HSCT. In HSCT, risk factors for EBV-PTLD revolve mainly around aspects of the conditioning regimen, graft manipulation, and the pharmacologic approach to GVHD prophylaxis. With regard to conditioning, the use of antithymocyte globulin (ATG) is associated with high risk of EBV-PTLD [102–105]. Grafts can be unmanipulated, both T and B cell depleted, or selectively T cell depleted, with selectively T-cell-depleted grafts being associated with a 15-fold higher incidence of EBV-PTLD [91, 102]. The pharmacologic approach to GVHD prophylaxis or its treatment also modulates EBV-PTLD risk, again with the highest risk being associated with agents that selectively deplete T lymphocytes [106]. A shorter duration of post-HSCT immunosuppression decreases the risk of EBV-PTLD. As an example, high dose, posttransplantation cyclophosphamide as GVHD prophylaxis seems to be associated with very low rates of PTLD in HSCT patients [107]. Chronic GVHD is associated with increased risk of EBV-PTLD [108–110].

Particular immunosuppressive regimens have been implicated in PTLD. However, because agents are mainly used in combination, the risks associated with particular drugs are often difficult to discern [101, 111, 112], and EBV-PTLD incidence may reflect the impact of a regimen than a particular drug. Historically, the incidence of PTLD has

risen as novel immunosuppressive drugs emerged and then fallen again with improved drug level monitoring and tighter therapeutic windows [113, 114]. Monoclonal antibodies that selectively deplete T cells, such as muromonab-CD3 (OKT3) and ATG, have been associated with heightened risk of EBV-PTLD, while those that deplete both B and T cells, such as alemtuzumab, may be associated with a lesser risk [115–118].

4.4 EBV-Associated Lymphoma in HIV Patients

Patients with HIV are 60- to 200-fold more likely to develop lymphoma than the general population [119]. A number of different B-cell lymphomas are increased in HIV patients. The first recognized were primary central nervous system lymphoma, BL, and DLBCL. These lymphoma types were thus recognized as "AIDS-defining" illnesses before HIV was recognized. More recently, HL has been recognized to be increased in HIV patients.

Primary central nervous system lymphoma is rare in the general population and generally not EBV associated. However, in AIDS patients in the era before highly active antiretroviral therapy (HAART), the incidence was increased 5,000-fold. In the mid-1990s, almost half of all primary central nervous system lymphoma in the United States were AIDS associated [120]. These tumors almost exclusively occurred in patients with very low CD4 counts and are virtually all EBV associated. With HAART therapy, their incidence substantially decreased. DLBCL (including immunoblastic lymphoma) is also increased in patients with HIV, but only approximately 98-fold. Less than half of these are EBV associated. The incidence of DLBCL dropped with HAART, but not nearly as dramatically as that of primary central nervous system lymphoma. The incidence of BL in the United States and Western Europe is also increased with HIV infection. In the United States, the estimate is a 57-fold increase. Approximately 30–50 % of AIDS-associated BL cases are EBV positive [34, 121]. Patients often have CD4 counts above 200 cells/μL at BL diagnosis [122]. In contrast to primary central nervous system lymphoma and DLBCL, the incidence of BL seems not to have changed with HAART. Little is known as to the specific impact of HAART on the proportion of EBV-associated DLBCL or BL among patients with AIDS.

AIDS primary central nervous system lymphoma seems to reflect profound end-stage cellular immune dysfunction. These lymphomas often express the full spectrum of EBV genes (latency 3 in Fig. 40.1), and as end-stage immune dysfunction is prevented by HAART therapy, the incidence has waned. At the other extreme are the BL, which when EBV associated express only EBNA-1 (latency 1). EBNA-1 is not as readily targeted by cytotoxic T cells as are some of the other EBV latency genes, and the incidence of these tumors may relate to other aspects of chronic HIV infection such as polyclonal B-lymphocyte activation and not simply loss of cellular immunity. The limits of our understanding of the interplay of viruses and parasites in the pathogenesis of BL are underscored by the report that in regions of Africa where malaria is endemic and vertical transmission of HIV to infants is common, there does not appear to be a heightened risk for endemic BL among those who are HIV positive [123].

Plasmablastic lymphoma (PBL) is a subtype of DLBCL that is typically EBV positive, tends to arise in the oral cavity and jaw, and is most commonly described in young men with HIV/AIDS, although they can arise in other settings of immunosuppression or immunosenescence [124]. As the name would suggest, the morphologic appearance is of blastoid plasma cells, CD20 staining is typically negative, the proliferative index is high, and plasma cell markers are expressed [124]. Translocations between the myc gene and immunoglobulin genes are a common finding [125, 126]. Similarities between PBL and plasmablastic transformation of multiple myeloma have been noted, with suggestion that these two diseases may be closely related [127]. Monoclonal immunoglobulins can be detected in the serum of patients with PBL and lytic bone lesions, classically seen in multiple myeloma, have also been reported [127].

There are also lymphomas that occur in HIV patients that harbor both EBV and KSHV genomes. These are discussed separately below.

4.5 DLBCL Associated with Chronic Inflammation

EBV-associated lymphomas have been recognized to arise in the setting of chronic inflammation [93]. The association between chronic inflammation and NHL was first reported in 1987 in Japanese patients with long-standing pyothoraces. These pyothoraces developed as a result of iatrogenic induction of pneumothoraces for the treatment for pulmonary tuberculosis before antituberculosis drugs were available [128]. These patients developed NHL decades after their tuberculosis treatment, most commonly of DLBCL histology, always involving the pleural cavity, and often with extensive necrosis and immunoblastic morphology [129]. The association with EBV was later noted upon examination of additional cases, with over 70 % of cases reported to harbor EBV [130–132]. Since the first description, EBV-positive lymphomas have been described to arise in other areas of foreign-body-associated chronic inflammation, including prosthetic cardiac valves, pacemaker pockets, surgical mesh implants, and joint prostheses [133–137]. Additional cases have also been described in areas of chronic fluid collections or infection, arising in renal, tes-

ticular, and adrenal gland pseudocysts, in inflamed synovial joints of patients with rheumatoid arthritis, in bones harboring chronic osteomyelitis, in empyemas, and in skin in areas of venous stasis ulcers, again developing after decades of inflammation and nearly all EBV positive [138–141]. These lymphomas typically have a latency 3 pattern of EBV gene expression [142].

4.6 EBV-Positive DLBCL of the Elderly

First described in older, apparently immunocompetent Japanese patients and referred to as age-related EBV-positive B-cell lymphoproliferative disorder [143], this entity has been given the provisional category "EBV-positive DLBCL of the elderly" by the World Health Organization in 2008 and has been reported in Western countries as well [93]. The incidence appears to be higher in Asian and Latin American countries, compared to European countries and the United States [144]. Typically, these lymphomas arise in older individuals (>50 years, median age 75 years) without known immune dysfunction and tend to be of non-germinal center, activated B-cell phenotype (CD10 and BCL6 negative, MUM1 positive) [145–148]. The association between these lymphomas and advanced age has led researchers to postulate that they arise in the setting of immune senescence and decreased control of latent EBV infection. Supporting this is the observation of less restricted, latency 2 or 3 patterns of viral gene expression in these lymphomas [143, 149]. Polymorphic, monomorphic, and lesions resembling classical HL have been described [125, 150]. These lymphomas are typically aggressive, and outcomes for these patients appear to be worse than for EBV-negative DLBCL, with older age (>70 years) an additional independent predictor of inferior outcomes among these patients with EBV-positive tumors [143, 146]. NF-κB activation has been described in these lymphomas [146].

4.7 Methotrexate-Associated Lymphoproliferative Disease

There may be increased risk for patients with autoimmune diseases that are treated with methotrexate to develop lymphoproliferative diseases, 40 % of which are EBV positive [151–153]. While it is difficult to distinguish the increased risk for lymphoma that is associated with autoimmune diseases from the potential increased risk for lymphoma associated with the immunosuppressive treatment [154], reports of spontaneous regression of these lymphomas upon cessation of methotrexate suggest that the latter has a possible role in lymphomagenesis [152]. Furthermore, spontaneous regression with methotrexate withdrawal was mainly observed in patients with EBV-positive lymphomas, suggesting that drug-induced decreases in immune control of EBV may have driven the lymphoproliferation [152]. The presence of immunoblasts and plasmablasts is often described [151, 153]. Patients treated with methotrexate have also been reported to subsequently develop LYG.

4.8 Congenital Immunodeficiency

Many congenital immunodeficiencies are linked with an increased risk of lymphoma. These include severe combined immunodeficiency, Wiskott-Aldrich syndrome, ataxia-telangiectasia, X-linked lymphoproliferative syndrome, and common variable immunodeficiency [155]. In some of these syndromes, such as severe combined immunodeficiency and X-linked lymphoproliferative syndrome, virtually all of the lymphoproliferative disease is EBV associated. In other syndromes such as ataxia-telangiectasia, approximately half of cases are EBV associated. Patients with X-linked lymphoproliferative disease (XLP, or Duncan's disease) have a rare, X-linked primary immunodeficiency in which patients are highly susceptible to severe EBV infection. It is the encounter with EBV that often leads to the diagnosis of XLP. Primary EBV infection is typically fulminant in XLP patients, and fatality rates are high due to either complications of lytic infection or the development of EBV-associated lymphomas. XLP patients have a mutation in the gene *SH2D1A*, which codes for a signaling lymphocyte activation molecule (SLAM)-associated protein (SAP). SAP is normally expressed on all T and NK cells. The deficiency of SAP in XLP leads to the inability of NK cells and T cells to recognize and eradicate EBV-infected B lymphocytes.

4.9 Lymphomatoid Granulomatosis

Lymphomatoid granulomatosis (LYG) is a rare EBV-associated lymphoproliferative disorder of B-cell origin that may involve the lungs, skin, central nervous system, and kidneys [156–158]. LYG is graded based on the number of EBV-positive cells in the biopsy specimen, and prognosis is inferior for higher-grade disease. Low-grade lesions often undergo spontaneous remission, but LYG can evolve into high-grade, aggressive EBV-associated DLBCL [158]. Patients typically have no known preexisting immunodeficiency but often have a history of autoimmune disease, recurrent viral infection, or low CD4 or CD8 cell counts [158]. Patients with Wiskott-Aldrich syndrome and common variable immunodeficiency or those receiving intensive chemotherapy for leukemia have been reported to develop LYG, again pointing to immune dysfunction as a predisposing factor [159]. Interestingly, LYG seems to be a very rare entity in HIV patients.

4.10 Extranodal NK-/T-cell Lymphoma and Aggressive NK-Cell Leukemia

Extranodal NK-/T-cell lymphoma is a very aggressive lymphoma that is more common in Asian countries and in Native American populations in parts of Central and South America, accounting for up to 10 % of all NHLs in some regions of the world [160–162]. Epidemiology studies in the United States have identified higher rates in Hispanics and Asian Pacific Islanders [163]. EBV is universally present in these lymphomas. Patients present with destructive lesions involving the nasal cavity, and historically, this disease entity was called "lethal midline granuloma." The prognosis is typically poor, especially for advanced stage disease.

Other high-risk groups for EBV-associated T-cell lymphoproliferative disorders and lymphomas include patients with a history of chronic active EBV (CAEBV) infection [164]. In Asians, particularly in the Far East, an association between CAEBV infection, mosquito bite hypersensitivity, and subsequent risk for NK-cell leukemia/lymphoma is noted, and Tokura-Ishihara disease is defined by this triad [165–169]. Patients with NK-cell leukemia often present with hepatosplenomegaly and cutaneous lesions. The presence of EBV in NK-cell leukemia distinguishes it from a more indolent disease, NK lymphoproliferative disease. Hemophagocytic syndrome is a common complication of disseminated disease or can precede the development of an EBV-associated T-cell malignancy in patients with CAEBV or congenital immunodeficiencies.

4.11 Angioimmunoblastic T-Cell Lymphoma (AITL)

Angioimmunoblastic T-cell lymphoma (AITL) is a mature T-cell lymphoma where patients classically have associated immune dysfunction (autoimmune cytopenias, hypergammaglobulinemia, and/or opportunistic infections). AITL tumors will often have polyclonal, or more rarely monoclonal, proliferations of EBV-positive B cells amid the malignant T cells. HHV6-positive B cells have also been reported to be present in AITL tumors, and coinfection with both herpesviruses can occur [170]. A reported phenomenon has been the development of a subsequent EBV-positive B-cell lymphoma after treatment for AITL [171]. One series noted that AITL patients with high EBV viral loads were more likely to have monoclonal, rather than polyclonal, B cells in their tumors; these patients were also more likely to subsequently develop an EBV-positive B-cell lymphoma [170].

4.12 KSHV and KSHV/EBV Lymphoma and Lymphoproliferative Disease

4.12.1 Primary Effusion Lymphoma (PEL)

Primary effusion lymphoma (PEL) is a very rare KSHV-associated body cavity-based lymphoma that occurs in the context of severe immunodeficiency, such as patients with AIDS and very low CD4 counts or rarely in solid organ transplant recipients [172]. PEL tumors are characterized by lymphomatous effusions involving the pleural, pericardial, or peritoneal spaces [172]. Lymphadenopathy is not present in the majority of cases, although spread to extracavitary sites does occur [173]. These tumors are always KSHV positive and 80 % are also EBV positive [172]. EBV gene expression is very restricted (type 1 latency), while KSHV gene expression includes latency and sometimes lytic viral genes. Although these tumors are quite rare, tumor-derived cell lines are readily established in vitro. These tumor cell lines have been extensively studied. It is not uncommon for patients with PEL to have other KSHV-associated diseases concurrently, such as Kaposi sarcoma or multicentric Castleman disease (MCD). Thus, lymphadenopathy or masses on staging evaluations should be thoroughly investigated. The lymphoma itself typically has a very aggressive course, and the prognosis for patients with PEL is extremely poor with median survival on the order of months [174]. Due to the rarity of the disease, treatment options are by no means standardized and rely on best available practices and expert opinion.

KSHV-positive solid lymphomas that are morphologically very similar to PEL have also been reported, again rare and predominantly in the setting of advanced HIV [173]. There is a tendency for these lesions to involve the gastrointestinal tract or skin, notably the same tissues of the body that tend to be involved in Kaposi sarcoma [173].

Cytologically, PEL tumor cells are large, monoclonal immunoblasts with prominent nucleoli. The tumor cells are of B-cell origin, exhibiting clonal immunoglobulin gene rearrangements, although they lack B-cell markers. On flow cytometry, CD45 positivity is typical, and CD30 or CD38 positivity is sometimes present. The association with KSHV can be confirmed by staining for a KSHV latent nuclear antigen, LANA-1.

KSHV is well recognized to produce viral IL-6, as well as potentially increase human IL-6 production, both contributing to the systemic inflammation seen in several KSHV-associated diseases [12, 15]. Viral IL-6 has been shown to activate various signaling cascades, including the STAT1, STAT3, and MAP kinase pathways, function as a pro-growth signal, and stimulate the production of acute phase proteins that reduce recognition of virally infected cells by the immune system [15].

4.12.2 Multicentric Castleman Disease (MCD) and KSHV/EBV Dual Positive Diseases

The plasma cell variant of MCD is usually associated with KSHV, and in HIV patients, it is virtually always KSHV associated [121]. MCD is a non-clonal lymphoproliferative disorder, although it characteristically has monotypic expression of the IgM heavy chain and the lambda light chain. In contrast to PEL, it is associated with lytic KSHV gene expression. Among the viral genes expressed is the viral IL-6 homologue, and this likely accounts for some of the constitutional symptoms such as fever and night sweats that are often associated with MCD. In some instances, it can progress to KSHV-positive lymphoma.

There are also reports of dually infected lymphomas. Germinotropic lymphoproliferative disorder, an indolent lymphoproliferative disease that harbors EBV and KSHV coinfection within involved cells, has been reported in a handful of immunocompetent, HIV-negative patients [175]. The disease has been reported to be localized, with good responses to a variety of therapies. An EBV-positive, KSHV-positive lymphoproliferative disorder similar in some ways to germinotropic lymphoproliferative disorder, but occurring in an HIV patient with a very aggressive clinical course, has also been reported [176, 177].

The presence of KSHV in tumor tissue is confirmed by immunohistochemistry for latency-associated nuclear antigen (LANA-1). MCD is often associated with high levels of lytic viral replication, and KSHV viral loads in the peripheral blood may be quite elevated.

5 Tools for Epidemiology: EBV or KSHV and Clinical Specimens

Tumor Specimens. Recognized EBV or KSHV lymphoma associations reflect the presence of viral genomes in tumor cells. A variety of techniques have been used to demonstrate the association. Extremely sensitive methods for detection of viral DNA in tumor tissues may be difficult to interpret because lymphocytes that harbor virus but are not malignant may be detected in tumor tissue. Histologic approaches that demonstrate the presence of viral DNA, RNA, or protein in tumor cells have become standard. Among the most useful tools for establishing EBV association has been the detection of EBER RNA by in situ hybridization. These noncoding short viral RNAs are expressed at high abundance and are readily detected in most EBV-associated tumors. Immunohistochemistry for viral antigens such as LMP-1 is also routinely performed to assess the EBV status of a tumor. This technique is particularly sensitive in HL but less so in other EBV-associated lymphomas where LMP-1 may not be expressed [178]. For detection of KSHV, the most standard assay has been immunohistochemistry for LANA-1. LANA-1 is expressed in all KSHV-associated malignancies.

Serology. Antibody responses to viral antigens have been a key tool in defining some of the virus-disease associations [43, 179]. Antibodies against viral antigens show clear evidence of past infection and, when appropriate serum collections are available, allow the timing of infection to be established. However, most individuals with EBV- and KSHV-associated lymphomas show antibody response patterns that do not readily distinguish them from healthy individuals also harboring the virus. Thus EBV serology at present does not allow individuals with EBV-associated HL to be distinguished from individuals with HL without EBV, although there are statistical associations with particular patterns of response. Thus among patients with HL with EBV in the tumor, the ratio of antibody titer against EBNA1 to antibody titer against EBNA2 is lower than among other patients with HL or among healthy controls [68]. New approaches to serology involving arrays of antigens may emerge as useful tools for investigation [180].

EBV and KSHV DNA. Viral DNA detected in the peripheral blood by real-time quantitative polymerase chain reaction (qPCR) techniques, both in lymphocytes (PBMCs – peripheral blood mononuclear cells) and in cell-free fractions (serum or plasma). However, detection of EBV DNA in the peripheral blood is not specific for any particular EBV-associated disease entity. As EBV infection is ubiquitous with lifelong infection in the majority of adults, even healthy adults with no EBV-related lymphoproliferative disorder will have EBV DNA detected by qPCR in a small percentage of circulating B lymphocytes. In cell-free fractions such as serum or plasma, EBV DNA may be tumor derived or virion derived. While healthy EBV-seropositive adults will rarely have EBV DNA detected in cell-free fractions, immunocompromised patients, such as patients with HIV, posttransplantation immunosuppression, or congenital immunodeficiencies, can often have elevated EBV DNA detected in the blood, with or without an associated EBV-positive malignancy [181]. EBV DNA quantification by qPCR is used clinically in some malignancies, such as EBV-positive undifferentiated nasopharyngeal carcinoma (NPC). Cell-free EBV DNA quantification has been shown to perform well as a clinical tool for staging NPC patients, assessing response to therapy, and prognostication [182–184]. EBV DNA monitoring is often used in the posttransplant setting to identify patients at high risk for developing EBV-PTLD. Recently, there has been growing evidence that plasma EBV DNA viral load may have prognostic and predictive significance in other EBV-associated lymphomas, such as ENKTL and HL [185–190].

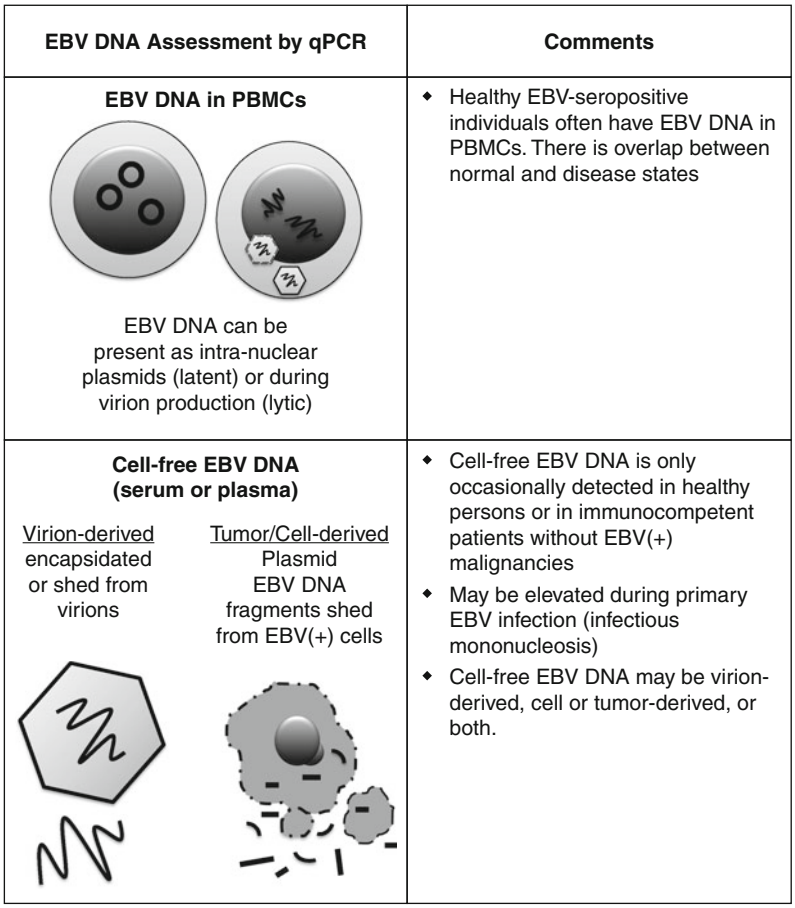

Fig. 40.4 Quantification of EBV DNA copy number by real-time polymerase chain reaction (*qPCR*). EBV DNA can be detected in peripheral blood mononuclear cells (*PBMCs*), as well as in cell-free specimens (serum or plasma). Viral DNA can be virion-derived or tumor/cell-derived

KSHV DNA can also be detected in PBMC or in cell-free fractions of blood. As with EBV, viral DNA may be detected in individuals without KSHV-associated tumors [191]. There have been few studies in patients with KSHV-associated lymphoma or lymphoproliferative disease, although very high viral DNA copy numbers have been reported in HIV patients with MCD and KSHV viral loads appear to be predictive of relapse [192]. Figure 40.4 outlines the sources of EBV detected in the blood by real-time polymerase chain reaction.

6 Vaccines for EBV-Positive Lymphomas

A vaccine that prevented primary infection might well reduce the incidence of EBV-associated disease including lymphoma [189]. A subunit vaccine targeting the viral glycoprotein gp350 has been evaluated in small clinical trials [194]. The vaccine did not prevent primary infection but did reduce the incidence of symptomatic infectious mononucleosis. Whether vaccination would have any impact on the incidence of EBV lymphoma is not clear. However, insofar as EBV-associated HL is more common in patients with history of symptomatic primary EBV infection, the argument has been made that reducing the incidence of infectious mononucleosis might reduce the incidence of EBV-associated HL [193]. An alternative approach to prevention has involved trials of polypeptides encoding epitopes from especially immunodominant latency viral antigens such as the EBNA-3 family of proteins [195]. Finally, there may also be a role for "therapeutic vaccines" for patients with EBV-associated tumors. Such an approach is being actively explored in another EBV-associated malignancy, nasopharyngeal carcinoma [196]. Underlying immune dysfunction in some target patient populations may hinder the efficacy of such vaccines [197].

7 Treatment of EBV-Associated Lymphoma

Generally, the approach to the treatment of EBV-associated lymphomas does not specifically target EBV and is similar to the approach to lymphomas that are not associated with EBV. However, there are few special situations in which targeting viral antigens are being used clinically [198]. In bone

marrow and hematopoietic stem cell transplantation, T-cell depletion and other manipulations to prevent graft-versus-host disease are associated with especially high rates of EBV-PTLD. Restoring T-cell immunity with infusion of donor lymphocytes or EBV-specific donor T-cell products is often effective in preventing or treating these tumors [199–206]. Similar adoptive cellular approaches have also been applied in SOT patients, although these have typically involved in vitro expansion and infusion of autologous or third-party EBV-specific cytotoxic T cells. These approaches are also being explored for the treatment of EBV-associated HL. However, since in EBV-associated HL the viral antigens being expressed by tumor cells are more limited than in EBV-associated PTLD, the activated T cells infused are typically targeted against particular viral antigens such as LMP-1 rather than latency viral antigens in general.

7.1 Unresolved Problems

The discovery of EBV in association with endemic BL seemed to provide a simple explanation for the geographic distribution of the tumor. The observation that the virus immortalized lymphocytes in vitro strengthened that conclusion. A half century later, the explanation for the association remains uncertain. While we suspect that both EBV and malaria play a role, another tumor that is similar in its histology and molecular characteristics occurs in the absence of either infection. At the same time, although the list of EBV-associated lymphomas continues to grow, it is not a list that includes every lymphoma, or every B-cell lymphoma, or every lymphoma arising in an immunocompromised patient. And when we sort out the answers to these mysteries, the mysteries of KSHV and the dual infection lymphomas and lymphoproliferative diseases await.

There is still much to be learned about how herpesviruses alter signaling pathways; mimic B-cell biology; drive or skew the production of cytokines, chemokines, and growth factors; influence noninfected cells of the microenvironment; and contribute to epigenetic and genetic changes. Emerging disease entities, such as EBV-positive DLBCL of the elderly, which occurs in patients that have no definable immune dysfunction but are postulated to be vulnerable to virally related lymphomas due to immune senescence of aging, require further investigation to understand the pathogenesis of the disease.

In HIV patients, the incidence of EBV-positive HL is rising despite the use of HAART, and most patients are diagnosed with reasonably robust CD4 counts. The abnormalities in the immune system that place these patients at ongoing risk for lymphoma, despite control of their HIV, are generally thought to relate to chronic B-cell activation and proliferation, but are not well characterized. Additionally, EBV-related T- and NK-cell lymphomas are much more common in Asian countries than in other parts of the world, yet the environmental, genetic, immune, and other factors that modulate this difference in EBV tropism and patterns of disease require further investigation. Insights into the factors that differ in patients that develop these virally related lymphomas may have implications for screening, diagnosis, therapy, and even prevention. Currently, there are no approved therapies specifically indicated for herpesvirus-associated lymphomas. In the research setting, clinical trials using EBV-specific CTLs, vaccines, and other targeted therapies are ongoing. However, the evasiveness of latent herpesvirus infection continues to present many challenges in the development of targeted therapies. There is much interest in the development and validation of EBV-related biomarkers to assess response to therapy, provide prognostic information, and serve predictive functions in the treatment of EBV-positive lymphomas.

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Epstein-Barr Virus: Nasopharyngeal Carcinoma and Other Epithelial Tumors

41

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1 Introduction

It is estimated that infection contributes to the development of around 20 % of human cancer – some two million cases per year [1]. Understanding the role of infection in cancer continues to provide fundamental insights into the underlying mechanisms responsible for driving the oncogenic process as well as highlighting opportunities for therapeutic and prophylactic intervention. Epstein-Barr virus (EBV) was the first human tumor virus to be discovered and has yielded significant insight into both the pathogenesis of cancer and the natural history of persistent herpesvirus infection.

EBV is one of the most common infections being found as a widespread and largely asymptomatic lifelong infection in all human populations [2, 3]. Early in the course of primary infection, EBV infects B lymphocytes, although it is not known where B lymphocytes are infected and whether this also involves epithelial cells of the upper respiratory tract. To achieve long-term persistence in vivo, EBV colonizes the memory B cell pool where it establishes latent infection, which is characterized by the expression of a limited subset of virus genes, known as the "latent" genes [4]. There are several well-described forms of EBV latency, each of which is utilized by the virus at different stages of the virus life cycle and which are also reflected in the patterns of latency observed in the various EBV-associated malignancies [2, 5]. Furthermore, during its life cycle EBV must periodically enter the replicative cycle in order to generate infectious virus for transmission to other susceptible hosts with epithelial sites in the oropharynx and salivary glands appearing to be the major site for viral replication. Thus, the natural history of persistent EBV infection appears to resemble that of other herpesviruses in requiring distinct cell lineages to manifest the latent and replicative forms of the virus life cycle.

The dual tropism of EBV infection is also reflected in the types of tumor associated with the virus. Colonization of the memory B cell pool in vivo, along with the unique ability of EBV to efficiently transform resting B cells into permanent latently infected lymphoblastoid cell lines (LCLs) in vitro, is mirrored by the various malignancies of B cell origin that are closely associated with EBV infection [2, 3, 5]. However, it is the EBV-associated undifferentiated form of nasopharyngeal carcinoma (NPC) that shows the most consistent worldwide association with EBV. Furthermore, a subset of gastric adenocarcinomas and certain salivary gland carcinomas are also infected with EBV. These tumors demonstrate the ability of epithelial infection with EBV to result in malignant transformation and provide opportunities to consider targeted approaches to preventive and therapeutic intervention.

2 Historical Background

In 1964 Tony Epstein and Yvonne Barr identified herpesvirus-type particles by electron microscopy in a subpopulation of Burkitt lymphoma (BL)-derived tumor cells in vitro. Only 2 years later Lloyd Old and colleagues described antibodies in the serum of African BL patients that recognized antigens prepared from BL cell lines [6]. A surprising observation was that similar BL antigen-specific antibodies were also present in the serum from patients with postnasal space carcinomas and these were found in a high proportion of patients from both Africa and the USA. This led the authors to suggest that "the high frequency of positive sera among patients with carcinoma of the postnasal space indi-

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cates the desirability of searching for similar particles in cultures of this class of tumor also." A more specific immunofluorescence assay for detecting antibodies against EBV-encoded replicative antigens was developed by George Klein, Werner and Gertrude Henle, and John H C Ho, and this confirmed the association of elevated antibody titers against EBV-encoded virus capsid antigen (VCA) and membrane antigen (MA) with NPC [7]. In 1970 Harald zur Hausen and colleagues used DNA hybridization technology to demonstrate the presence of EBV DNA in extracts from NPC tumors [8]. Further serological analysis showed an association between EBV antibody titers and NPC tumor stage [9] and identified VCA-specific IgA levels as a potential prognostic indicator [10]. In 1973, EBV DNA was detected by in situ hybridization in the tumor cells of NPC and not infiltrating lymphoid cells [11]. The apparent specificity of the association between VCA IgA levels and NPC led Zeng Yi to embark on a mass serological screening program in Wuzhou City in China where these EBV-specific antibodies were found to be useful in the early detection of NPC and where subsequent prospective analysis revealed the presence of IgA VCA up to 41 months prior to the clinical diagnosis of NPC [12]. Analysis of the terminal repeats (TRs) of the EBV genome in NPCs by Southern blot hybridization demonstrated the monoclonality of the resident viral genomes providing important evidence that EBV infection had taken place before the clonal expansion of the malignant cell population [13].

3 EBV Infection and Nasopharyngeal Carcinoma

The World Health Organization (WHO) has classified NPC into two main histological types – keratinizing squamous cell carcinoma (type I) and nonkeratinizing squamous cell carcinoma (types II and III) – based on the light microscopic appearance of the tumor cells. The nonkeratinizing type is further subdivided into differentiated nonkeratinizing (type II) and undifferentiated carcinomas (type III), and it is these tumors that are predominantly EBV positive [14, 15]. The well-differentiated keratinizing NPC (type I) accounts for less than 20 % of all NPC cases worldwide, and this tumor type is relatively rare in Southern China [15, 16]. However, the association of EBV with the more differentiated WHO type I form of NPC has been found particularly in those geographical regions with a high incidence of undifferentiated NPC [14]. In NPC the virus exists in a latent state, exclusively in the tumor cells and absent from the surrounding lymphoid infiltrate. However, the interaction between the prominent lymphoid stroma found in undifferentiated NPC and adjacent carcinoma cells appears to be crucial for the continued growth of the malignant NPC cells.

Carcinomas with similar features to undifferentiated NPC have been described at other sites including the thymus, tonsils, lungs, stomach, skin, or uterine cervix and are often referred to as undifferentiated carcinomas of nasopharyngeal type (UCNT) or "lymphoepitheliomas." The morphological similarities of UCNTs to undifferentiated NPC prompted several groups to examine such cases for the presence EBV. UCNTs of the stomach are consistently EBV positive [17], whereas the association of the other UCNTs with EBV is less strong. EBV has been demonstrated in thymic epithelial tumors from Chinese but not Western patients [18]. Salivary gland UCNTs are EBV-associated in Greenland Eskimos and Chinese but not in Caucasian patients [19], and several case reports have demonstrated the absence of EBV from UCNTs arising in the uterine cervix and breast [20, 21].

4 Biological Characteristics

4.1 Patterns of EBV Latent Gene Expression in Virus-Associated Tumors

Much of our understanding of the biology of EBV relates to its interaction with B lymphocytes. This is reflected in the ability of EBV to readily infect and transform normal resting B lymphocytes in vitro, a model system which has provided important insights into the biology and behavior of the virus. The lymphoblastoid cell lines (LCLs) generated by in vitro infection of B lymphocytes carry multiple copies of circular extra chromosomal viral DNA (episomes) in every cell and express a limited set of latent proteins, including six nuclear antigens (EBNAs 1, 2, 3A, 3B, and 3C and EBNA-LP) and three latent membrane proteins (LMPs 1, 2A, and 2B) [2, 3, 5]. In addition to the latent proteins, LCLs also show abundant expression of the small non-polyadenylated RNAs, EBERs 1 and 2; these are expressed in all forms of latent EBV infection and have served as excellent targets to detect EBV in tumors. Transcripts, originally referred to as BARTs (BamHI A rightward transcripts), from the *Bam*HI A region (Bam A) of the viral genome are also detected in LCLs and these, along with transcripts from the BHRF1 region, encode microRNAs (miRNAs). This pattern of latent EBV gene expression is referred to as the "latency III" (Lat III) form of EBV infection and is characteristic of the majority of EBV-associated lymphomas arising in immunosuppressed patients.

The examination of EBV latent gene expression in virus-associated tumors and in cell lines derived from Burkitt lymphoma (BL) biopsies identified at least two additional forms of EBV latency. EBNA1 is the only EBV protein consistently observed in EBV-positive BL tumors along with the EBER and *Bam*HIA transcripts; this form of latency is referred to latency I or Lat I [2, 3, 5]. A variant of Lat I is

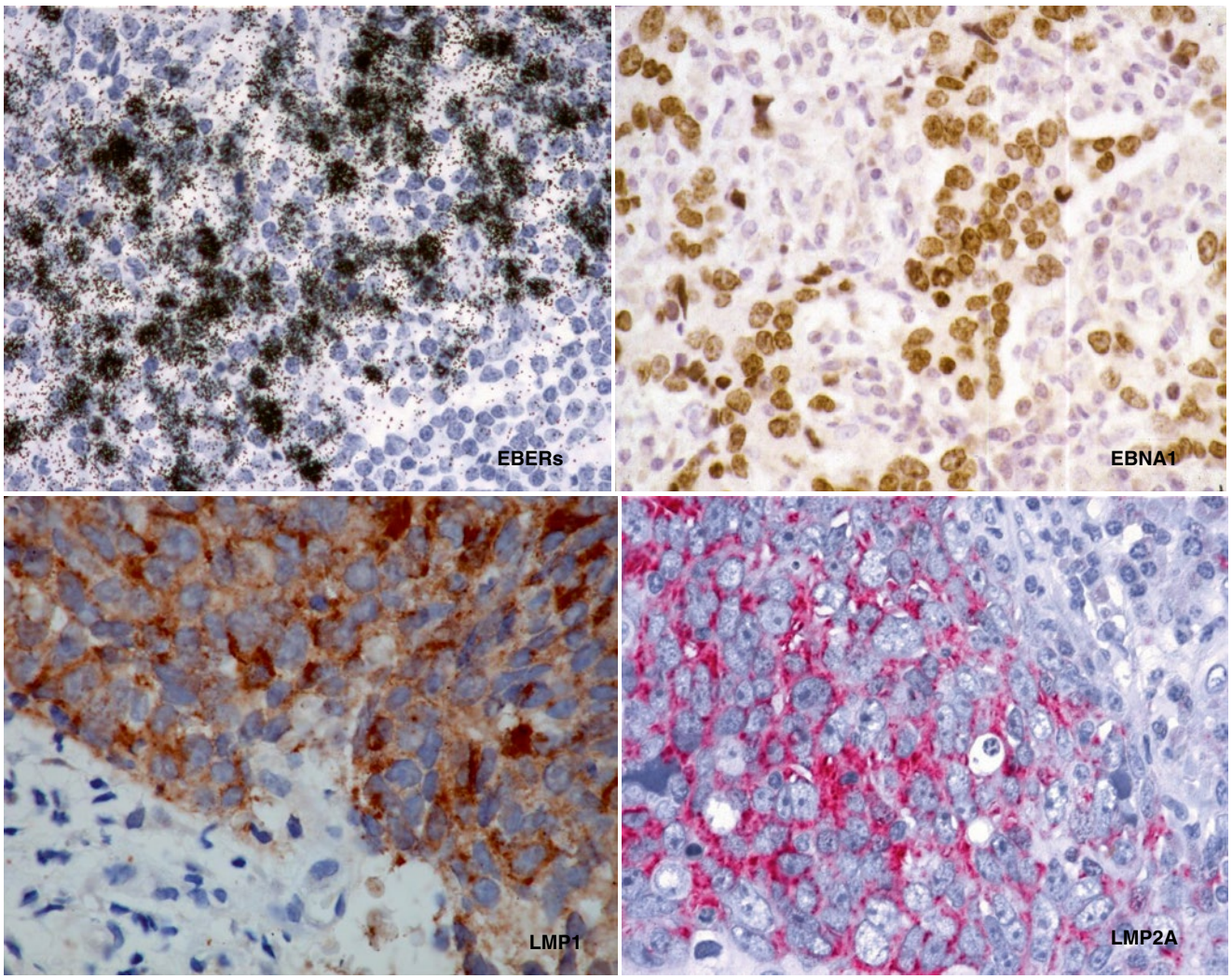

Fig. 41.1 EBV latent gene expression in NPC. In situ hybridization to the abundant EBV-encoded *EBER* transcripts (*left, upper panel*) is the standard approach to detect EBV infection in cells and tissues. Immunohistochemical staining of NPC confirms *EBNA1* expression in every tumor cell (*right, upper panel*). The expression of *LMP1* and *LMP2A* in NPC biopsies (*lower panels*) is more variable. Note the prominent lymphoid infiltrate in NPC, which is believed to contribute to the growth and survival of the tumor cells

observed in around 5–10 % of EBV-positive BL tumors where expression of BHRF1, EBNA3A, EBNA3B, and EBNA3C is observed [22]. It appears that in these tumors the selective pressure to downregulate EBNA2 expression has occurred via deletion of the EBNA2 gene rather than through the switch in viral promoter usage observed in the conventional BL scenario. Another form of EBV latency, Lat II, was originally identified in biopsies of NPC and subsequently found in cases of EBV-associated Hodgkin's lymphoma (HL) (Fig. 41.1) [23–26].

Here, expression of the EBERs, EBNA1, and *Bam*HI A transcripts is accompanied by expression of LMP1 and LMP2A/B. While this Lat II pattern of EBV latent gene expression is a consistent feature of virus-associated HL, LMP1 expression in NPC is variable with only around 20 % of biopsies being unequivocally positive for LMP1 at the protein level [3]. The mechanisms underlying differential LMP1 expression in NPC and the consequent effects on the NPC phenotype remain unknown.

These different forms of EBV latent infection are a reflection of the cellular environment and the complex interplay between host regulatory factors and the viral promoters driving EBV latent gene expression. In Lat III, the different EBNAs are encoded by individual mRNAs generated by differential splicing of the same long (over 100 kb) "rightward" primary transcript expressed from one of two promoters (Cp or Wp) located close together in the *Bam*HI C and W region of the genome [27]. A switch from Wp to Cp occurs early in B cell infection as a consequence of the transactivating effects of both EBNA1 and EBNA2 on Cp. The LMP transcripts are

expressed from separate promoters in the *Bam*HI N region of the EBV genome, with the leftward LMP1 and rightward LMP2B mRNAs apparently controlled by the same bidirectional promoter sequence (ED-L1) which also responds to transactivation by EBNA2 [5, 27]. The LMP2A promoter is also regulated by EBNA2. Both LMP2A and LMP2B transcripts cross the TRs into the U1 region thus requiring the circularization of the genome for transcription. Circularization occurs by homologous recombination of the TRs resulting in fused termini of unique length, and this has been used as a measure of EBV clonality on the assumption that fused TRs with an identical number of repeats denote expansions of a single infected progenitor cell [13]. This contention has been challenged by the observation that EBV clonality postinfection may be a consequence of the selective growth advantage achieved by optimal LMP2A expression over a minimal number of TRs [28]. In the more restricted forms of EBV latency observed in NPC and HL, EBNA1 transcription is driven from the TATA-less Qp promoter [29] and an alternative promoter, L1-TR, located in the terminal repeats is responsible for LMP1 expression [30]. It is widely accepted that these forms of EBV latency reflect the different transcription programs that EBV employs as the virus transits through the germinal center B cell reaction to finally colonize the resting memory B cell compartment [4]. The adoption of these forms of latency in EBV-associated tumors is therefore assumed to represent the aberrant manifestation of inappropriate virus transcription programs as a consequence of both the host cell environment and the selection pressures associated with oncogenic progression.

4.2 The Function of EBV Latent Genes Expressed in NPC

4.2.1 EBNA1

EBNA1 is a DNA-binding protein that is required for the replication and maintenance of the episomal EBV genome; a function that is achieved through the binding of EBNA1 to oriP, the plasmid origin of viral replication [5]. EBNA1 also interacts with two sites downstream of Qp to negatively regulate its own expression [29]. EBNA1 also acts as a transcriptional transactivator and upregulates Cp and the LMP1 promoter [5]. The EBNA1 protein contains a glycine-glycine-alanine (gly-gly-ala) repeat sequence, which varies in size in different EBV isolates. This gly-gly-ala repeat domain is a *cis*-acting inhibitor of MHC class I-restricted presentation and appears to function by inhibiting antigen processing via the ubiquitin-proteosome pathway [31]. While this effect may result in poor presentation of EBNA1-derived epitopes to cytotoxic T lymphocytes (CTLs), it does not prevent the induction of EBNA1-specific CD8+ T cell responses.

Aside from its role in EBV replication, segregation, and transcriptional activation, EBNA1 has recently been found to protect cells from apoptosis, enhance cell survival, and directly contribute to the tumorigenic phenotype [32]. These effects are manifest through a variety of mechanisms including the destabilization of p53, the disruption of PML nuclear bodies, and the modulation of various signaling pathways.

4.2.2 LMP1

LMP1 is the major transforming protein of EBV behaving as a classical oncogene in rodent fibroblast transformation assays and being essential for EBV-induced B cell transformation in vitro [33, 34]. LMP1 has pleiotropic effects when expressed in cells resulting in induction of cell surface adhesion molecules and activation antigens, upregulation of anti-apoptotic proteins (Bcl-2, A20), and stimulation of cytokine production (IL-6, IL-8) [35]. LMP1 functions as a constitutively activated member of the tumor necrosis factor receptor (TNFR) superfamily activating a number of signaling pathways (NF-κB, ERK-MAPK, JNK/AP1, PI3-K) in a ligand-independent manner [36]. Functionally, LMP1 resembles CD40, a member of the TNFR superfamily, and can partially substitute for CD40 in vivo providing both growth and differentiation signals to B cells [37]. The LMP1 protein consists of six hydrophobic transmembrane loops which mediate the self-aggregation of LMP1 into oligomers and a C-terminal cytoplasmic tail which contains two distinct functional domains referred to as C-terminal activation regions 1 and 2 (CTAR1 and CTAR2) originally identified on the basis of their ability to activate the NF-κB transcription factor pathway [35]. This effect contributes to the many phenotypic consequences of LMP1 expression including the induction of various antiapoptotic and cytokine genes. LMP1 is also able to engage the MAP kinase cascade resulting in activation of ERK, JNK, and p38 and to stimulate the JAK/STAT pathway [38–41]. Many of these effects result from the ability of TNFR-associated factors (TRAFs) to interact either directly with CTAR1 or indirectly via the death domain protein TRADD to CTAR2 [42]. LMP1 can also activate the phosphatidylinositol 3-kinase (PI3-K) pathway resulting in a variety of effects including cell survival mediated through the Akt (PKB) kinase, actin polymerization, and cell motility [43]. Expression of LMP1 in epithelial cells is associated with a range of phenotypic effects including hyperproliferation, induction of pro-inflammatory cytokines, resistance to apoptosis, and enhanced motility [35].

The role of LMP1 in the pathogenesis of NPC remains speculative. There is considerable variation in the reported expression of LMP1 in NPC biopsies and a general consensus that around 20–40 % of tumors express LMP1 at the protein level [3]. In one study all six early preinvasive NPC (NPC in situ) lesions analyzed expressed the LMP1 protein,

arguing for a critical role of LMP1 in the early pathogenesis of NPC [44]. The possibility of geographical variation in LMP1 expression in NPC has been suggested and is supported by studies in North Africa where LMP1 has been found to be more prevalent in the juvenile form of NPC [45].

4.2.3 LMP2A/B

The two proteins encoded by the LMP2 gene, LMP2A and LMP2B, share 12 hydrophobic membrane-spanning domains and a short cytoplasmic C-terminus, but it is the unique cytoplasmic N-terminal domain of LMP2A that appears to be responsible for the functional effects of this molecule. This domain contains an immunoreceptor tyrosine-based activation motif (ITAM), similar to that in the B cell antigen receptor (BCR), which interacts with and negatively regulates members of the *src* family of protein tyrosine kinases (PTKs) [46]. The LMP2A ITAM blocks BCR-stimulated calcium mobilization, tyrosine phosphorylation, and activation of the EBV lytic cycle in B cells [47]. Expression of LMP2A in the B cells of transgenic mice abrogates normal B cell development allowing immunoglobulin-negative cells to colonize peripheral lymphoid organs [48]. This suggests that LMP2A can drive the proliferation and survival of B cells in the absence of signaling through the BCR, an effect that may be relevant to the ability of EBV to colonize the memory B cell pool. While LMP2A and LMP2B may share common or overlapping functions, studies suggest that the primary function of LMP2B is to negatively regulate LMP2A signaling [49].

In non-B cells, the LMP2 proteins localize to various intracellular compartments (perinuclear endosomes, the endoplasmic reticulum (ER), the trans-Golgi network (TGN), and CD63-positive lysosomes) [50–52], and LMP2A is also associated with lipid rafts, microdomains that form membrane-associated signaling platforms [53]. While much is known about the so-called LMP2A signalosome in the context of B cells, its role in the epithelial environment is less clear. LMP2A is required for the successful outgrowth of EBV-infected epithelial cells in vitro [28] and can induce anchorage-independent growth, enhance cell adhesion and cell motility, and inhibit epithelial cell differentiation [54–58]. Many of these effects are a consequence of the ability of LMP2A to activate the PI3-K/Akt and β-catenin pathways [55]. LMP2A can also induce an epithelial-mesenchyme transition (EMT) and this is associated with the acquisition of stem cell-like properties [59]. A role of LMP2A and LMP2B in resistance to the antiviral effects of interferons α and β in epithelial cells has been identified [60], and LMP2A is able to modulate NF-κB and STAT signaling in EBV-infected epithelial cells through effects on IL-6 transcription and secretion [61].

Unlike LMP1, LMP2A expression is more consistent in NPC. Studies using RT-PCR confirmed expression of LMP2A mRNA in greater than 98 % of NPC cases, while expression of LMP2B was lower and mirrored that of LMP1 [23, 62]. Immunohistochemical staining has confirmed expression of LMP2A protein in greater than 50 % of NPC cases [59, 63].

Interestingly, this consistent expression of LMP2 in NPC is reflected in serological responses such that the majority of NPC patients have detectable IgG responses to LMP2A/2B [64].

4.2.4 EBERs

Two small non-polyadenylated (noncoding) RNAs, EBERs 1 and 2, are highly expressed in all forms of EBV latency and serve as sensitive targets for detecting EBV infection in cells and tissues [2, 3, 5]. The EBERs assemble into stable ribonucleoprotein particles with the autoantigen La, with ribosomal protein L22, and bind the interferon-inducible, double-stranded RNA-activated protein kinase PKR [65]. The interaction of EBERs with RIG-1 induces type I interferon, an effect that may be counteracted by other viral genes such as LMP1 and LMP2A/B [66]. A role for the EBERs in inducing insulin-like growth factor 1 (IGF-1) in NPC cell lines has been described [67]. This effect is associated with the enhanced growth of NPC cell lines and its relevance to NPC is supported by the frequent expression of IGF-1 in tumor biopsies.

4.2.5 BARTs and BARF1

A group of abundantly expressed RNAs encoded by the BamHIA region of the EBV genome were originally identified in NPC but subsequently found to be expressed in other EBV-associated malignancies as well as in the peripheral blood of healthy individuals [2, 3, 5, 68, 69]. These highly spliced transcripts are commonly referred to as either BARTs (*Bam*HIA rightward transcripts) or complementary strand transcripts (CSTs). It was originally suggested that the BARTs might function as antisense transcripts by interfering with transcription from the complementary DNA strand of the EBV genome thereby inhibiting viral mRNAs associated with the productive cycle of the virus [70]. This possibility was refuted by the discovery of EBV as the first human virus to be found to encode microRNAs (miRNAs) and that the BARTs are a cluster of 22 miRNA precursors that generate 44 mature miRNAs [71]. These miRNAs target and thereby regulate a variety of viral and cellular transcripts [69, 72]. Thus, specific BART miRNAs have been found to protect from apoptosis and to contribute to immune evasion. The BART miRNAs also appear crucial in the regulation of EBV gene expression targeting both latent (LMP1, LMP2A) and lytic genes (BALF5). Because of their stability in serum, miRNAs have been recognized as potential cancer biomarkers and recent studies support the possible use of serum BART miRNAs in the detection and prognosis of NPC [73, 74].

It appears that the BART miRNAs in serum reside in cell-derived exosomes and that these are able to deliver miRNAs to other non-EBV-infected cells [75, 76]. This raises the intriguing possibility that BART miRNAs may be able to contribute to the development of NPC by influencing the behavior of stromal and immune cells.

Another transcript generated from the *Bam*HIA region is BARF1 that encodes a 31 kDa protein originally identified as an early antigen expressed upon induction of the EBV lytic cycle. However, BARF1 is a secreted protein which is expressed as a latent protein in EBV-associated NPC and gastric carcinoma [77, 78]. BARF1 shares limited homology with the human CSF-1 receptor (*c-fms* oncogene) and competes for its natural ligand, macrophage colony-stimulating factor (M-CSF or CSF-1), thereby modulating immune cell growth and function [79]. BARF1 displays oncogenic activity when expressed in rodent fibroblasts and simian primary epithelial cells [80]. Engineered expression of BARF1 in the context of a recombinant EBV enhanced the growth and tumorigenicity of virus-negative NPC cell lines implicating BARF1 in the pathogenesis of NPC [81]. It has been suggested that serum BARF1 could be a useful diagnostic marker for NPC [82].

4.3 EBV Strain Variation

EBV isolates from different regions of the world or from patients with different virus-associated diseases are remarkably similar when their genomes are compared by restriction fragment length polymorphism analysis [3, 5, 83]. However, variations in repeat regions of the EBV genome are observed among different EBV isolates. Analysis of the EBV genome in a number of Burkitt lymphoma (BL) cell lines revealed gross deletions, some of which account for biological differences, i.e., P3HR-1 virus which is non-transforming has a deletion of the EBNA2-encoding gene [3, 5, 83]. Strain variation over the EBNA2-encoding (BamHI WYH) region of the EBV genome permits all virus isolates to be classified as either "type 1" (EBV-1, B95.8-like) or "type 2" (EBV-2, Jijoye-like) [83]. This genomic variation results in the production of two antigenically distinct forms of the EBNA2 protein with only 50 % amino acid homology. Similar allelic polymorphisms (with 50–80 % sequence homology depending on the locus) related to the EBV type occur in a subset of latent genes, namely, those encoding EBNA-LP, EBNA3A, EBNA3B, and EBNA3C [84]. These differences have functional consequences as EBV-2 isolates are less efficient in in vitro B lymphocyte transformation assays compared with EBV-1 isolates [85]. A combination of virus isolation and seroepidemiological studies suggests that type 1 virus isolates are predominant (but not exclusively so) in many Western countries, whereas both types are widespread in equatorial Africa, New Guinea, and perhaps certain other regions [83, 86].

In addition to this broad distinction between EBV types 1 and 2, there is also minor heterogeneity within each virus type. Individual strains have been identified on the basis of changes, compared with B95.8, ranging from single base mutations to extensive deletions [83]. While infection with multiple strains of EBV was originally thought to be confined to immunologically compromised patients, more recent studies demonstrate that normal healthy seropositives can be infected with multiple EBV isolates and that their relative abundance and presence appears to vary over time [87]. Coinfection of the host with multiple virus strains could have evolutionary benefit to EBV enabling the generation of diversity by genetic recombination. Such intertypic recombination has been demonstrated in HIV-infected patients and in the Chinese population and appears to arise via recombination of multiple EBV strains during the intense EBV replication that occurs as a consequence of immunosuppression [88].

The possible contribution of EBV strain variation to virus-associated tumors remains contentious. Many studies have failed to establish an epidemiological association between EBV strains and disease and suggest that the specific EBV gene polymorphisms detected in virus-associated tumors occur with similar frequencies in EBV isolates from healthy virus carriers from the same geographical region [3, 86]. However, this does not exclude the possibility that variation in specific EBV genes is responsible for the distinct geographic distribution of virus-associated malignancies. In this regard, an LMP1 variant containing a 10 amino acid deletion (residues 343–352) was originally identified in Chinese NPC biopsies and has oncogenic and other functional properties distinct from those of the B95.8 LMP1 gene [89–91]. It is therefore likely that variation in LMP1 and other EBV genes can contribute to the risk of developing virus-associated tumors, but more biological studies using well-defined EBV variants are required.

5 Descriptive Epidemiology

NPC has a very distinctive geographic and ethnic distribution. Although the incidence of this cancer is less than 1 per 100,000 person-years in most parts of the world, rates of between 20 and 30 per 100,000 have been reported for populations in Southern China and Southeast Asia with intermediate rates recorded for those living in the Arctic region, North Africa, and parts of the Middle East. However, recent analyses of temporal trends suggest that incidence and

mortality rates from NPC are falling in both high- and low-risk countries [92–96].

5.1 Comparisons Between Low- and High-Incidence Countries

Although only limited comparisons are possible between high- and low-risk countries, because most of the areas at high risk have as yet underdeveloped cancer registration systems, such comparisons as are possible reveal intriguing differences in the age-specific incidence curves of NPC [97]. Whereas in high-risk countries, incidence increases monotonically reaching a peak at around 50 years of age, a bimodal distribution is observed in low-risk populations, irrespective of geographic location. In these countries, the first peak in incidence is seen in late adolescence or early adulthood and is followed by a second peak around the age of 70. Bray et al. [97] suggest that whereas the early peak in incidence point to a role for major susceptibility genes in disease pathogenesis, possibly mediated by other factors including EBV infection, for which age at infection may be critical, the later peak is a consequence of heavy smoking, alcohol consumption, and possibly long-term exposure to occupational carcinogens.

5.2 Immigrant Studies

Studies of immigrant populations have shown that NPC incidence among the Chinese is higher than among African Americans and Caucasians in the USA and is lower in second- and third-generation Chinese immigrants to the USA than in first-generation immigrants while remaining higher than those of other ethnic groups [95]. Compared with the indigenous population, the standardized incidence rate of NPC in Sweden was found to be significantly higher in male (SIR = 35.6) and female (24.6) Southeast Asians, male (12.4) and female (34.7) North Africans, and male (4.9) and female (10.9) Asian Arabs [98]. McDermott et al. commenting on the healthy immigrant effect has pointed out that while immigrants to Canada have a lower overall cancer risk than the indigenous population, they have a significantly higher incidence of nasopharyngeal, liver, and cervical cancer, three cancers which can be related to earlier viral infection in the country of origin [99]. While these immigrant studies suggest that both genetic factors and lifestyle contribute to the etiology of NPC, a recent systematic review has confirmed a decreasing trend in the incidence of NPC in Chinese migrants living in countries with a low risk of the disease, providing further evidence that the excess risk associated with migrant status becomes attenuated over time [100].

5.3 Tobacco Use and Risk of Nasopharyngeal Cancer

Case-control studies have reported an increased risk of NPC related to the duration and intensity of cigarette smoking in both low- and high-incidence regions [101–105]. In those analyses in which cases could be stratified by histological types, the risk associated with smoking behavior was found to be stronger for differentiated as compared to undifferentiated tumors in studies conducted in both low- and high-incidence countries [16, 103, 106]. A fall in the incidence of this histological type have been reported in Singapore, in the Netherlands, and in Chinese men living on the Western seaboard of the USA and has been attributed to a decline in cigarette smoking [94, 95, 107]. In the Netherlands, a low-risk country, the decrease in the incidence of smoking-related keratinizing carcinoma coincided with an increase in the incidence of differentiated nonkeratinizing NPC, a pattern which the authors attribute to recent waves of migration [96].

Compelling evidence of an exposure-disease relationship is provided by cohort studies in which smoking behavior and other risk factors for NPC are ascertained prior to the diagnosis of disease. Such studies have confirmed that prolonged cigarette smoking is associated with an increased risk of developing NPC [108–111]. Consistent with the impression left by earlier cross-sectional and cohort studies, a recent case-control study has shown that not only are cigarette smoking and EBV seropositivity associated with an excess risk of NPC but also that smoking was the only risk factor for NPC associated with elevated titers of VCA IgA antibody in disease-free subjects recruited from both low- and high-incidence areas [102]. The investigators show in vitro that cigarette smoke extract promotes EBV replication, induces the expression of the immediate-early transcriptional activators Zta and Rta, and increases the transcription of BFRF3 and gp350 in the lytic phase. These findings suggest an alternative role for cigarette smoking in the pathogenesis of NPC other than through direct DNA damage.

5.4 Dietary Factors

The consumption of salted fish is an established risk factor for NPC [102, 112]. There is also compelling evidence to suggest that whereas the risk of NPC increases with increasing consumption of preserved foods, it falls with increasing consumption of fresh fruit and Vegetables [101, 112–114]. More recently case-control studies have shown a reduced risk of NPC related to consumption of green tea [113, 115]. Epigallocatechin-3-gallate, the principal polyphenol present in green tea, has been shown to inhibit stemlike characteristics and epithelial-mesenchymal transition in NPC cell lines

when grown in spheres [116]. A meta-analysis of four case-control studies conducted in China revealed a modest but significant excess risk of NPC associated with alcohol consumption [117]. Although these findings are consistent with the results of an earlier systematic review, which concluded that heavy alcohol consumption is associated with an increased risk of NPC, the exposure-disease relationship is complex with some studies revealing a J-shape dose-response trend, with NPC risk decreasing with modest consumption and increasing with higher intake [118].

5.5 Wood Dust and Formaldehyde

At least two million workers are routinely exposed to wood dust in the work environment worldwide. A pooled analysis of cancer mortality among five cohorts of workers in wood-related industries has shown that workers exposed to wood dust may have an excess risk of death from sinonasal cancers and NPC [119]. More recently, case-control studies, which included detailed assessments of occupational exposure, have shown that the risk of NPC is highest for individuals exposed to wood early in life. Consistent with a possible interaction between environmental exposures and genetic predisposition, these studies have shown that the excess risk of NPC associated with wood exposure stronger in families with two or more members already affected by NPC is strongest in those families with early age-onset of disease [120, 121]. However, an association between wood dust and NPC may be more difficult to reveal in low-incidence populations. A recent cohort study with more than 25 years of follow-up did not find an increased risk of NPC in Finnish men in whom estimates of exposure to wood dust had been generated using a job-exposure matrix [122]. There is no compelling evidence of an increased risk of NPC following formaldehyde exposure; a recent meta-analysis of case-control and cohort studies found no excess risk after excluding a single plant with an unexplained cluster of NPC cases [123]. A more recent study which included information on occupational exposure in a cohort of Finnish men also found no evidence to suggest that chronic exposure to formaldehyde increases the risk of NPC [122].

5.6 Genetic Susceptibility

As comprehensively discussed in a recent review [124], there have been many studies exploring the role of genetic polymorphisms in NPC development, but these have been hampered by small cohort sizes, poor study design, and a lack of confirmatory studies. Genome-wide association studies (GWAS) have, however, confirmed previous observations suggesting a strong association of NPC with the MHC locus on chromosome 6p21 [125, 126]. The association of specific HLA alleles with NPC risk implies a possible contribution of differential immune responses to EBV, and this is further supported by linkage of NPC to other immune-related genes. Other studies implicate DNA repair genes, cell cycle control genes, and cell adhesion/migration genes in the development of NPC [124]. It is clear that larger, well-powered, and better coordinated studies are required to confirm these observations and to determine the real impact of gene-environment interactions on the development of NPC.

6 Mechanisms and Routes of Transmission

Transmission of EBV is addressed more extensively in Chap. 38 – EBV and Infectious Mononucleosis. EBV infects the majority of the world's adult population, and following primary infection, the individual remains a lifelong carrier of the virus [2, 3, 5]. In underdeveloped countries, primary infection with EBV usually occurs during the first several months to few years of life and is often asymptomatic. However, in developed populations, primary infection is more frequently delayed until adolescence or adulthood, in many cases producing the characteristic clinical features of infectious mononucleosis (IM). EBV is orally transmitted, and infectious virus can be detected in oropharyngeal secretions from IM patients, from immunosuppressed patients, and at lower levels from healthy EBV seropositive individuals. These observations, together with the fact that EBV-transformed LCLs in vitro tend to be poor producers of the virus and B lymphocytes permissive of viral replication have not been demonstrated in vivo, suggest that EBV replicates and is shed at epithelial sites in the oropharynx and/or salivary glands. This is supported by the demonstration of replicating EBV in the differentiated epithelial cell layers of oral "hairy" leukoplakia, a benign lesion of the tongue found in immunocompromised patients [127]. Replicative EBV infection has been also been found, albeit rarely, in normal squamous epithelial cells at the tongue margin but not in salivary glands (Fig. 41.2) [128]. However, it is also likely that EBV-infected B cells are reactivated within the local mucosal environment and that this contributes to virus shedding at oropharyngeal sites.

The inability to routinely detect EBV in normal epithelial cells and the demonstration that EBV can be completely eradicated by irradiation in bone marrow transplant recipients suggests that B lymphocytes are the main site of EBV persistence [129]. This is supported by the B lymphotropism of EBV that is mediated by the binding of the major viral envelope glycoprotein gp350 to the CR2 receptor on the surface of B cells [130]. Virion penetration of the B cell membrane requires further interactions between

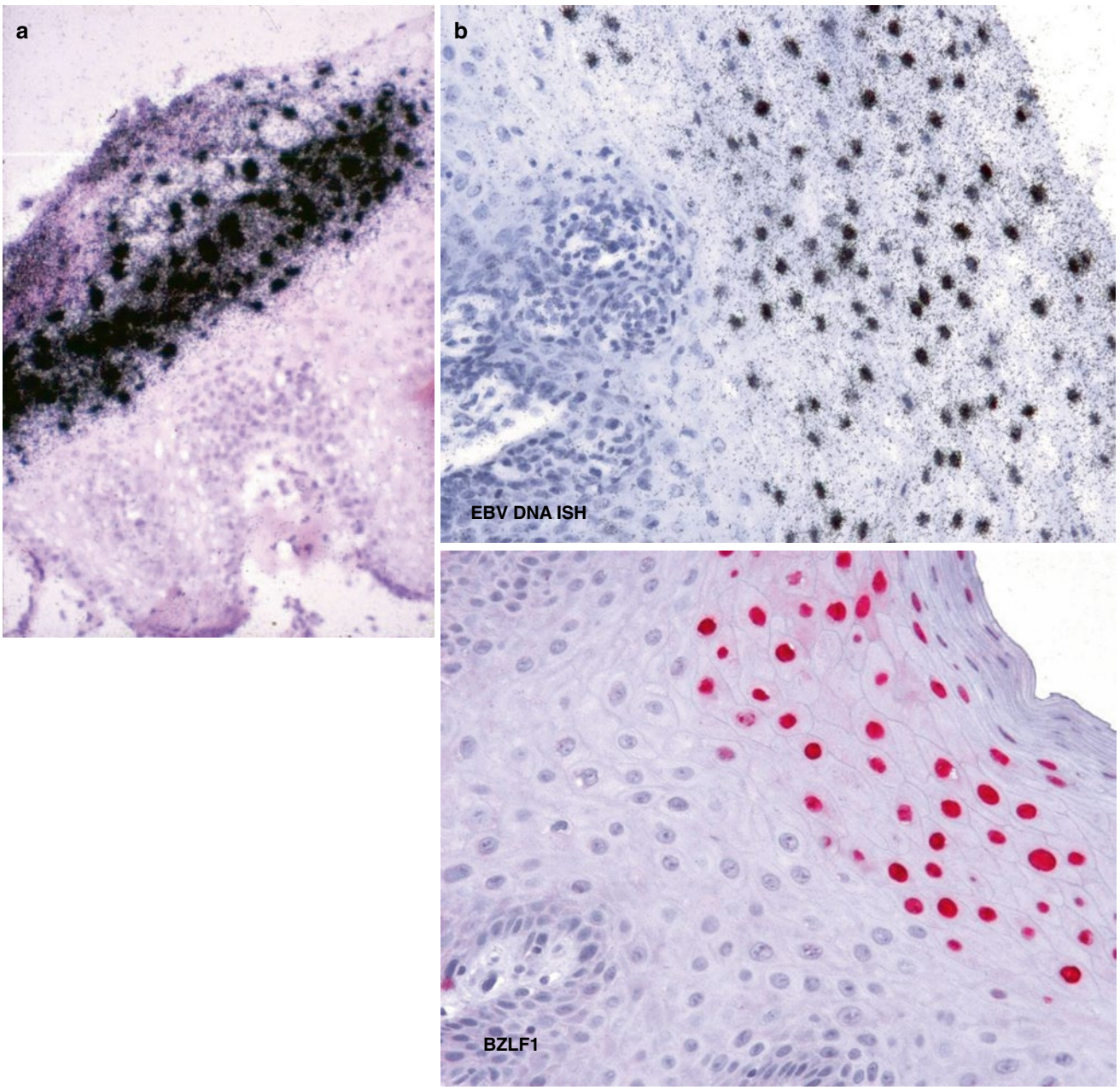

Fig. 41.2 In situ hybridization for EBV DNA reveals rampant virus replication in the differentiating squamous epithelium of oral "hairy" leukoplakia (**a**) and rarely in the epithelial cells of normal tonsil (**b**, upper panel [128]). Staining for the BZLF1 immediate early viral protein confirms EBV replication of EBV in normal tongue epithelium (**b**, lower panel [128])

the EBV glycoprotein gp42 (which forms a ternary complex with gH and gL viral glycoproteins) and HLA class II molecules [130]. It appears that the presence or absence of HLA class II in virus producing cells influences the tropism of EBV for B cells or epithelial cells by affecting the availability of gp42 [131]. Other CR2-independent pathways may be responsible for EBV infection of epithelial cells, including secretory component-mediated IgA transport, integrin interactions with polarized epithelium, and direct cell-to-cell contact, but these are relatively inefficient and of unknown relevance to EBV infection in vivo [130].

7 NPC Pathogenesis

The presence of monoclonal EBV episomes in NPC indicates that virus infection precedes the clonal expansion of the malignant cell population [13]. However, the lack of epithelial EBV infection in tonsils from IM patients and in

normal nasopharyngeal biopsies from individuals at high risk of developing NPC suggests that epithelial infection may not be the initiating event in virus-associated carcinogenesis [2]. EBV infection as detected by in situ hybridization to the abundantly expressed non-polyadenylated EBER RNAs has been demonstrated in high-grade (severe dysplastic and carcinoma in situ) preinvasive lesions in the nasopharynx but not in low-grade disease or histologically normal nasopharyngeal epithelium [44, 132]. Both the high-grade and carcinoma in situ lesions carry monoclonal EBV genomes [44]. Multiple genetic changes have been found in NPC with frequent deletion of regions on chromosomes 3p, 9p, 11q, 13q, and 14q and promoter hypermethylation of specific genes on chromosomes 3p (RASSF1A, RARβ2) and 9p (p16, p15, p14, DAP-kinase) [133, 134]. Both 3p and 9p deletions have been identified in low-grade dysplastic lesions and in normal nasopharyngeal epithelium from individuals at high risk of developing NPC in the absence of EBV infection suggesting that genetic events occur early in the pathogenesis of NPC and that these may predispose to subsequent EBV infection [132, 135]. This possibility is supported by in vitro data demonstrating that stable EBV infection of epithelial cells requires an altered, undifferentiated cellular environment [136] and that cyclin D1 overexpression (a consequence of p16 deletion on chromosome 9p and amplification of the cyclin D1 locus on chromosome 11q) facilitates persistent EBV infection of immortalized nasopharyngeal epithelial cells [137]. Thus, a scheme has been proposed whereby loss of heterozygosity occurs early in the pathogenesis of NPC possibly as a result of exposure to environmental cofactors such as dietary components (i.e., salted fish) creating low-grade preinvasive lesions that after additional genetic and epigenetic events become susceptible to EBV infection (Fig. 41.3). Once infected, EBV latent genes provide growth and survival benefits resulting in the development of NPC. Additional genetic and epigenetic changes occur after EBV infection.

8 Immunity and Patterns of Host Response

As mentioned previously, the association of EBV infection with NPC was originally identified as a consequence of the observation that these patients had significantly elevated antibody responses to EBV-encoded antigens associated with virus replication. The serological response to EBV infection is manifest in all virus-positive individuals and targets both latent and lytic antigens. In healthy individuals these antibody titers are stable, consistent with the nature of asymptomatic persistent EBV infection. However, immune suppression (e.g., HIV, immunosuppressive drugs) or chronic inflammatory conditions (e.g., rheumatoid arthritis, multiple sclerosis) can result in elevated antibody levels to EBV, and this can sometimes confuse the etiological contribution of EBV infection to various diseases. Unique to NPC and other EBV-associated carcinomas is a robust mucosal (IgA) immune response to certain lytic antigens (VCA, EA, MA); these can be seen several years prior to the development of NPC and correlate with tumor burden, remission, and recurrence [3, 138].

Primary EBV infection elicits strong cellular immune responses that then bring the infection under control. Thus the lymphocytosis typifying acute IM reflects the hyperexpansion of cytotoxic CD8+ T lymphocytes (CTLs). The majority of these CTLs are specific for epitopes derived from immediate-early and early antigens of the lytic cycle, while some are specific for latent antigens, particularly to epitopes drawn from the immunodominant EBNA3A, EBNA3B, and EBNA3C proteins [139]. Both sets of reactivities are subsequently maintained in CD8+ T cell memory at levels that, collectively, may constitute up to 5 % of the total circulating CD8 T cell pool. This level of commitment to a single virus, apparent even in EBV carriers with no prior history of IM, implies a crucial role for CD8+ T cell memory in controlling persistent EBV infection. EBV-induced CD4+ T cell responses are also detectable both in acute IM patients and in long-term virus carriers but at much lower levels than their CD8 counterparts. The role of these CD4+ T cells, whether in maintenance of effective CD8 immunity or possibly as antiviral effectors in their own right, remains to be determined. NPC patients are not immunosuppressed and maintain robust EBV-specific T cell responses suggesting that the tumor cells are able to evade immune recognition. This is likely to reflect the poor immunogenicity of the latent antigens expressed in NPC (EBNA1, LMP1, LMP2) and the immune milieu in the tumor microenvironment [139].

9 Control and Prevention

9.1 Molecular Biomarkers

While EBV-specific IgA antibodies are useful in the early detection and prognostic monitoring of NPC, the circulating levels of cell-free EBV DNA in the peripheral blood of NPC patients have been found to be a more sensitive and specific marker [140–142]. The pre-therapy levels of plasma EBV DNA are able to identify prognostically distinct subsets of patients and thus contribute to the staging and subsequent management of NPC. Other studies have highlighted the potential of analyzing nasal swabs for EBV DNA or methylated tumor suppressor genes (TSGs) for the diagnosis and local monitoring of NPC [143, 144]. More recent work has identified serum EBV miRNAs as diagnostic biomarkers in

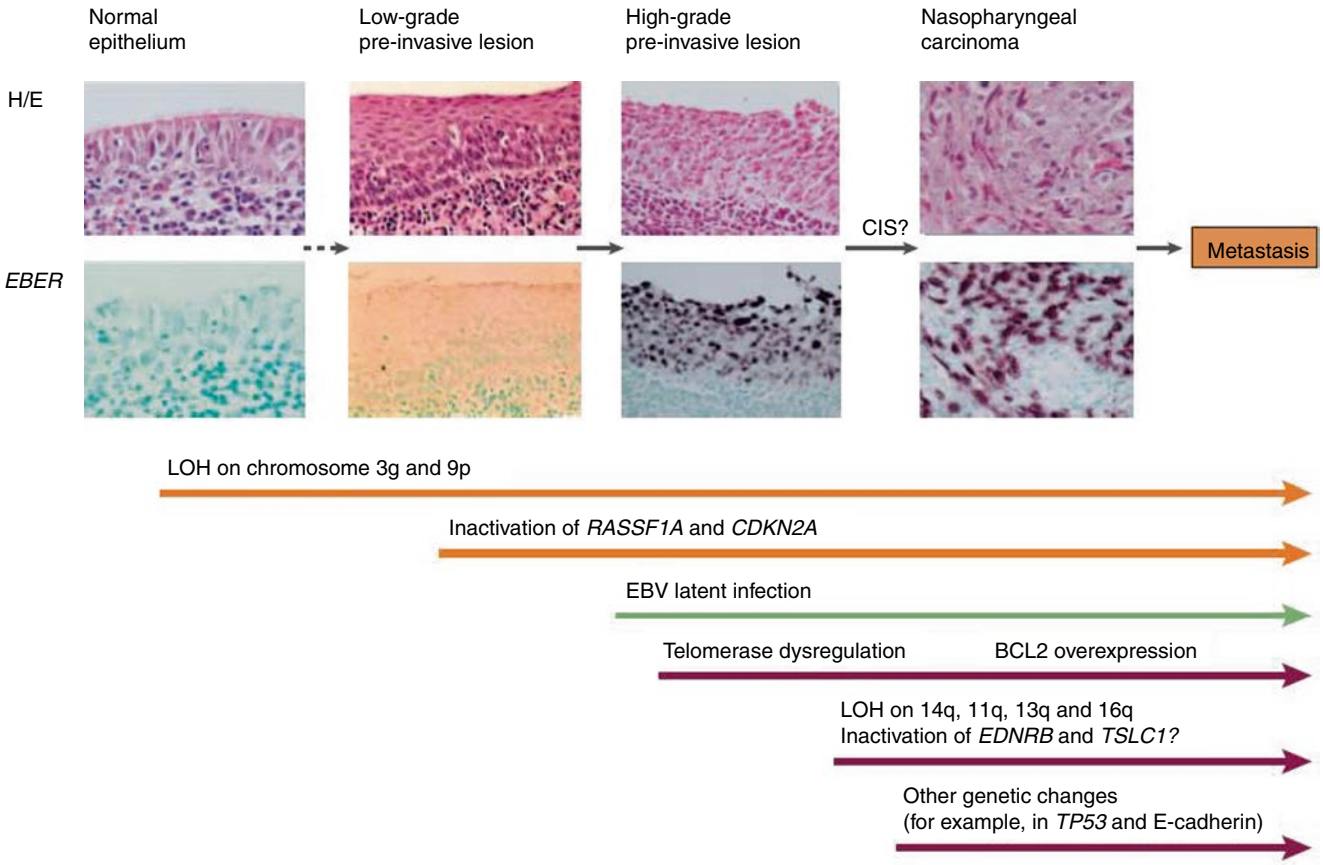

Fig. 41.3 Schematic representation of the pathogenesis of NPC. This model proposes that loss of heterozygosity (*LOH*) occurs early in the pathogenesis of NPC, possibly as a result of exposure to environmental cofactors such as dietary components (such as salted fish). This results in low-grade preinvasive lesions that, after additional genetic and epigenetic events, become susceptible to EBV infection. Once cells have become infected, EBV latent genes provide growth and survival benefits, resulting in the development of NPC. Additional genetic and epigenetic changes occur after EBV infection

NPC [73, 145]. As with other cancers, the advent of technologies to provide more detailed molecular classification of NPC tumors will provide opportunities to develop more personalized approaches to managing this disease [146].

9.2 Conventional Treatment

Early stage NPC is highly sensitive to radiotherapy (so-called intensity modulated radiation therapy), and locally advanced disease responds well to platinum-based chemotherapy in conjunction with radiotherapy [140]. Locally advanced recurrent NPC is more difficult to manage, but nasopharyngectomy has yielded excellent outcomes [147]. While there have been significant improvements in the primary treatment of NPC, 30 % of patients with locoregionally advanced disease will subsequently succumb to distant metastasis [140]. While first-line therapy with cisplatin-based chemotherapy is effective in some of these patients, novel approaches using targeted drugs and antibodies in conjunction with conventional radiochemotherapy are providing encouraging results [148].

9.3 EBV-Targeted Therapy

The success of the adoptive transfer of EBV-specific CTLs for the treatment of virus-driven posttransplant B cell lymphomas has led to a number of clinical trials using this same approach in the context of NPC. While these studies have demonstrated some limited clinical efficacy in patients with locoregional disease, the response in patients with metastatic tumors has been disappointing [149]. This likely reflects: (a) the broad specificity of the CTLs which are polyclonal populations containing low frequencies of T cells specific to the EBV latent antigens expressed in NPC and (b) the tumor microenvironment which is likely to suppress CTL activity. Approaches are being developed to enhance the generation of LMP1- and LMP2-specific CTLs [150] and to modify the cytokine milieu by using modified EBV-specific T cells [151, 152].

Alternative approaches to the treatment of NPC that target either the function of EBV-encoded proteins or exploit the transcriptional regulation of the virus are being developed. One approach is the use of gene therapy to deliver either cytotoxic proteins or proteins that interfere with EBV latent gene function. Selective expression of cytotoxic proteins (e.g., FasL) or wild-type p53 exploiting the ability of EBNA1 to activate transcription downstream of OriP has been demonstrated in in vitro and in vivo models of NPC [153, 154]. Induction of the EBV lytic cycle by either pharmacologic agents or delivery of EBV immediate-early genes results in the expression of virus-encoded kinases (EBV-TK, BGLF4) that phosphorylate ganciclovir (GCV) into its active cytotoxic form [155]. The ability of chemotherapy to confer sensitivity to GCV has been demonstrated in in vitro and in vivo NPC models [156] and was more recently shown to have therapeutic benefit in patients with recurrent and metastatic NPC [157]. Demethylating agents such as 5-azacytidine are also able to induce lytic EBV infection as well as expression of the immunodominant EBNAs (EBNAs 2, 3A, 3B, 3C) in EBV-associated tumors [158]. Such demethylation of EBV latent and lytic genes has been demonstrated in vivo in a clinical trial of 5-azacytidine therapy in patients with NPC [159].

More specific targeted approaches to inhibit the EBV transforming proteins, particularly LMP1, have also been investigated. Inhibiting the downstream consequences of LMP1 expression such as NF-κB activation by either genetic or pharmacologic approaches also has the potential to be effective in the management of LMP1-positive NPC [160]. Given the central role of EBNA1 in supporting EBV genome replication, the use of dominant-negative forms of EBNA1 to directly inhibit interaction of the wild-type protein with OriP may prove useful in eradicating EBV infection from tumor cells. This approach has been tested in various experimental systems using a recombinant adenovirus to deliver a dominant-negative version of EBNA1 [161]. OriP function has also been exploited to generate a conditionally replicating oncolytic adenovirus that, in conjunction with local radiotherapy, was able to eradicate established NPC tumors in an in vivo model [162].

9.4 Prophylactic and Therapeutic Vaccines

A vaccine to either protect from primary EBV infection or boost existing antiviral immunity in patients with NPC is an obvious approach that has been explored for over 40 years. The major EBV envelope glycoprotein gp340 is a target for neutralizing antibody responses and, when delivered as purified protein, has been shown to prevent lymphomagenesis in animal models. This approach has also been tested in phase I and phase II clinical trials and shown to be safe and well tolerated in EBV seronegative individuals [163, 164]. Whether such vaccination results in long-term sterilizing immunity and will prevent NPC is questionable. Therapeutic vaccines aimed at boosting EBV-specific T cell responses are being developed that use either peptides or viruses (adenovirus, vaccinia) expressing intact or modified (polyepitopes, fusion proteins).

9.4.1 Is EBV Associated with Other Common Epithelial Malignancies?

A number of other more common carcinomas such as breast cancer [165] and liver cancer [166] have been reported to be infected with EBV. Difficulties in confirming these associations have raised concerns about the use of PCR analysis alone to define EBV association and about the specificity of certain monoclonal antibody reagents. Definitive designation of a tumor as "EBV-associated" should require unequivocal demonstration of the EBV genome or virus gene products within the majority of the tumor cell population. This is not the case with breast cancer where it is clear that a small and extremely variable proportion of tumor cells are susceptible to EBV infection in vivo resulting in a low level lytic EBV infection [167, 168]. It is possible that a subset of EBV-infected breast carcinoma cells undergoing the virus lytic cycle produce soluble factors that are able to influence the growth and survival of surrounding EBV-negative tumor cells, but this remains to be demonstrated. The association of EBV with liver cancer, which was originally described in Japanese cases, has not been confirmed in cases from Europe and the USA raising the possibility of geographical variation [169].

Gastric Cancer

Gastric cancer remains the second leading cause of death from cancer (Global Cancer Statistics 2007). Although incidence and mortality rates have been falling for over 50 years, the number of deaths from this tumor will inevitably continue to increase globally because of population growth and aging in high-risk countries. As approximately 10 % of gastric carcinomas comprise cells latently infected with EBV, EBV-associated gastric cancers (EBV-GC) may represent the most common form of EBV-associated malignancy [170, 171]. These EBV-GC tumors display a restricted pattern of EBV latent gene expression (EBERs, EBNA1, LMP2A, the BART miRNAs, BARF1) similar to that observed in NPC [78, 172]. There is significant geographical variation in the association of EBV with GC that may be attributed to ethnic and genetic differences [170, 171]. EBV-GCs have distinct phenotypic and clinical characteristics compared to EBV-negative GC tumors including loss of p16 expression, p73 promoter methylation, wild-type p53, a different pattern of allelic loss, and improved patient survival [173–176]. As in NPC, the precise role of EBV in the pathogenesis of gastric carcinoma remains to be determined, but the absence of EBV

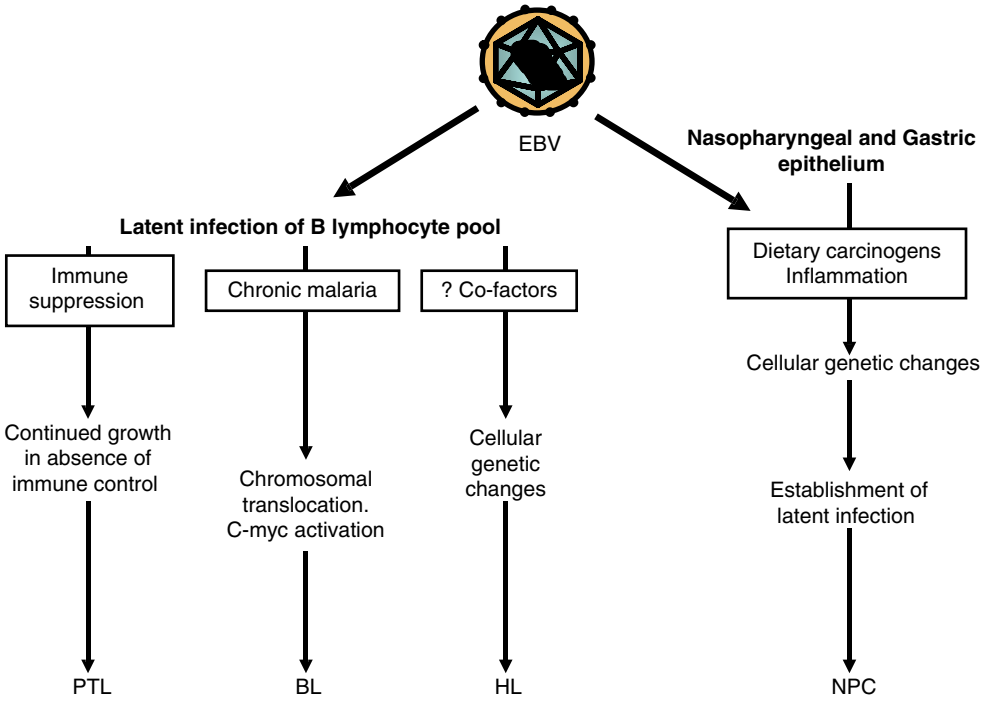

Fig. 41.4 This schematic representation highlights the different role of EBV infection in the pathogenesis of B cell-derived lymphomas as compared to carcinomas arising in the nasopharynx and stomach. It is proposed that latently infected B cells, arising from the persistent infection of the memory B cell pool, are influenced by immunosuppression and/or various cofactors to develop into either posttransplant lymphomas (*PTL*), Burkitt lymphoma (*BL*), or Hodgkin's lymphoma (*HL*). By contrast, stable latent EBV infection can only be sustained in epithelial cells of the nasopharynx or stomach once genetic and/or epigenetic changes have created a conducive cellular environment. These changes may be induced by dietary carcinogens along with a chronic inflammatory milieu

infection in premalignant gastric lesions supports the contention that virus infection is a relatively late event in gastric carcinogenesis [177]. Epidemiological studies have suggested that EBV-GC is related to birth order, high salt intake, and exposure to metal dust, but likely geographical variation in these factors (e.g., between Japan and Colombia) supports the need for more detailed investigation [170, 171]. It has been suggested that distinct EBV strains contribute to the development of EBV-GC, although this could reflect geographical variation in the prevalence of different EBV strains in the population [178].

10 Future Perspectives

EBV was discovered almost 50 years ago and its DNA was fully sequenced in 1984. It remains the most common persistent virus infection in humans with over 95 % of the population sustaining an asymptomatic lifelong infection – testimony to the intimate interaction between EBV and the immune host. This relationship relies on the ability of EBV to persist in the memory B cell pool of normal healthy individuals, and perturbation of this interaction results in virus-associated B cell tumors. Much work has contributed to the unequivocal identification of EBV as oncogenic in humans, but the precise role of EBV in the pathogenesis of NPC and EBV-GC remains unclear.

Little is known about the replicative life cycle of EBV in vivo particularly with regard to the relative role of B cells versus epithelial cells in this process. The impact of EBV infection on the development of NPC and EBV-GC may be a consequence of the aberrant establishment of virus latency in epithelial cells that have already undergone premalignant genetic changes. This appears to be a distinct pathogenic process from that associated with EBV-induced lymphomagenesis where the virus appears to be the initiating driver (Fig. 41.4).

The development of more efficient in vitro systems for studying EBV infection and replication in different cell types is helping to unravel the complex interplay between the virus and the cell. The use of EBV recombinants continues to shed light on the role of latent genes in the transformation process, on the requirements for the efficient production of progeny virus, and on the role of membrane glycoproteins in the infection process. Understanding the host cell–virus interaction will be dependent on the generation of appropriate in

vitro and in vivo models, particularly systems which allow a more detailed understanding of the tumor microenvironment and the role of the local cytokine milieu.

Whatever the precise role of EBV in the carcinogenic process, there is clearly the opportunity to exploit this association for the clinical benefit of patients. EBV is the ultimate biomarker and routine application of serum testing for virus DNA not only provides invaluable prognostic information in NPC patients but will also facilitate the implementation of mass screening programs to identify patients with the early stages of NPC. This approach will be further enhanced by the development of additional adjunctive tests (e.g., EBV miRNAs, methylated TSGs). Novel therapeutic approaches using targeted drugs, gene therapy [60], or therapeutic vaccination [61] augur well for our ability to effectively target the clinically challenging aspects of locally recurrent and metastatic disease. Alongside these therapies, the advent of personalized medicine raises the possibility of using molecular classification to subdivide NPC and EBV-GC and thereby improve patient management and outcomes. Our growing understanding of EBV-associated oncogenesis provides paradigms for the development of targeted cancer therapies and diagnostics and further confirms the far-reaching value of tumor virology to the whole cancer field.

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Suggested Reading

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Human Herpesviruses: Varicella and Herpes Zoster

42

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1 Introduction

Varicella-zoster virus (VZV) causes two clinically distinct diseases in humans [1]. Varicella (or chickenpox), which results from primary infection of a susceptible individual, is a common, extremely contagious, and usually benign acute illness that occurs in seasonal epidemics and is characterized by a generalized vesicular rash. Like all herpesviruses, VZV establishes latency following primary infection. Reactivation of latent VZV results in herpes zoster (or shingles), a localized cutaneous eruption in a dermatomal distribution that occurs most commonly among elderly individuals. Thus, varicella results from primary exogenous contact with VZV, while herpes zoster represents reactivation of latent VZV that usually occurs decades after the primary infection. Both varicella and herpes zoster can cause serious morbidity and mortality in immunocompromised populations. Both manifestations of VZV can now be prevented through the use of vaccines containing live, attenuated virus [2].

2 Historical Background

Varicella has been recognized as a distinct disease since at least the ninth century AD when the Persian physician Rhazes described a vesicular rash that was not protective against smallpox. However, confusion of varicella with other pustular skin diseases persisted into the nineteenth century. Indeed, the name "varicella" is a diminutive form of the term "variola," or smallpox, indicating that the two diseases were formerly thought to be etiologically related. The common term "chickenpox" has been variously attributed either to the old English word *gican* which means "to itch" or "to scratch" or to the old French word *chiche-pois* which likens the appearance of a vesicle to a chickpea.

Because of its distinctive dermatologic distribution, herpes zoster has been recognized as a unique entity since antiquity, although the observation that varicella and herpes zoster were etiologically related was not made until the nineteenth century. *Herpes* is derived from the Greek word meaning "to creep." *Zoster* and *shingles* are derived from the Greek and Latin terms, respectively, for "girdle," referring to the band-like distribution of lesions.

Heberden was among the first to definitively distinguish varicella from smallpox in 1767. In 1875, Steiner demonstrated the infectious nature of varicella when he was able to transmit the disease to volunteers by inoculating them with vesicular fluid from patients with chickenpox. The relationship between varicella and herpes zoster began to be elucidated by von Bokay in 1888 when he observed that susceptible children acquired varicella after contact with patients with herpes zoster. In 1925 Kundratitz was able to produce varicella-like lesions in volunteers by experimentally inoculating them with fluid from patients with herpes zoster. The careful work of Tyzzer in 1908 and Lipschüv in 1921 demonstrated that the histopathologic characteristics of varicella and herpes zoster lesions were identical. The important intuitive leap that herpes zoster represented a reactivation of latent varicella virus was made by Garland in 1943. In the 1950s, Weller and colleagues were able to propagate the etiologic agent in vitro and provide final definitive proof that varicella and herpes zoster are caused by the same viral pathogen. Hope-Simpson is credited with theorizing that declining immunity allowed latent VZV to reactivate, producing herpes zoster, as reviewed [3, 4].

3 Methodology Involved in Epidemiologic Analysis

Varicella has been added to and deleted from the nationally notifiable disease list several times since 1972 [5]. Most recently, varicella deaths became nationally notifiable in 1999.

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To track varicella incidence after implementation of the varicella vaccine in 1995, the CDC established sentinel sites for active surveillance in California and Pennsylvania [5]. With the success of varicella vaccination and reduction in the number of cases, the sentinel sites were no longer able to provide sufficient incidence data. In 2003, case-based surveillance for varicella was reestablished in all states via the National Notifiable Disease Surveillance System (NNDSS). By 2010, a total of 39 states required reporting of varicella cases, and 38 states were conducting passive case-based surveillance [5].

Vaccination data are collected via the National Immunization Survey (NIS), which is a random telephone survey that gathers vaccination coverage data from households with children age 19–35 months in all 50 states and in selected urban areas. Additional data on vaccination are available from the Vaccine Adverse Event Reporting System and from the Vaccine Safety Data Link.

Herpes zoster has never been a reportable disease. Data regarding the incidence of herpes zoster are gathered from several sources including large managed care databases such as the Keiser Permanente System [6] and the US Department of Veterans Affairs databases [7]. Insurance company databases (both private and federally sponsored insurers such as Medicare) are also a key source of incidence data [8]. Valuable data have been derived from studies of large population-based cohorts such as the Rochester Epidemiology Project [9] and the Olmsted County cohort [10]. Other methodologies include searches of electronic hospital diagnosis codes and prescription records for evidence of herpes zoster encounters [11]. Ongoing surveillance systems that can provide disease incidence data include the National Hospital Discharge Survey, the National Ambulatory Medical care Survey, and the National Hospital Ambulatory Care Survey.

4 Biological Characteristics of the Virus That Affect the Epidemiologic Pattern

Varicella-zoster virus (human herpesvirus 3), along with herpes simplex viruses type I and type II, makes up the human alpha herpesvirus group. Sequencing analysis demonstrates genetic differences among VZV strains isolated in different epidemiologic situations. These strains can be grouped into clades, but there is only a single VZV serotype [12–14]. At least 5 (and perhaps as many as 9) genotypically distinct clades of VZV have been identified and used for epidemiologic mapping [15].

The alpha herpesviruses are characterized by short replication cycles, rapid spread in tissue culture, destruction of host cells during replication, and latency in sensory nerve ganglia (including cranial nerve, dorsal root, and autonomic ganglia) [16]. VZV shares structural characteristics with other members of the family *Herpesviridae*. Complete VZV virions are pleomorphic and measure approximately 180–200 nm in diameter. The nucleocapsid measures about 100 nm in diameter and is composed of 162 capsomeres arranged in icosahedral symmetry. An amorphous tegument layer surrounds the capsid. The virion is enclosed in a lipid-containing laminated envelope with external glycoprotein spikes, which are key antigenic determinants. The VZV genome consists of a linear, double-stranded DNA molecule containing about 125,000 base pairs with a guanosine-plus-cytosine content of about 46 %. The genome is organized in a manner similar to that of other herpesviruses, having unique long (UL; 104.5 kb) and unique short (US; 5.2 kb) regions flanked by inverted terminal repeat sequences. VZV encodes about 70 genes, including 7 that encode glycoproteins [17, 18]. VZV can be propagated in vitro in a limited number of continuous and discontinuous cell culture monolayers, mostly of human or simian origin. In human embryonic fibroblasts, cytopathic effects begin as a focal process with subsequent cell-to-cell spread. Approximately 8–10 h after infection, virus-specific immunofluorescence can be detected in cells adjacent to the initial focus of infection. Viral cytopathic effects are visible after about 3 days. In vitro, VZV replication is highly cell associated, with very limited release of infectious virions into the fluid medium.

5 Descriptive Epidemiology

5.1 Epidemiology of Varicella

In temperate climates such as that of the USA, varicella epidemics occur annually in the late winter and early spring, with peak numbers of cases reported in March. Before the availability of the varicella vaccine, approximately 3.8 million cases of chickenpox occurred each year in the USA (about 15 cases per 1,000 population), which approximately equaled the annual birth cohort. In the absence of a systematic vaccination program, varicella is predominantly a disease of school-aged children. Historically, in the USA, about 50–60 % of varicella cases occurred in children between 5 and 9 years of age; over 90 % of cases occurred in persons younger than 15 years. Previous surveys indicated that greater than 90 % of the US. population was VZV seropositive by age 20 years [19–22]. Sex and race have no apparent impact on the incidence of varicella in susceptible populations. Mortality estimates among children (ages 1–14 years) with varicella were 1.4 deaths per 100,000 cases. In temperate countries where routine varicella vaccination has not been adopted, this epidemiologic pattern continues (Fig. 42.1) [23]. In countries such as the USA, widespread use of the varicella vaccine has produced dramatic changes in the epidemiology of varicella and varicella-related mortality, as discussed in detail below.

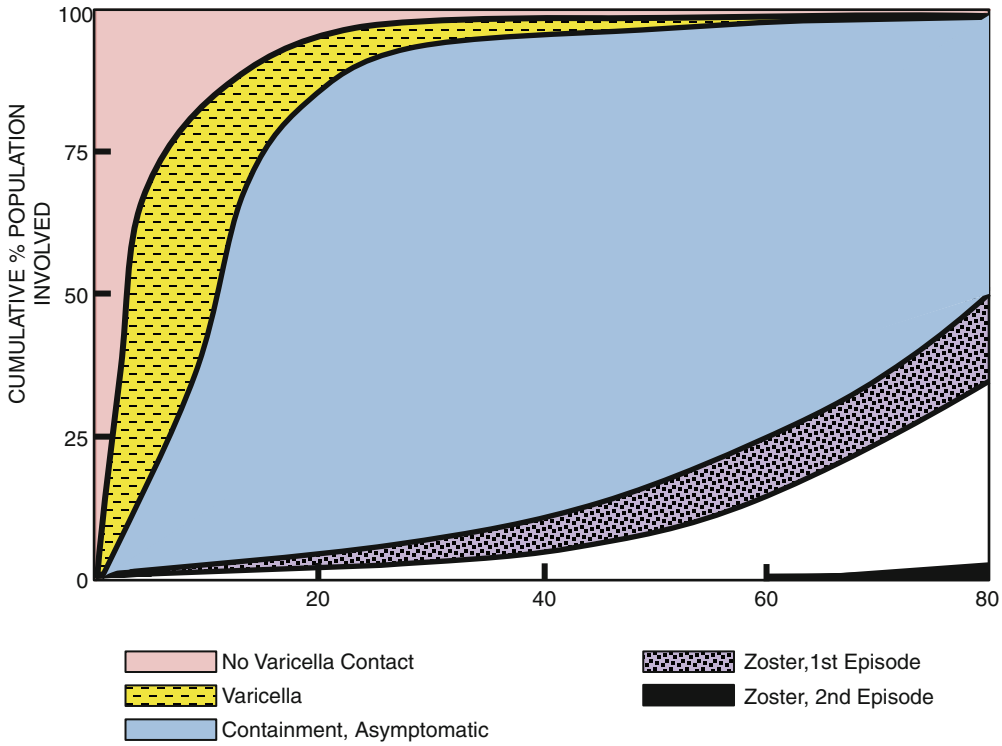

Fig. 42.1 Age-related occurrence of VZV disease in a population in a temperate climate without varicella or herpes zoster vaccination [302]

An interesting observation regarding the geographic distribution of varicella is the lower incidence of chickenpox among children in tropical areas of the world. This phenomenon is not yet fully understood, although it may be related to decreased transmission efficiency of VZV due to climate (temperature, humidity, etc.) or sociological factors [24]. The consequence is that a higher proportion of persons growing up in tropical areas may remain VZV seronegative after reaching adulthood. When those susceptible individuals subsequently travel to areas with higher rates of VZV transmission (e.g., young adults from Puerto Rico reporting to mainland USA for military training), they are at increased risk to acquire chickenpox. This has significant consequences, because the morbidity of varicella is much higher in adolescence and adults than it is in young children.

6 Epidemiology of Herpes Zoster

Herpes zoster results from reactivation of latent VZV [25–27]. Over 90 % of adults in the USA have serologic evidence of prior VZV infection and are at risk for herpes zoster. In the general population (including individuals of all ages), the annual incidence rate for herpes zoster is about 3.6 cases per 1,000 population, with a range of 3.2–5.2 cases per 1,000 person-years in various studies (Fig. 42.2) [6, 8, 10, 28–38]. This incidence rate would predict about one million cases of herpes zoster annually in the USA [6, 10, 27].

Increasing age is a well-defined risk factor for herpes zoster. Herpes zoster is not a rare disease in young adults, but the incidence clearly begins to increase in individuals over 55 years of age. The median age of patients with herpes zoster is estimated to be 64 years [39]. By decades, the risk for herpes zoster is about 7, 9.5, and 11 cases per 1,000 person-years for populations aged 60–69, 70–79, and >80 years, respectively [6, 28–30]. In the prospective Shingles Prevention Study that evaluated the efficacy of the herpes zoster vaccine, the incidence of shingles was 11.8 cases per 1,000 person-years among the 18,357 placebo recipients ≥60 years of age who completed the study [40].

A key component of Hope-Simpson's original hypothesis regarding the pathogenesis of herpes zoster was that intermittent reexposure of adults to VZV (primarily via children with chickenpox) resulted in the boosting of VZV-specific immunity and immune containment of latent virus. While attractive, this hypothesis has been difficult to support with observation. For example, the hypothesis would predict that adults who are not intermittently exposed to VZV should have earlier onset and perhaps increased incidence of herpes zoster. However, studies of individuals who are members of cloistered monastic orders have not shown that they have an increased risk or earlier onset of herpes zoster compared to the general population [41]. While studies suggest that the incidence of herpes zoster in adults has increased since the implementation of varicella vaccination, a causal relationship has been difficult to establish [7, 8, 42]. For example, age-standardized surveys conducted in the states of Washington (1997–2002) and

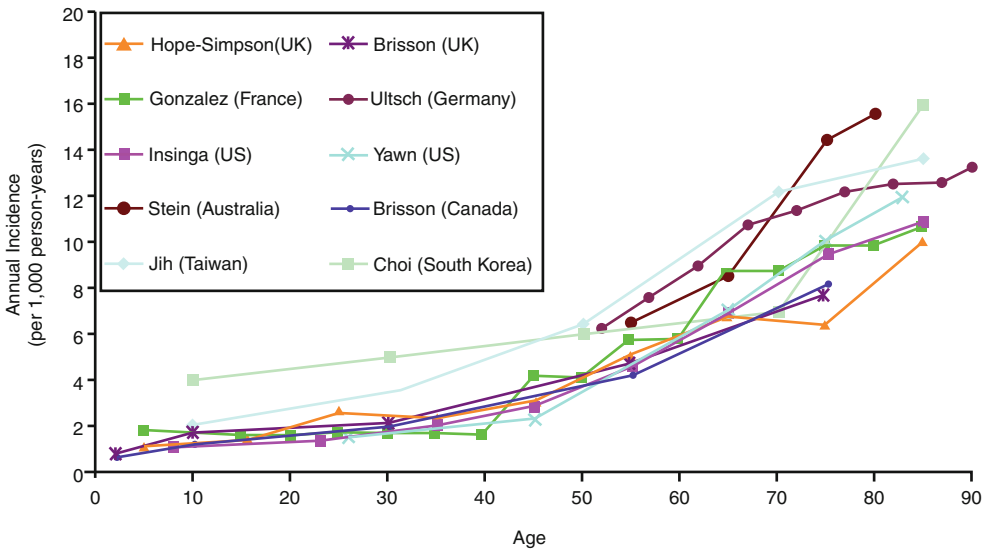

Fig. 42.2 Increasing age has a positive correlation with incidence of herpes zoster. Data displayed from 10 incidence studies conducted in 8 countries (compiled by Eddie Bresnitz, M.D., Merck and Co., Inc.) [10, 28, 32, 38, 297–301, 303]

Minnesota (1996–2005) demonstrated increases in herpes zoster incidence over the surveillance periods from 3.28 to 4.29 cases and from 3.2 to 4.1 cases per 1,000 person-years, respectively [6, 10]. An analysis of large medical claim databases for the period 1993–2006 showed an increase in herpes zoster incidence as well as herpes zoster-related hospital discharges [8, 43]. However, herpes zoster incidence did not vary by state on the basis of varicella vaccination coverage [8]. Similar studies in Australia demonstrated an increase in herpes zoster incidence [44], while herpes zoster incidence in Canada appeared to be stable [45]. In neither case was it possible to attribute observed trends in herpes zoster incidence to the varicella immunization program. Thus, while there is compelling evidence that the incidence of herpes zoster is increasing among adults, it has not been possible to link this observation to increased varicella vaccine coverage [46]. Contributing factors to the increasing incidence may be the growing proportion of the population in the developing world that is over 65 years of age and the steadily increasing numbers of persons who are immunocompromised due to disease or medical interventions. The complex epidemiologic interactions between pediatric varicella vaccination and incidence of adult herpes zoster remain to be fully elucidated [47]. Notably, the incidence of herpes zoster among children covered by varicella vaccination has declined substantially [48]. In a cohort of California children monitored from 2000 to 2006, the incidence of herpes zoster in children under 10 years of age declined by 55 %, from 42 cases reported in 2000 to 2018 reported in 2006 [49]. The authors estimated that children aged 10 years who had received varicella vaccination had a 4–12 times lower risk for developing herpes zoster compared with children who had experienced varicella disease [49].

In addition to age, the other well-described risk factor for herpes zoster is immunodeficiency, specifically depressed cell-mediated immune responses. Groups clearly identified as being at high risk for herpes zoster include patients taking immunosuppressive medications (e.g., high-dose corticosteroids), organ transplant recipients receiving immunosuppressive antirejection therapy, and patients with lymphoproliferative malignancies or HIV infection. For example, 15–30 % of patients with Hodgkin disease experience herpes zoster compared to 2 % of patients with non-hematologic solid tumor malignancies. However, the converse is not true: herpes zoster is not a sensitive enough marker for malignancy that it warrants a search for underlying cancer [50]. Shingles occurs in 3–10 % of renal transplant recipients, 20–25 % of cardiac transplant recipients, and 20–50 % of allogeneic hematopoietic stem-cell transplant recipients [51–54]. In a recent US study using the Department of Veterans Affairs databases, the incidence of herpes zoster among solid organ transplant recipients was 22.2 cases per 1,000 person-years [55]. Herpes zoster also occurs with increased frequency among patients with immunologically mediated connective tissue diseases, including rheumatoid arthritis and systemic lupus erythematosus [56–59]. The zoster risk may be further increased when these patients are treated with biological therapies such as anti-TNF-alpha drugs [60, 61]. In surveillance studies conducted in the USA, Europe, and Africa, the incidence of herpes zoster among HIV-seropositive patients was shown to be approximately 30–50 cases per 1,000 person-years, with a cumulative incidence over 10 years of approximately 30–40 %. In contrast, the shingles incidence among HIV-seronegative controls was two to three cases per 1,000 person-years, with a 10-year cumulative incidence of about 3 %. These observations confirm that the incidence of herpes zoster is approximately 15-fold higher among HIV-infected individuals than among age-matched HIV-seronegative controls. From a clinical perspective, the possibility of HIV infection should be considered in otherwise healthy younger patients who present with herpes zoster. In

African populations where the prevalence of HIV infection is high, more than 90 % of patients presenting with a new diagnosis of herpes zoster were found to be HIV infected [62]. The incidence of herpes zoster in HIV-infected children and adults has declined substantially since the availability of effective antiretroviral therapy and varicella vaccination [63, 64], but shingles continues to occur at higher frequency, even among patients with well-controlled HIV infection [65].

Unlike varicella, there is no seasonal predilection for herpes zoster. In age-adjusted cohorts, female sex and Caucasian race appeared to be risk factors for slightly increased incidence of herpes zoster [8, 66]. Investigations of a possible familial or genetic predisposition to herpes zoster have yielded conflicting results. In studies conducted in the USA, case patients were more likely to report blood relatives with a history of herpes zoster than controls (43.5 % vs. 10.5 %; $p<0.001$), with evidence of a dose-dependent effect (risk was increased with multiple blood relatives) [67, 68]. A study in Italy, however, found no evidence of family history as a risk factor for herpes zoster [69]. Larger prospective cohort studies will be needed to definitely determine whether family history is a risk factor for herpes zoster.

The lifetime risk of herpes zoster is estimated to be 10–20 %. The frequency of second episodes of herpes zoster in immunocompetent persons is not well defined, but appears to be low (≤5 %) [70]. Persons who experience a first episode at a relatively young age may have a second episode as older adults. In contrast, multiple recurrences of herpes zoster are frequently observed in HIV-seropositive patients.

7 Mechanisms and Routes of Transmission

7.1 Varicella

Chickenpox is acquired by person-to-person transmission of VZV from an infected individual to a susceptible host. The incubation period is about 14 days with a range of 10–21 days. Patients with chickenpox are capable of transmitting virus from 48 to 72 h before the onset of rash until all cutaneous lesions have crusted. The fact that transmission has been documented to occur during the incubation period prior to rash onset suggests that respiratory secretions are an important source of viral transmission. While VZV can be difficult to recover from respiratory secretions by culture during the incubation period, the presence of virus is readily demonstrated by polymerase chain reaction (PCR). Virus is present in high concentrations in vesicular cutaneous lesions, and aerosolized virus from vesicular fluid is also a likely mechanism for transmission. Varicella is a highly contagious infection, and attack rates of 60–90 % have been observed among susceptible household contacts of chickenpox patients.

Herpes zoster can also serve as a source for viral transmission [71]. Exposure of a susceptible host to a patient with active vesicular herpes zoster can result in VZV transmission and development of chickenpox. The source of virus is not completely clear. Aerosolization of vesicular fluid seems the most obvious mechanism. Studies using PCR methodology have clearly demonstrated the presence of VZV DNA (and presumably infectious virus) in the air from a room where a patient with herpes zoster is housed. However, VZV DNA can also be detected in the saliva of patients with acute dermatomal herpes zoster, suggesting that transmission via respiratory secretions might be possible [72, 73]. Because vaccination has resulted in such a dramatic decline in varicella incidence in the USA, herpes zoster may prove to be an increasingly important source of VZV transmission and new infections [74].

Another unusual route of VZV infection is organ transplantation. Rare cases of transmission of VZV via the donor organ have been reported when the donor had a very recent history of chickenpox [75].

Varicella occurring during pregnancy can result in congenital or neonatal infections. Development of maternal chickenpox during the first half of pregnancy introduces the risk of the rare congenital varicella syndrome (CVS), which results from viremic intrauterine transmission and VZV infection of the fetus [76]. VZV infection of neonates may result from either vertical or horizontal transmission [77–79]. If maternal varicella develops during the time window from 4 to 5 days before until 2 days after delivery, the baby is exposed to VZV (usually by transplacental viremia) without the benefit of transplacental maternal anti-VZV Immune globulin indicates a drug G (IgG). In this situation, varicella will develop in an estimated 17–48 % of neonates. Conversely, infants who have varicella lesions present at birth or within the first 5 days following delivery (thus having received transplacental maternal anti-VZV IgG prepartum) are unlikely to develop severe disease. In addition, infants (particularly low-birth-weight babies) born to VZV-seronegative mothers are at high risk for severe varicella if infected during their first few days of life (e.g., in the nursery).

7.2 Herpes Zoster

Shingles always results from reactivation of endogenous latent VZV. Thus, there is no transmission of herpes zoster from one individual to another (although exposure to herpes zoster can result in chickenpox in a susceptible host, as described above). Despite frequent anecdotal reports, there is no convincing evidence that exogenous exposure to VZV (e.g., contact with a person with varicella) can serve as a trigger for reactivation of latent VZV resulting in herpes zoster.

8 Pathogenesis and Immunity

8.1 Pathogenesis of Varicella

Humans are the only known reservoir for VZV. Primary infection occurs when a susceptible individual is exposed to airborne virus, presumably via the respiratory route. Since there is no easily manipulated animal model for varicella, much of the pathogenesis story is based on clinical observations and analogies with other exanthamatous viral pathogens. Varicella is most often acquired following exposure to another person with active chickenpox, but infection can also result from close exposure to a patient with herpes zoster. Virus and airborne droplets enter the susceptible host via mucosal surfaces of the conjunctiva, oropharynx, or upper respiratory tract. Like HSV, VZV demonstrates tropism for epithelial cells of mucosal and cutaneous surfaces as well as for neural tissues. VZV undergoes an initial round of replication, presumably in tonsillar or cervical lymph nodes. Studies have demonstrated that subpopulations of activated memory T lymphocytes in tonsils are readily infected by VZV [80]. According to the long-held theory, when local immune responses are overcome, a primary round of viremia occurs with widespread dissemination of VZV to the reticuloendothelial system. Following additional cycles of replication, a second viremic phase occurs approximately 1 week after the initial viremia and is accompanied by the onset of clinical symptoms. A newly proposed hypothesis is that VZV may be transported to the skin primarily via infected peripheral blood mononuclear cells (PBMCs), particularly infected T lymphocytes. T cell subpopulations that express the cutaneous homing antigen and chemokine receptor CCR4 are highly permissive for VZV infection and may serve to deliver virus to the cutaneous epithelial cells. VZV initially localizes to endothelial cells of cutaneous capillaries and then extends to epithelial cells of the epidermis. Innate immune mechanisms appear to establish early control of viral replication. Cell-to-cell spread of virus is contained for the first week by production of interferon-alpha in adjacent epithelial cells [81]. Localized viral replication in epithelial cells is subsequently facilitated by VZV-mediated downregulation of interferon-alpha within the infected cells and failure of induction of inflammatory adhesion molecules [80, 81]. Eventually, the virus overcomes the innate immune defenses and undergoes epithelial replication with the appearance of cutaneous vesicles. The production of cytokines and upregulation of capillary endothelial adhesion factors attract activated T cells that may become infected by VZV and serve to further spread infection before replication is eventually contained [82]. In the normal host, viremia and new vesicle formation continue for 3–5 days until suppressed by developing humoral and cellular immune responses. Cutaneous vesicles contain cell-free infectious virus. Sensory nerve exons that terminate in the dermis may be infected with VZV, allowing viral transport to sensory ganglia and subsequent establishment of latent infection [83]. VZV may also reach neurons via hematogenous spread.

VZV replication in the skin results in ballooning degeneration of epithelial cells in the prickle cell layer (stratum spinosum) of the epidermis. Local collections of extracellular edema fluid result in acantholysis, with elevation of the stratum corneum to form a clear vesicle. Multinucleated giant cells are found at the base of the lesion. Infected cells contain eosinophilic intranuclear inclusion bodies (Cowdry type A inclusions) surrounded by a clear zone [84]. Perivascular infiltration of mononuclear cells is seen around cutaneous vessels. The vesicular fluid becomes cloudy as it accumulates inflammatory cells and desquamated epidermal cells. Vesicular fluid is resorbed, resulting in drying and crusting of the lesion. Healing occurs with regeneration of the epithelial cell layers.

8.2 Immune Responses in Varicella

Control of VZV replication in the early stages of primary infection is mediated by innate immune responses, including production of interferon-alpha by epidermal cells [81]. VZV-specific IgM and IgG can be detected in serum as early as 1–3 days after the appearance of skin lesions. Antibodies induced during primary infection are directed against nucleic capsid proteins and glycoproteins and have viral neutralizing activity. Humoral immune responses are thought to contribute to recovery from primary VZV infection, but are not essential. This is supported by the observation that children with humoral immune deficiency syndromes tend to have uncomplicated courses of varicella. On the other hand, passive immunotherapy with immunoglobulin preparations containing high titers of anti-VZV IgG within 96 h of VZV exposure clearly ameliorates the clinical symptoms of varicella in immunocompromised patients. The roles of humoral and cellular immunity have been demonstrated in the simian varicella virus (SVV) animal model. Animals depleted of CD4+ cells had higher viral load, prolonged viremia, and disseminated SVV infection, while B cell-depleted animals demonstrated no alteration in disease severity [85]. In humans, adaptive cell-mediated immune (CMI) responses appear to be essential for resolving primary VZV replication. This is demonstrated by the severe and potentially life-threatening clinical courses of varicella in children with congenital or acquired T cell immunodeficiency syndromes. VZV-specific T cell proliferative responses are detectable in most healthy children within a week of appearance of rash. The CMI response consists of both CD4+ and CD8+ effector cells as well as memory T cells. T cell proliferation results in elaboration of cytokines with both direct and indirect

antiviral activity, including interferon-gamma. The termination of VZV replication in varicella is marked by upregulation of human leukocyte antigen (HLA) class II expression, permitting lysis of VZV-infected cells by CD4+ CTLs as well as interferon-gamma-enhanced HLA class I-restricted cellular cytotoxicity mediated by CD8+ T lymphocytes [86–88]. These memory T cell responses are also important contributors to protection following reexposure to VZV [87]. Adaptive cell-mediated immune responses can be boosted by exposure to endogenous (reactivation of latent virus) or exogenous (environmental exposure) VZV, which is thought to be important in long-term immune suppression of latent virus in the ganglia.

8.3 Pathogenesis of Herpes Zoster

In many ways, VZV should be considered a neurotropic virus [89]. As VZV replicates in the skin during acute varicella, virions are transported via sensory nerves to the corresponding sensory ganglia. In the ganglion, the virus establishes a latent infection within neurons and probably also in satellite cells [90]. An alternative explanation is that VZV may reach the ganglion by viremic spread [91, 92].

Difficulty in obtaining large quantities of cell-free virus and the lack of suitable small animal models for varicella and herpes zoster have complicated investigations of mechanisms of latency. Recently developed rodent models allow study of some aspects of VZV latency [90]. Latent VZV is located predominately in human ganglionic neurons where VZV DNA is found in a circular or concatameric (end-to-end) configuration at a copy frequency of 2–9 genomic copies per neuron. In situ hybridization studies have shown the latent VZV genome to be present in 1–7 % of the sensory ganglia neurons [93–96]. Animal model experiments have demonstrated the presence of VZV genomic DNA, viral proteins, and virion production in both neurons and satellite cells [97]. Transcription of VZV genes during latency is restricted to expression of 6 genes (4, 21, 29, 62, 63, and 66), with expression of gene 63 the most prevalent and considered to be the marker for latency [98–100]. A recently developed model of SVV infection in rhesus macaques appears to simulate human varicella and results in latent ganglionic infection with expression of ORF63 protein [101]. The complex mechanisms that control latency and virus reactivation in ganglia remain incompletely understood.

Following reactivation and replication in the ganglion, virus is transported along the sensory nerve at a rate estimated to be 13 cm/day to the skin where it again replicates in epithelial cells, producing the dermatomal vesicular rash characteristic of shingles [102, 103]. Unlike the lesions of varicella, in which different stages are seen simultaneously, most herpes zoster lesions are in the same stage of development. Replication of VZV in the sensory ganglion results in intense inflammation, neuronal destruction, and focal hemorrhagic necrosis. Less severe inflammatory changes often occur in adjacent ganglia. Occasionally, inflammation and necrosis also extend to the anterior nerve root, resulting in localized motor neuropathy. These changes are accompanied by lymphocytic pleocytosis and virus may be detectible in the CSF. Movement of VZV from the ganglion down to the sensory nerve to the skin produces acute inflammation in the nerve. Inflammatory changes in the sensory nerve persist for months and may result in demyelination, Wallerian degeneration, and sclerosis. Virus reaching the skin replicates in epithelial cells of the epidermis, producing pathologic changes identical to those described for varicella above. Viremia occurs during most episodes of herpes zoster. VZV can be demonstrated both in plasma and PBMCs during acute shingles and can, in some cases, persist for weeks [104, 105].

8.4 Immune Responses in Herpes Zoster

The specific immune responses that limit reactivation of VZV from the sensory ganglia are poorly understood, although the role of VZV-specific memory T lymphocytes is critical [86, 87]. VZV may periodically reactivate and undergo limited replication, but replication is suppressed by the immune response before any clinical symptoms result. The most important factor that predisposes to the development of herpes zoster is decline or suppression of VZV-specific cellular immunity [106]. Lower frequency of circulating VZV-specific responder T cells characterizes all conditions associated with increased incidence of VZV reactivation, while levels of humoral immunity are well maintained, even in the elderly [107]. VZV responder cell frequency peaks in early adulthood and then begins to decline [87]. Elderly individuals and renal transplant recipients have lower frequency of VZV-reactive effector memory T cells when compared with controls [108]. Specificity of these responder T cells in latently infected individuals is directed against VZV glycoproteins (E, H, B, and I) as well as against transcriptional activators encoded by ORF 4, 10, 62, and 63. Currently, there is no assay available that identifies an exact immunologic correlate of protection for herpes zoster.

Following reactivation of latent VZV and clinical herpes zoster, patients demonstrate enhanced levels of VZV-specific humoral and CMI responses [107, 109]. The observation that second episodes of herpes zoster are rare in immunocompetent individuals suggest that this boosting of immunity that accompanies clinical herpes zoster is sufficient to maintain the virus in its latent state and prevent subsequent reactivation for at least several years.

9 Patterns of Host Response

9.1 Clinical Presentation of Varicella

Symptoms of varicella develop after an incubation period that averages about 15 days (range 10–21 days). Varicella is generally a benign disease in healthy children, but symptoms are often more severe in adolescents and adults. Symptomatic visceral organ infection (pneumonia, encephalitis, hepatitis, etc.) is rare in immunocompetent patients, but occurs at much higher frequency in the setting of immune suppression, especially in children with hematologic malignancies or organ transplant recipients. Fewer than 5 % of primary VZV infections are asymptomatic or subclinical. A prodrome of fever, malaise, headache, and anorexia is variably present, occurring more commonly among older children and adults and lasting 1–2 days. A transient scarlatina-like rash is occasionally noted just before or coincident with the appearance of the varicella lesions. Lesions begin as pink macules that quickly evolve into fragile vesicles 1–4 mm in diameter surrounded by a zone of erythema. The lesions first appear on the face and scalp, then on the trunk, and finally on the extremities, with the greatest concentration of lesions seen on the trunk and proximal extremities. Vesicles also appear on mucosal surfaces and rapidly evolve into shallow ulcerations. The rash of chickenpox is characterized by a rapid evolution of lesions over 8–12 h and by successive crops of new lesions. Consequently, lesions of all stages are present simultaneously on the involved skin surfaces. New lesion formation continues for 2–4 days, accompanied by pruritus, fever, headache, malaise, and anorexia. The rash peaks on about the fifth day with an average lesion count of 250–500 lesions. Fewer lesions are seen in children younger than 5 years. Older children and adults, as well as secondary cases within a household, tend to have more severe disease (higher lesion counts and higher fever) and a higher frequency of complications [110]. As inflammatory cells migrate into the vesicular fluid, the lesions become pustules that are often centrally umbilicated. The pustules become crusted and the scabs detach after 1–3 weeks, usually healing without scarring.

9.2 Complications of Varicella

9.2.1 Skin and Soft Tissue Infections

The most common complication of pediatric varicella is cellulitis. The bacterial superinfection is most often caused by *Staphylococcus aureus* or *Streptococcus pyogenes* (group A streptococcus) and is attributed to scratching [111]. The skin infection can range from simple cellulitis (which is common) to more severe manifestations, including necrotizing fasciitis (which is rare) [112]. In the prevaccine era, it was reported that up to one-third of all cases of group A streptococcal cellulitis in children were temporally associated with chickenpox. Toxin-induced diseases (scalded skin and toxic shock syndromes) have been reported as a consequence of bacterial skin infections in children with varicella [113].

9.2.2 Varicella Pneumonia

Pneumonitis caused by VZV is rare in children with chickenpox but occurs with increased frequency in adolescents and adults [114]. In studies of military recruits with chickenpox, 15–20 % showed pulmonary infiltrates on chest radiograph, although most did not have clinical signs or symptoms of pneumonia. Women who contract varicella while pregnant have an estimated 10–20 % risk of developing symptomatic VZV pneumonia, which is a much higher rate than observed in nonpregnant women [115]. Some studies have suggested that both the frequency and the severity of VZV pneumonia are higher when chickenpox is acquired during the third trimester of pregnancy. Before the availability of acyclovir, mortality among pregnant women with severe VZV pneumonia (those requiring mechanical ventilation) was about 40 %, but is now less than 15 % with the availability of effective antiviral therapy and improved supportive care [77].

9.2.3 Neurologic Complications

The CNS manifestations most frequently associated with chickenpox are cerebellar ataxia and encephalitis [116, 117]. Uncommon neurologic complications include transverse myelitis, aseptic meningitis, vasculopathy syndromes, and Guillain-Barré syndrome. Optic neuritis has been reported as a rare complication of varicella in both pediatric and adult patients with good visual recovery expected in most cases. Reye syndrome, a triad of acute hepatic failure, encephalopathy, and hypoglycemia, previously associated with varicella and other viral infections, is now known to be more specifically related to salicylate therapy in febrile children.

Cerebellitis with ataxia, the most common neurologic abnormality associated with chickenpox, is diagnosed in approximately 1:4,000 cases of varicella. Children can develop ataxia from a few days before to 2 weeks after the onset of the rash, although neurologic symptoms most often occur simultaneously with the rash. Symptoms include vomiting, headache, and lethargy accompanied by ataxia. Chickenpox-associated cerebellar ataxia is usually self-limited, and most abnormalities resolve completely within 1–3 weeks. Mortality is essentially zero.

Encephalitis is a less common but more severe complication of chickenpox. The incidence of encephalitis is estimated to be one to two cases per 10,000 cases of varicella. The largest number of cases of encephalitis occurs in children, but the incidence is highest in adults and young infants. Neurologic symptoms most often appear about 1 week after the onset of the varicella rash (but may actually precede the

rash or follow much later in some cases). The usual presenting symptoms are headache, fever, vomiting, and altered sensorium. Seizures occur in 29–52 % of patients. The reported mortality for varicella encephalitis has ranged from 5 to 35 %, but is probably less than 10 %, with complete or near-complete recovery expected in most cases. Long-term sequelae may be present in 10–20 % of survivors.

Recent studies have emphasized that large- and small-vessel vasculitis is a pathologic hallmark of CNS VZV infection. Arterial ischemic stroke syndromes can occur in children (and adults) following varicella [118, 119]. By some estimations, young children with arterial ischemic strokes are threefold more likely than controls to have recently had varicella [120, 121]. The syndrome typically occurs in otherwise healthy children, average age 5 years, who present with hemiplegia. The median interval between varicella infection and the onset of neurologic deficits is 2 months [122, 123]. Angiography reveals vasculopathy of the branches of the middle cerebral artery. In one fatal case, histopathologic examination demonstrated active granulomatous arteritis of the middle cerebral artery with lymphocytic inflammatory infiltrates and VZV antigens in the smooth muscle layer [123]. Most children with hemiplegia following varicella have a good neurologic outcome.

9.2.4 Congenital and Neonatal Varicella

As noted above, varicella infection early in pregnancy may result in CVS [76], a syndrome characterized by limb hypoplasia, ocular and neurologic abnormalities, and distinctive cicatricial skin scarring in a dermatomal pattern [124]. Thirty percent of babies born with CVS will die during the first few months of life. The pathogenesis of the syndrome is thought to be invasion of the fetal nervous system by VZV during a critical state of development. The risk of CVS with maternal chickenpox occurring during gestational weeks 2–12 is 0.4–0.6 % and weeks 13–28 is 1.4–2.0 %, and the risk beyond 28 weeks is essentially zero [125, 126]. The period of highest risk appears to be gestational weeks 13–20. When maternal varicella occurs during the third trimester, fetal infection can occur despite passively acquired maternal antibody, but the outcome is usually good with no associated birth defects.

VZV infection of neonates may result from either vertical or horizontal transmission [77]. If maternal varicella develops during the time window from 4 to 5 days before until 2 days after delivery, the baby is exposed to VZV via transplacental viremia without the benefit of transplacental maternal anti-VZV immunoglobulin G. In this situation, varicella will develop in approximately 17–48 % of neonates. The babies seem healthy at birth but develop signs and symptoms of varicella 5–10 days postpartum. Historically the mortality rate for neonatal varicella was about 30 %, but the availability of VZV immune globulin, antiviral therapy, and intensive supportive care has reduced the mortality to about 7 %. Conversely, infants who have varicella lesions at birth or within the first 5 days of life (thus having received transplacental maternal anti-VZV antibody before delivery) are unlikely to have severe disease. In addition, infants (particularly low-birth-weight babies) born to VZV-seronegative mothers are at risk for severe varicella if infected during the first few days of life (e.g., in the nursery). Infants born to seropositive mothers who experience chickenpox early in life tend to have mild disease (perhaps due to partial protection from maternally acquired antibody), but are at increased risk to develop childhood herpes zoster [127].

9.3 Diagnosis of Varicella

The appearance of varicella is quite distinctive, and a clinical diagnosis made by an experienced healthcare provider is usually accurate and reliable. The presentation of an unvaccinated child with mild constitutional symptoms, a diffuse vesicular rash, and no history of chickenpox is strongly suggestive of the diagnosis, especially if there has been a documented exposure within the previous 2 weeks. However, with the dramatic decline of varicella in the American pediatric population, new medical trainees have much less experience with clinical diagnosis of chickenpox. Furthermore, cases of chickenpox in immunocompromised patients or "breakthrough" varicella in vaccinated patients may have an atypical appearance, necessitating laboratory confirmation.

In the past, viral culture has been the benchmark method for laboratory diagnosis of active VZV infections. VZV can be cultured by inoculating vesicular fluid onto monolayers of human fetal diploid kidney or lung cells. Unlike HSV, VZV is extremely labile and every effort should be made to minimize the time spent in specimen transport and storage. Ideally, fluid should be aspirated from clear vesicles into a tuberculin syringe containing 0.2 ml of viral transport medium, inoculated directly into tissue culture at the bedside (or taken directly to the laboratory).. If no vesicles or pustules are available for aspiration, the clinician should carefully remove overlying crusts from the freshest lesions available, swab the underlying ulcer, and place the swab directly into viral transport medium for rapid delivery on ice to the laboratory. Characteristic cytopathic effects are usually seen in tissue culture in 3–7 days, although cultures should be held for 14 days before they are declared negative. The culture process can be accelerated by using centrifugation cultures in shell vials. Identification of the isolate can be confirmed by staining the cell monolayer with VZV-specific monoclonal antibodies. In general, viral culture for VZV is highly specific but slow, insensitive, and expensive. However, culture remains essential if in vitro testing of susceptibility of the VZV isolate to antiviral drugs is desired.

The use of the PCR to detect VZV nucleic acids in clinical specimens has emerged as an important diagnostic tool and has replaced viral culture in many settings [128–131]. PCR overcomes the difficulties inherent in culturing labile VZV and has been used successfully to detect viral DNA not only from cutaneous lesions but also in cerebrospinal fluid from patients with CNS infection and in ocular fluids and tissues from VZV retinitis cases [132, 133]. Unlike HSV or CMV, VZV is not known to be shed asymptomatically (except possibly in saliva). Therefore, identification of VZV nucleic acids from cutaneous lesions or tissue biopsies is diagnostic of active infection. PCR is a reliable diagnostic tool for cases with atypical or vaccine-modified skin lesions [131]. PCR of blood is likely the diagnostic method of choice for patients with visceral VZV disease (e.g., pneumonia, hepatitis, etc.) without cutaneous involvement [134–136]. In addition, the use of PCR methods makes it possible to distinguish between diseases caused by wild-type VZV or by the VZV vaccine virus, which is not routinely possible using viral culture.

VZV can be identified in infected tissues by histopathology or electron microscopy, but visualization of multinucleated giant cells with inclusion bodies or herpesvirus virions does not distinguish between HSV and VZV. Immunohistochemical staining of viral antigens can provide a more specific diagnosis. Directly fluorescent antigen (DFA) staining using fluorescein-conjugated monoclonal antibodies to detect VZV glycoproteins in infected epithelial cells is especially helpful for making a rapid diagnosis when the clinical presentation is atypical [137]. This simple, rapid technique is more sensitive than viral culture (especially in later stages of VZV infection when virus isolation becomes more difficult) and can be performed more rapidly than PCR. To perform the DFA assay, epithelial cells are gently scraped from the base of a vesicle or ulcer with a scalpel blade, smeared on a glass slide, fixed with cold acetone, stained with fluorescein-conjugated monoclonal antibodies, and then examined using a fluorescent microscope. By using virus-specific monoclonal antibodies, HSV can be readily distinguished from VZV; DFA staining is therefore a much more powerful technique than a conventional Tzanck preparation.

Serologic assays are helpful for determining susceptibility to VZV but are not generally useful for diagnosing acute infections. VZV-specific serum IgG becomes detectible several days after the onset of varicella, with titers peaking at 2–3 weeks, so routine serologic tests provide only a retrospective diagnosis. The detection of VZV-specific serum IgM does not reliably distinguish among primary infection, reinfection, or reactivation. A variety of techniques have been used to detect VZV IgG antibodies, but most laboratories have now adopted an ELISA or latex agglutination (LA) assay for VZV serodiagnosis [130, 138]. The ELISA is capable of detecting IgG or IgM responses, is a reliable indicator of immune status following natural infection, and is readily automated. However, the ELISA may not be sufficiently sensitive to measure vaccine-induced immunity [139]. The LA is rapid, simple, inexpensive, and highly sensitive, but cannot be automated or used to detect IgM. The fluorescent antibody to membrane antigen (FAMA) test is also highly sensitive, but not widely available. The best tests for documenting vaccine-induced protection against varicella appear to be FAMA or a purified VZV glycoprotein ELISA (gpELISA), although further validation is necessary [130].

9.4 Clinical Presentation of Herpes Zoster

Herpes zoster classically presents as a painful cutaneous eruption in a dermatomal distribution [140, 141]. The inflammatory changes that occur when latent VZV reactivates in the sensory ganglion produce discomfort in the corresponding dermatome. The patient may report unpleasant prodromal sensations that range from mild itching or tingling to severe pain that precede the development of the skin lesions by 1–5 days (or occasionally longer) [142]. The cutaneous eruption appears in the skin segment innovated by a single sensory ganglion, is unilateral, and does not cross the midline. Overlap of lesions into ipsilateral adjacent dermatomes occurs in at least 20 % of cases. The most common sites for herpes zoster are the thoracic dermatomes (50 % of cases), followed by cranial nerve (15 %), cervical (15 %), lumbar (15 %), and sacral (5 %) dermatomes. During the acute phase of herpes zoster, most patients experience dermatomal pruritus and pain, which can be quite severe. Patients may also complain of headache, photophobia, and malaise, but significant fever is uncommon. Skin changes begin with an erythematous maculopapular rash followed by the appearance of clear vesicles (Fig. 42.3).

New vesicle formation typically continues for 3–5 days, followed by lesion pustulation and scabbing. The extent of cutaneous involvement can range from a few vesicles to a confluent eruption filling the entire dermatome. Skin necrosis in the involved dermatome can occur but is more commonly encountered in immunocompromised patients. Skin lesions heal within 2–4 weeks, often leaving scarring and permanent pigmentation changes. Immunocompetent children and young adults with herpes zoster tend to have less extensive cutaneous eruptions, less severe pain, and a much lower risk for chronic pain compared with elderly adults. Disseminated VZV infection with visceral involvement is extremely rare in immunocompetent patients with herpes zoster, although occasional cases of encephalitis and myelitis have been reported. In contrast, immunocompromised patients (especially organ transplant recipients and those receiving myeloablative chemotherapy) are at significant

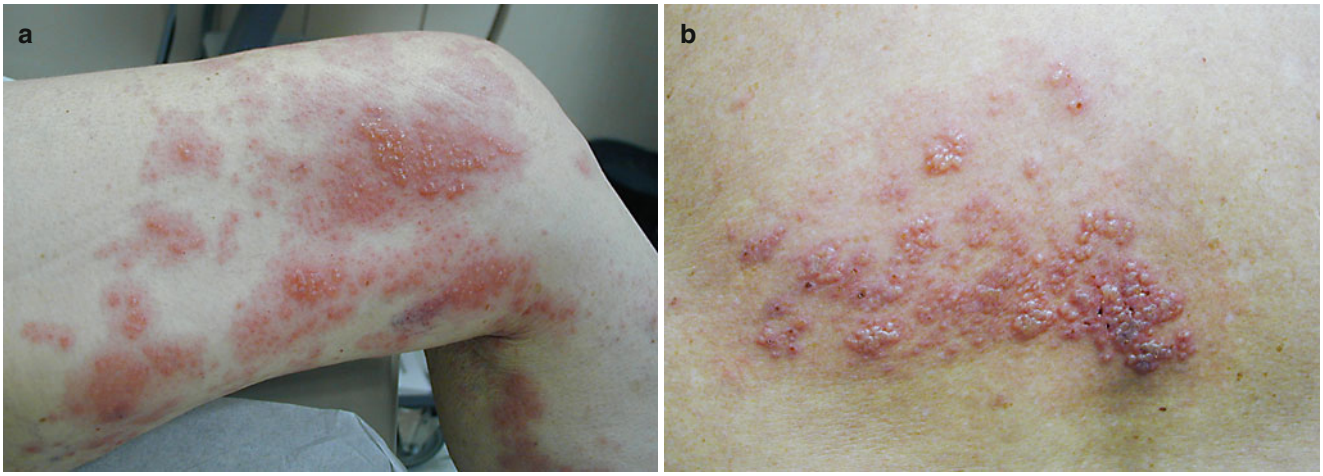

Fig. 42.3 Clinical presentation of herpes zoster. (**a**) Acute herpes zoster involving the L3 dematome. (**b**) Clusters of vesicles characteristic of acute herpes zoster

risk for cutaneous and visceral dissemination complicating dermatomal herpes zoster. In unusual cases, patients develop typical dermatomal neuralgic pain or even focal neurologic syndromes but do not progress to the typical cutaneous eruption, a condition termed "zoster sine herpete."

9.5 Complications of Herpes Zoster

9.5.1 Acute Pain

While the cutaneous manifestations of herpes zoster are unpleasant, pain is the defining symptom for most patients with shingles. As the virus replicates within the ganglion and travels down the afferent sensory nerve, the resulting intense inflammation and neuronal necrosis produce pain and related symptoms that can be very severe. The range of symptoms can vary from numbness or tingling, to a deep aching discomfort, to sharp stabbing or burning sensations. A significant number of patients also report prominent itching which may occur along with or instead of pain [143].

9.5.2 Postherpetic Neuralgia

Pain that persists after healing of the skin lesions, termed postherpetic neuralgia (PHN), is the most feared complication of herpes zoster. The quality and severity of the pain varies among patients, with common descriptions of constant deep aching or burning pain, or intermittent sharp stabbing pain or electric shock sensations, or severe itching [39, 144]. The precise incidence of PHN is difficult to state with certainty because of the wide variety of definitions employed by various investigators. In an analysis that combined data from several large clinical trials of antiviral therapy for herpes zoster in outpatients >50 years of age, 68 % of placebo recipients had pain 30 days after rash onset, 46 % had pain at 120 days, and 35–40 % had pain at 180 days [145]. Analyses of the severity and duration of pain in herpes zoster patients enrolled in clinical trials demonstrate three distinct phases of pain. Acute pain can last as long as 30 days after rash onset, followed by subacute neuralgia that lasts 30–120 days after rash onset and PHN which is defined as pain that persists for at least 120 days after rash onset [146, 147]. The implication is that these different stages of pain may be induced by different pathophysiologic mechanisms. Most pain experts favor a theory of altered excitability of the ganglionic or spinal cord neurons as the etiology of persistent zoster-associated pain [39, 148]. Other investigators have suggested persistent ganglionitis caused by ongoing low-level viral replication [149].

The strongest risk factor for development of long-term postherpetic pain is age [150]. Just as older adults are at increased risk for development of herpes zoster, older adults demonstrate a much higher incidence of PHN [151]. In a study conducted in Iceland, postherpetic pain of >3 months duration was documented in 1 % of patients aged 0–49 years, 13 % of persons 60–69 years of age, and 29 % in those ≥70 years of age [152]. In addition to older age, other well-defined risk factors for PHN include the presence and severity of prodromal pain preceding the rash, greater severity of acute pain, and greater severity of the rash (as measured by lesion surface area) [145, 147, 153, 154]. Like other chronic pain syndromes, PHN is a potentially debilitating and expensive affliction that can have a long-term impact on the quality of life of elderly patients [155–157].

9.5.3 Central Nervous System Complications

CNS involvement with VZV may occur simultaneously with the cutaneous shingles eruption or follow the acute episode by weeks or months. A variety of neurologic syndromes attributed to VZV infection have been described including encephalitis, myelitis, cranial and peripheral nerve palsies,

and vasculopathies, including a syndrome of delayed contralateral hemiparesis [133]. VZV neurologic disease has also been described in patients with no history of VZV cutaneous lesions [158]. Large- and small-vessel vasculitis is now recognized as the pathologic mechanism underlying most CNS manifestations of VZV infection.

Acute VZV encephalitis is a rare complication of herpes zoster that develops a few days after the onset of rash and occurs most frequently in immunocompromised patients. Herpes zoster involving a cranial nerve dermatome and the presence of cutaneous VZV dissemination are other markers for increased risk of encephalitis. The clinical presentation is most often an acute or subacute delirium accompanied by few focal neurologic signs. The mortality rate due to herpes zoster-associated acute encephalitis is around 10 % and may be higher in more severely immunocompromised patients. A chronic form of multifocal VZV encephalitis is seen almost exclusively in immunocompromised patients, especially AIDS patients with low levels of CD4+ T cells, and is thought to be due to small-vessel vasculitis. The onset of chronic encephalitis may occur months after an episode of herpes zoster and up to 40 % of these patients have no recognized history of shingles. The clinical presentation is usually subacute with headache, fever, mental status changes, and seizures. Patients may have focal neurologic defects including, aphasia, hemiplegic, and visual field cuts. The clinical course is often one of progressive deterioration and death, but responses to intravenous acyclovir therapy have been reported [159, 160].

Herpes zoster-associated myelitis is thought to result from direction invasion of the spinal cord by VZV, with virus spreading along the central axons of infected primary sensory neurons. The most severe forms are seen in immunocompromised patients, especially those with AIDS. Myelitis usually follows herpes zoster involving thoracic dermatomes, with weakness developing in the same spinal cord segment as the rash. Neurologic symptoms begin to develop an average of 12 days after the onset of rash. The most common initial manifestations are bladder dysfunction, lower extremity weakness, asymmetrical reflexes, and sensory disturbances [161]. In severe cases, myelopathy can progress to a partial Brown-Séquard syndrome or total cord transection.

When herpes zoster involves the seventh cranial nerve (geniculate) ganglion, the result is weakness of ipsilateral facial muscles with rash in the external auditory canal and sometimes on the ipsilateral anterior two-thirds of the tongue or hard palate. This clinical presentation of peripheral facial weakness with zoster oticus is termed the Ramsay-Hunt syndrome. Some patients also report nausea and vomiting, nystagmus, tinnitus, diminished hearing, and vertigo, indicating involvement of the eighth cranial nerve as well [162].

Delayed contralateral hemiparesis following herpes zoster ophthalmicus (HZO; shingles involving the first division of the trigeminal nerve) is a rare but devastating vasculopathy syndrome that has been reported in both immunocompetent and immunocompromised patients [163]. The pathogenesis of this unusual disorder is thought to be direct VZV invasion of large cerebral arteries by extension of virus from smaller vessels that transverse the trigeminal-innovated meninges. This produces a necrotizing arteritis that can result in vascular thrombosis or hemorrhage [164]. The most frequently described presentation is headache and contralateral hemiplegia that follow herpes zoster ophthalmicus by an average of 7 weeks. The mortality rate among adults is 20–25 %, with a high probability of permanent neurologic sequelae among survivors. In some studies, the overall risk for strokes was increased in the 1-year interval following an episode of herpes zoster [165].

9.5.4 Ocular Complications

HZO can be associated with several potentially sight-threatening conditions. While not all patients with shingles involving the first division of the trigeminal nerve develop ocular complications, the consequences are so serious that ophthalmologic consultation and close follow-up are warranted. VZV infection of ocular structures can range from conjunctivitis (which is extremely common) to serious forms of keratitis, iridocyclitis, glaucoma, and palsies of cranial nerves III, IV, and VI [166].

Syndromes of acute retinal necrosis (ARN) caused by VZV have occasionally been described in immunocompetent patients, but more aggressive variants of this infection are now recognized in immunocompromised patients, especially those with AIDS. These syndromes, termed "progressive outer retinal necrosis" (PORN) or "rapidly progressive herpetic retinal necrosis" (RPHRN), occur most frequently in AIDS patients with CD4+ T lymphocyte counts of <100 cells/mm^3 [167]. This syndrome of necrotizing VZV retinitis may occur concurrently with active herpes zoster or, more frequently, develop weeks or months after the acute episode of herpes zoster has resolved. The retinitis begins with multifocal necrotizing lesions involving the peripheral retina. Most patients present with unilateral involvement, but progression to bilateral disease occurs frequently. PORN rapidly progresses to confluent full-thickness retinal necrosis and results in blindness in 75–80 % of involved eyes. The prognosis for preservation of vision is better when ARN occurs in immunocompetent patients.

The etiologic role of VZV in PORN occurring in immunocompromised patients has been established by demonstrating the virus by culture or PCR from ocular fluids or tissues (although HSV occasionally causes an identical syndrome) [168]. PORN can follow either HZO or herpes zoster in a remote dermatome. Furthermore, retinal involvement is bilateral in more than half of cases, suggesting that VZV reaches the retina via hematogenous spread, possibly with extension along nerve pathways within the anterior visual system.

9.5.5 Pregnancy

CVS is not observed after herpes zoster during pregnancy, possibly due to preexisting maternal anti-VZV immunity that limits viremia and protects the fetus. Similarly, development of maternal herpes zoster in the peripartum period does not put the healthy infant at increased risk for neonatal varicella [169].

9.6 Diagnosis of Herpes Zoster

The appearance of a classic case of herpes zoster is sufficiently distinctive that a clinical diagnosis is usually accurate. Characteristic features include pain (or other abnormal sensations) and clustered vesicular lesions in a dermatomal distribution that does not cross the midline (Fig. 42.4).

One important alternative diagnosis to be considered is "zosteriform" HSV rash appearing in a pseudodermatomal distribution, most commonly in the sacral area. However, many presentations of herpes zoster are not "classic" and may require laboratory confirmation. Patients may have very few cutaneous lesions, or the lesions may have an atypical appearance (which is especially common in immunocompromised patients). The laboratory diagnostic methods discussed above with varicella also apply to the diagnosis of herpes zoster. The DFA and PCR assays are especially useful [170]. Most patients will demonstrate an increase in VZV-specific antibody titers following an episode of herpes zoster, which is occasionally useful for retrospective confirmation, but not very helpful for real-time diagnosis.

10 Control and Prevention

10.1 Treatment of Varicella

In healthy children, chickenpox is generally a benign disease associated with low rates of morbidity and mortality. For most children, supportive care is sufficient. Astringent soaks, antipruritics, and antipyretics (preferably acetaminophen rather than aspirin) provide symptomatic relief. Trimming the fingernails closely helps prevent bacterial superinfections related to scratching. If bacterial cellulitis develops (especially infection caused by group A streptococcus), antibiotic therapy may be necessary.

As antiviral therapy, oral acyclovir has been evaluated for treatment of uncomplicated varicella in immunocompetent children (Table 42.1)

When initiated within 24 h of rash onset, acyclovir results in shorter duration of fever, fewer skin lesions, and accelerated lesion healing. Oral acyclovir is well tolerated and reduces the overall duration of symptomatic illness by about 24 h. The populations that have been studied in clinical trials were not sufficiently large to assess the impact of acyclovir therapy on the incidence of varicella complications such as pneumonitis or encephalitis. Unlike acyclovir, valacyclovir and famciclovir have not been as extensively evaluated as therapy for varicella in children but should be efficacious. Many pediatricians still view antiviral therapy as optional for otherwise-healthy children with chickenpox.

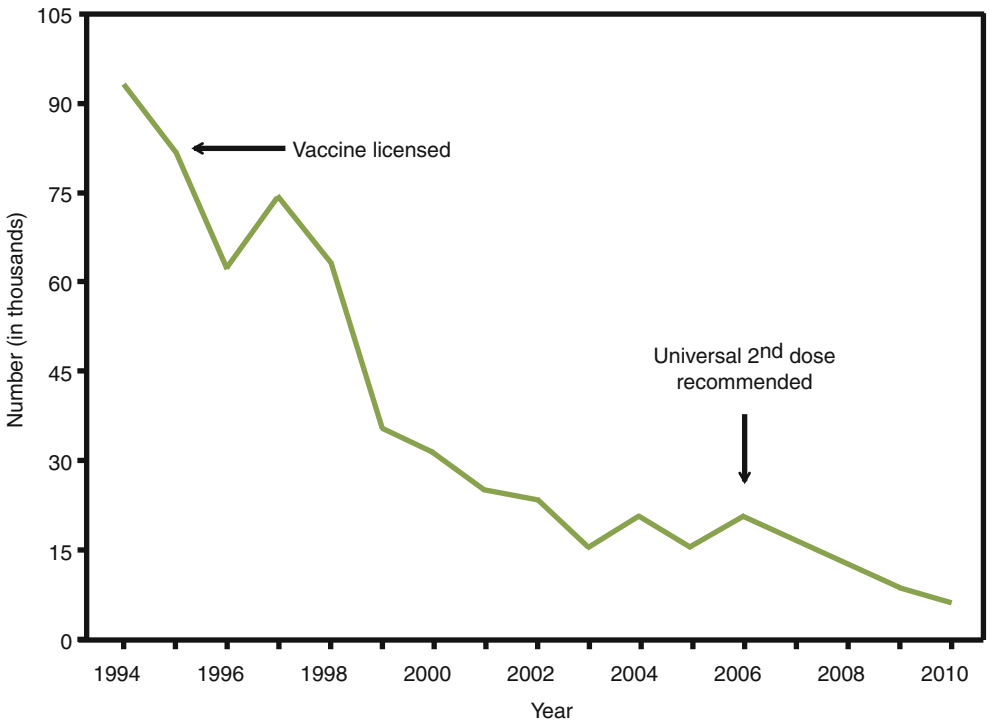

Fig. 42.4 Decline in chickenpox in the U.S. after institution of vaccination. Number of reported cases from the states of Illinois, Michigan, Texas, and West Virginia, 1994–2010 (Centers for Disease Control and Prevention, Summary of Notifiable Diseases-United States, 2010. MMWR 59 (No. 53): 89, June 1, 2012)

Table 42.1 Recommended antiviral therapy for VZV infections

	Drug	Route and dose[a]	Major toxicities
Immunocompetent patients			
Varicella	Acyclovir	20 mg/kg (800 mg max.) po five times daily × 5 days[b]	None, minor nausea or headache
Herpes zoster	Acyclovir	800 mg po five times daily × 7–10 days	As above
	Valacyclovir	1,000 mg po every 8 h × 7 days	None, minor nausea or headache
	Famciclovir	500 mg po every 8 h × 7 days	None, minor nausea or headache
	Brivudin[c]	125 mg po once daily × 7 days	Potentially serious interaction with fluoropyrimidines (e.g., 5-fluorouracil)
Immunocompromised patients			
Varicella	Acyclovir	10–15 mg/kg (or 500 mg/m^2) intravenously every 8 h for ≥7 days	Nephrotoxicity (rare), CNS disturbances (rare)
Herpes zoster	Acyclovir	IV (dose as above)[d]	As above
Disseminated VZV syndromes (e.g., encephalitis, pneumonitis)	Acyclovir	IV (dose as above)	As above
Infection caused by acyclovir-resistant VZV	Foscarnet	60–90 mg/kg intravenously every 12 h until healed (≥10 days)[e]	Nephrotoxicity (common): electrolyte disturbances (common), seizures, arrhythmias, anemia, genital ulcers

[a]Doses given are for adults with normal renal function
[b]Famciclovir and valacyclovir will also likely be effective
[c]Not licensed for use in the United States
[d]Mildly to moderately immunocompromised patients, including most AIDS patients, can be treated with oral therapy
[e]Alternative – intravenous cidofovir

On the other hand, adolescents and adults with varicella can be seriously ill, with high fever, hundreds of skin lesions, debilitating constitutional symptoms, and a higher risk of complications (especially VZV pneumonia) [171]. Given the risk of significant morbidity and resultant absenteeism from school or work, therapy that will reduce the severity and duration of the acute illness is warranted. In clinical trials enrolling adolescents and adults with varicella, oral acyclovir therapy was shown to reduce the duration of new lesion formation, reduce the maximum number of lesions, accelerate cutaneous healing, and shorten the duration of fever. In these studies, the benefit of acyclovir administration was minimal when therapy was initiated later than 24 h after rash onset. Overall, acyclovir reduced the duration of symptomatic illness of about 2 days. Valacyclovir and famciclovir are also likely to be effective for chickenpox in adolescents and adults, but data from controlled clinical trials are limited. The decision whether to initiate antiviral therapy in a patient with chickenpox will be influenced by the patient's age, the underlying medical conditions, and the risk of complications [172]. While antiviral therapy is considered optional for healthy children with chickenpox, the higher potential for morbidity clearly favors treatment in adolescents and adults.

10.1.1 Pregnant Women

Although based more on case reports than on prospectively acquired data, the evidence that varicella in pregnancy is associated with enhanced morbidity, especially pneumonia, is compelling [78]. Any pregnant woman who presents with signs and symptoms suggestive of chickenpox should be started immediately on oral acyclovir (800 mg five times daily) or valacyclovir (1 g three times daily) and undergo further evaluation with chest radiograph and blood oxygen saturation measurement. If she has significant respiratory symptoms (including cough or dyspnea), pulmonary infiltrates on chest radiograph, or low oxygen saturation, hospitalization for therapy with intravenous acyclovir (10 mg/kg every 8 h) should be strongly considered. No data are available to indicate whether treating the mother will alter the risk of the congenital varicella syndrome [77, 126].

10.1.2 Immunocompromised Patients

The availability of safe and effective antiviral drugs has greatly reduced the high mortality rate previously associated with varicella in immunocompromised patients. Populations at high risk include organ transplant recipients, patients with cancer (especially hematologic malignancies), and other patients receiving immunosuppressive medications (including high-dose corticosteroids). Small studies of intravenous acyclovir in immunocompromised children with varicella demonstrated a dramatic reduction in the frequency of VZV complications, especially pneumonitis. In this setting, therapy with intravenous acyclovir (10 mg/kg or 500 mg/m^2 every 8 h for 7–10 days) should be initiated at the first sign of infection. A switch to oral antiviral therapy (acyclovir, valacyclovir, or famciclovir) can be considered when the

patient is afebrile and new lesion formation has ceased. Initial treatment with oral therapy may be efficacious in modestly immunocompromised patients (e.g., those with solid tumor malignancies or taking low-dose corticosteroids), but prospectively acquired data are limited. Because of the high frequency of visceral involvement in immunocompromised children or adults with chickenpox, antiviral therapy should be considered mandatory.

Varicella does not appear to be especially severe in most HIV-seropositive children, although some investigators have reported a longer duration of new lesion formation and higher median lesion counts. Deaths attributable to chickenpox in children with HIV infection are rare and are usually due to pneumonitis. Most clinicians prescribed oral antiviral therapy for chickenpox in HIV-infected children, reserving intravenous acyclovir for patients with unusually severe or complicated infections.

10.2 Varicella Postexposure Prophylaxis

10.2.1 Postexposure Vaccination

The administration of varicella vaccine within the first few days after exposure to VZV will produce a protective (or partially protective) immune response in VZV-seronegative individuals [173–175]. About 20 % of patients receiving postexposure immunization within 3 days of exposure may still develop some signs and symptoms of chickenpox, but the disease manifestations are usually very mild (<50 skin lesions) [176]. Postexposure vaccination appears to be more effective and less expensive than preemptive therapy with antiviral drugs. This postexposure vaccination approach is useful for managing VZV exposures that occur in a family, in the workplace, or in a medical care setting.

10.2.2 Passive Immunotherapy

Advisory committees have recommended the administration of varicella-zoster immune globulin (VariZIG™) to VZV-susceptible immunocompromised patients, pregnant women, or neonates who have been exposed to a patient with active chickenpox or herpes zoster [177]. In many cases, varicella-zoster immune globulin administration will not prevent infection in the susceptible host, but will significantly reduce the severity of the resulting illness [178]. Placebo-controlled trials in immunocompromised children have clearly demonstrated that varicella-zoster immune globulin ameliorates the severity of chickenpox and significantly reduces the risk of disseminated infection. A single treatment will reduce the risk of disseminated infection by about 75 % and will provide 4 weeks of passive immunity. No controlled clinical trials of VZV immune globulin prophylaxis have been conducted in populations of pregnant women. The primary rationale for immunoprophylaxis in pregnant women is to prevent varicella complications in the mother, although there are some data to suggest that timely administration of varicella-zoster immune globulin may also reduce the risk of CVS [179].

For maximal efficacy, varicella-zoster immune globulin must be administered as soon as possible after exposure. The window for administration has traditionally been 96 h (4 days), but this was recently extended by the FDA to 10 days [180]. Unfortunately, in this time-critical scenario, the true serologic status of the immunocompromised patient or pregnant woman with a negative history of varicella is often not known. The clinician may be faced with the decision to initiate passive immunoprophylaxis empirically or to wait for the results of serologic testing. Given the time pressure to administer varicella-zoster immune globulin as soon as possible after exposure, it may not be feasible to await serologic test results. In that situation, the recommended course is to proceed with varicella-zoster immune globulin administration after first obtaining a blood specimen for VZV serologic testing. Administration of varicella-zoster immune globulin to an individual who is subsequently shown to be immune will result in no adverse consequences (other than unnecessary expense). If varicella-zoster immune globulin cannot be obtained in a timely fashion, administration of standard intravenous immunoglobulin (IVIG) at a dose of 400–600 mg/kg is an option. Most IVIG contain significant titers of anti-VZV IgG, but the amount varies from lot to lot and may decline in the coming years as more plasma contributions come from younger donors who never had chickenpox.

The VZV immune globulin product available in the USA since 2006 is VariZIG™ (Cangene Corporation, Winnipeg, Canada) [181]. It is available on a patient-specific basis under an FDA-approved expanded access protocol from FFF Enterprises (Temecula, CA; 24-h hotline 1-800-843-7477). VariZIG™ is a lyophilized preparation of purified human immunoglobulin made from plasma containing high levels of anti-VZV IgG. Each vial of VariZIG™ contains 125 units and must be reconstituted to a concentration of 5 % IgG and then administered intramuscularly. The recommended dose of VariZIG™ is 125 units/10 kg of body weight (with a minimum dose of 125 units and a maximum dose of 625 units).

10.2.3 Varicella Chemoprophylaxis

Studies in healthy children have shown that oral acyclovir administration during the latter half of the varicella incubation period (i.e., 7–14 days after exposure) successfully protects 80–90 % of recipients from clinical disease [182]. About 80 % of subjects receiving prophylactic acyclovir demonstrate a humoral immune response, indicating that acyclovir prophylaxis does not reliably prevent infection but does significantly ameliorate symptomatic disease. For healthy children and healthy nonpregnant adults,

most authorities consider postexposure vaccination to be a more effective approach and chemoprophylaxis has not been widely used. Acyclovir and valacyclovir seem to be safe for use in pregnancy, and antiviral chemoprophylaxis in pregnant women can be considered if VZV immune globulin immunotherapy is not available or not feasible. However, this approach has not been adequately studied and is not routinely recommended [183].

10.3 Prevention of Varicella by Vaccination

A live, attenuated varicella vaccine was developed in Japan in 1974 by serial passage of a clinical isolate by Takahashi and colleagues [184]. The genetic differences between wild-type VZV and the vaccine strain (called VZV_{oka}) that account for the attenuation are relatively minor [185, 186]. As a result, the vaccine-strain virus is replication competent and relatively immunogenic [187]. However, VZV_{oka} can still be readily distinguished from wild-type virus using molecular methods [188]. In 1995, the USA became the first country to adopt a universal one-dose childhood varicella vaccination program [46] which was changed to a routine two-dose childhood immunization schedule in 2006 [189]. In the USA, the CDC Advisory Committee on Immunization Practices (ACIP) currently recommends that all immunocompetent children receive their first dose of varicella vaccine at age 12–15 months, followed by a second dose at age 4–6 years before entering school [177]. For adolescents and adults (≥13 years) without evidence of varicella immunity, varicella vaccine should be administered as 2 doses given 4–8 weeks apart. Adolescents who received only a single dose in childhood should receive a second "catch-up" vaccination. The varicella vaccine has proven to be very safe and effective in children, and its widespread use has resulted in substantial reduction in the incidence of varicella and varicella-related complications in the USA (Fig. 42.4) [177].

By 2011, varicella vaccine coverage (receiving at least one dose of vaccine) for children aged 19–35 months was 90.8 % [190], according to the National Immunization Survey (NIS). Between 1995 and 2005, varicella incidence in the USA declined 90.4 % according to the data from the NIS [191] and the CDC Varicella Active Surveillance Project. According to the data from the NNDSS, the national incidence of varicella decreased 43 % between 2000 and 2005 (the one-dose era) and declined another 72 % between 2006 and 2010 (the two-dose era) [5]. Between 2000 and 2010, the national incidence of varicella declined from 43 cases to 9 cases per 100,000 population, a decline of 79 % [5]. The single dose of varicella vaccine has been estimated to be about 84 % effective in preventing varicella disease and 100 % effective for preventing severe varicella [46, 175, 192]. The vaccine is well tolerated and has an excellent safety profile.

However, the decline in numbers of varicella cases plateaued between 2003 and 2006; outbreaks continued to occur even among highly vaccinated school populations [46]. Testing using the highly sensitive FAMA assay indicated that only about 76 % of vaccinees seroconverted after a single dose of varicella vaccine [193]. Multiple studies showed that a single dose of vaccine elicited low humoral and CMI responses that might not be protective [194]. It became apparent that many new cases of varicella occurred in recipients of a single vaccine dose, likely due to primary vaccine failure.

By 2005, 57–64 % of varicella cases occurred in vaccinated patients [191]. About 20 % of children vaccinated with a single dose of varicella vaccine may develop breakthrough varicella if exposed to the virus [195]. It should be emphasized that "breakthrough varicella" is caused by a wild-type virus, not by VZV_{oka} vaccine virus [196]. Among vaccinated children aged 1–14 years, breakthrough varicella is a modified disease and typically very mild; 75 % of patients had <50 lesions [195]. However, these children were able to transmit virus to other susceptible individuals and so outbreaks of varicella continue to occur, although dramatically less frequently than in the prevaccine era [197, 198]. Surveillance in Oregon public schools in 2000–2007 showed that breakthrough varicella was occurring in 0.2–0.3 % of public elementary school students annually [199]. While single-dose vaccination produced an impressive reduction in varicella incidence, the regimen was not sufficiently effective to provide population immunity to prevent continuing transmission, even in highly vaccinated populations [46, 192].

Multiple studies demonstrated that, compared to a single-dose, a two-dose regimen was more efficacious and resulted in a higher proportion of vaccinees with protective antibody titers [46, 194]. Humoral and CMI responses seen after two doses of varicella vaccine approximate those seen after wild-type infection [194]. Compared to single-dose vaccinees, children receiving two doses were 95 % less likely to develop breakthrough varicella [46, 200]. The CDC ACIP changed their recommendation to a two-dose vaccination regimen for varicella prevention in 2006. As of the school year 2011–2012, all 50 states require at least one dose of varicella vaccine before beginning childcare or elementary school. Two doses of varicella vaccine prior to school entry are required by 36 states and District of Columbia. Audit of the Kaiser Permanente database in southern California demonstrated that coverage with the second dose of the varicella vaccine increased from 42.1 % in 2007 to 74.6 % in 2009 [201]. Addition of the second dose has significantly reduced the problem of breakthrough infection and resulted in a continuing decline in varicella incidence [202]. Occasional chickenpox outbreaks continue to be reported, even among vaccinees who received two doses [203].

The declining incidence of varicella has produced benefits even for populations not actively vaccinated. Between 1995 and 2008, the incidence of varicella in infants aged 0–11 months fell 89.7 %, even though this age group is not routinely vaccinated [204]. Similar declines in congenital and neonatal varicella have been reported from Australia following introduction of universal vaccination [205]. Adults also benefited from herd immunity with a 74 % decline in the incidence of varicella between 1995 and 2005, despite a very low frequency of vaccination in adults [110].

Widespread adoption of varicella vaccination in the USA has resulted in a decrease in varicella-related outpatient visits, hospitalizations, and deaths [46, 206, 207]. Comparing the prevaccine era (1988–1995) with the single-dose vaccine era (2000–2006), the frequency of varicella-related hospitalizations in the USA declined from 0.42 per 10,000 population to 0.12 per 10,000 population [208]. This 65 % reduction in hospitalization was seen across all age groups (but was highest in the 0–4-year group) and predicts that varicella vaccination prevented approximately 50,000 varicella-related hospitalizations in the USA between 2000 and 2006 [206, 208]. Similar reductions in hospitalization rates following institution of a varicella vaccine program have been described in other countries including Canada [209] and Australia [210]. In the USA, deaths due to varicella have been nearly eliminated by wide adoption of varicella vaccination. The varicella death rate fell from 0.41 deaths to 0.05 deaths per million population between 1990 and 1994 and 2005–2007 [211]. This represents a reduction in deaths of 97 and 96 % for patients less than 20 years of age and less than 50 years of age, respectively [211]. The decline in the incidence of chickenpox and its complications has resulted in substantial reductions in medical expenditures for chickenpox [46]. Compared with no varicella vaccination, both the single-dose and two-dose vaccination regimens are estimated to result in substantial cost savings from the societal prospective [212].

Post-marketing surveillance studies have demonstrated that the VZV_{oka} varicella vaccine is generally safe and well tolerated [213]. The most common adverse effect is a mild vesicular rash seen in 2–3 % of recipients. Vesicular rashes occurring within 2 weeks after vaccination are usually caused by wild-type virus, while vesicular rashes seen 14–42 days post vaccination are usually caused by VZV_{oka} [214]. VZV_{oka} shed from these lesions is potentially infectious, and rare cases of secondary transmission of VZV_{oka} to susceptible household contacts have been reported [214]. In addition, rare cases of disseminated VZV_{oka} disease have been described in immunocompromised patients. The vaccine virus is markedly less neurovirulent than wild-type VZV [103]. Retrospective database reviews have not demonstrated any association between varicella vaccination and neurologic complications such as ischemic stroke or encephalitis in children [214, 215]. Rare cases of herpes zoster caused by VZV_{oka} have been observed, sometimes involving the same arm where the vaccine was administered [103, 216]. This demonstrates that VZV_{oka} is capable of establishing latency. Furthermore, cases of herpes zoster caused by VZV_{oka} occurring in remote (non-C5) dermatomes are consistent with viremic delivery of vaccine virus to multiple ganglia [92]. There have been occasional reports of the onset of wild-type herpes zoster temporally associated with varicella vaccination [217, 218]. It is not currently clear whether this is coincidence or whether induction of reactivation of latent wild-type virus may occur after exposure to VZV_{oka}.

A data registry of women exposed to VZV_{oka} during pregnancy has not documented any occurrence of fetal varicella syndrome or other birth defects linked to vaccine virus [219]. VZV-susceptible children living in a household with a susceptible pregnant woman should be vaccinated. If the pregnant woman is known to be VZV susceptible and if the vaccinee develops a rash, it may be prudent to separate the vaccinee and the pregnant woman until the rash resolves [177]. The risk of having a susceptible child in the household who might acquire wild-type varicella in the community almost certain exceeds the risk of transmission of attenuated vaccine virus.

VZV_{oka} varicella vaccine has been combined with measles, mumps, and rubella vaccine (MMR) to constitute a quadrivalent vaccine (MMRV) that has been generally safe and effective [220]. Based on reports of increased risk of fever and febrile seizures (eight cases per 10,000 vaccinations, occurring 5–12 days after vaccine administration) when MMRV is used as the first vaccination at 12–15 months, some guidelines recommend that MMR and varicella vaccine be administered separately for the first vaccination [221]. The risk of febrile seizures does not seem to occur among older children, and MMRV can be used for the second vaccination at age 4–6 years [222, 223].

Since varicella vaccine contains replicating virus, it is generally contraindicated in immunocompromised patients, including pregnant women. Concerns about the use of live attenuated vaccines in immunocompromised patients have focused on the potential for vaccine virus to cause disease and on the possibility that immunocompromised patients will fail to mount a protective immune response [224]. However, there are scenarios where varicella vaccine can be safely and effectively used in patients with impaired immunity [225]. Varicella vaccine is recommended for susceptible HIV-seropositive children and adults with CD4-positive T lymphocyte counts of >200 cells/mm^3 (or >15 %) [226] and is safe and well tolerated in this population [227]. Prospective studies have demonstrated that 11.8 % of HIV-seropositive children with preserved CD4+ T lymphocyte counts seroconverted after the first dose of varicella vaccine and 79.4 % seroconverted after the second dose [228]. Retrospective

case reviews suggest that varicella vaccine is about 82 % effective for preventing chickenpox and 100 % effective for preventing herpes zoster when administered to HIV-seropositive children [229].

Other clinical situations where varicella vaccine is being tested on an investigational basis include children with leukemias and other malignancies and children undergoing hematopoietic stem-cell transplant or solid organ transplantation [230, 231]. Multiple studies have demonstrated that varicella vaccine is safe and effective when given to solid organ transplant candidates prior to transplantation [232–235]. Small studies have demonstrated that varicella vaccine can be safely administered after organ or bone marrow transplantation in selected clinical settings, although much larger clinical trials will be required to fully validate the safety and efficacy of this approach [236–238]. Compared with the expense of varicella-zoster immune globulin or treatment of varicella, immunization would be highly cost-effective if shown to be safe and effective [239]. An alternative approach being taken by other investigators is development ofnon-replication competent subunit vaccines (e.g., a glycoprotein E vaccine) that will be highly immunogenic, yet safe to use in seriously immunocompromised populations [240].

10.4 Treatment of Herpes Zoster

The therapeutic goals for herpes zoster in immunocompetent adults are to accelerate the events of cutaneous healing, to reduce the severity of acute neuritis, and, most importantly, to reduce the incidence, severity, and duration of chronic pain [1]. Ganglionic and neuronal inflammation and necrosis begin well before the appearance of skin lesions; thus, the observation of suboptimal response to antiviral therapy initiated after onset of rash is not surprising. Even without antiviral therapy, the cutaneous lesions of herpes zoster almost always resolve within a month. However, chronic pain can persist for months or even years and is the most significant clinical manifestation of herpes zoster in the normal host [241]. In the USA, three oral antiviral drugs (acyclovir, valacyclovir, and famciclovir) are approved and have been demonstrated to reduce the duration of viral shedding, promote resolution of skin lesions, and limit the duration of pain when therapy is initiated within 72 h of lesion onset [242, 243] (Table 42.1). In prospective controlled clinical trials, these antiviral drugs reduced the duration of new vesicle formation by about 1.5 days and reduced the time to 50 % lesion healing by about 2.5 days. The percentage of patients still experiencing pain at 6 months after the onset of shingles was reduced from about 25–35 % in placebo groups to 15–20 % in groups receiving antiviral therapy. It should be noted that, even with appropriate and timely antiviral therapy, some patients will still develop PHN. Acyclovir, valacyclovir, and famciclovir are all well tolerated and appear to be approximately comparable in clinical efficacy for managing herpes zoster in the immunocompetent host. Because their enhanced pharmacokinetic properties allow simpler dosing regimens, valacyclovir and famciclovir are preferred over acyclovir for this indication. Another potent antiviral drug, brivudin, has also been demonstrated to be effective for reducing the duration of new lesion formation and improving pain outcome [244, 245]. Brivudin is commercially available in several countries but has not been approved in the USA because of concerns about potential drug-related toxicities [246]. There is currently no role for topically applied antiviral medications in management of herpes zoster.

Characteristics have been defined which identify immunocompetent patients at highest risk for complications of shingles and thus most likely to benefit from antiviral therapy. Several studies have clearly shown that older age, greater skin surface area involved with rash, and severity of pain at the time of clinical presentation are all predictors of more severe and longer-lasting pain. Patients meeting these criteria should be targeted with therapy with antiviral drugs and potent analgesics. Conversely, patients under 50 years of age are at lower risk for severe or prolonged pain and an argument can be made that antiviral therapy in this group is optional.

Available efficacy data from published clinical trials describe patients who present within 72 h of lesion onset, although, in practice, patients frequently present for medical care well beyond that window. The presence of new vesicles correlates with recent viral replication and may be a marker for patients who would benefit from antiviral therapy, even beyond 72 h. In addition, patients presenting with the high-risk clinical characteristics described above should be considered for antiviral treatment, even when presenting beyond 72 h after lesion onset. However, patients whose lesions have all begun to crust are unlikely to derive benefit from antiviral treatment.

Symptomatic measures should be suggested to keep the patient with herpes zoster more comfortable. Patients should keep the cutaneous lesions clean and dry to reduce the risk of bacterial superinfection. Patients may wash the skin lesions with soap and water in the shower and then carefully pat the skin dry with a clean towel. Some patients find warm or cool astringent soaks to be soothing. A sterile nonocclusive, nonadherent dressing placed over the involved skin will protect the lesions from contact with clothing, which may be especially helpful for patients with increased skin sensitivity (i.e., allodynia). Pain is the most important symptom of herpes zoster and should be managed aggressively. Short-acting narcotic analgesics should be prescribed on a scheduled (rather than as-needed) basis [247]. Prospective clinical trials have demonstrated that corticosteroid administration within 72 h of lesion onset leads to a reduction of pain during the acute phase of herpes zoster, but does not impact the risk of PHN. This modest benefit is balanced against the potential

adverse effects of high-dose corticosteroids in elderly patients, and most physicians do not routinely prescribed steroids for herpes zoster. In patients with severe neuralgic pain unresponsive to oral analgesics, sympathetic nerve blocks can provide rapid but temporary relief [248].

10.4.1 Postherpetic Neuralgia

Medical management of established PHN is complex and often requires a multifaceted approach [140, 247, 249–251]. Opioid analgesics are the mainstay of therapy during the early phase of neuralgic pain. Treatment of PHN may require combinations of analgesics, anticonvulsants (e.g., gabapentin or pregabalin), antidepressants (e.g., nortriptyline or duloxetine), and topical anesthetics (e.g., lidocaine patches) [252]. Patients with severe or prolonged PHN may benefit from referral to a pain management specialist. Most experts do not think that prolonged administration of antiviral drugs has any role for treatment of established PHN [253], although some small studies have suggested benefit [254].

10.4.2 Herpes Zoster Ophthalmicus

Because of the high potential for ocular complications, special emphasis should be given to patients presenting with herpes zoster involving the first division of the trigeminal nerve. Without antiviral therapy, 50 % of patients with HZO will develop significant ocular complications (which can include neurotropic keratopathy, episcleritis, iritis, and epithelial or stromal keratitis). Controlled prospective clinical trials have clearly demonstrated that oral antiviral therapy with acyclovir, valacyclovir, or famciclovir reduces the frequency of late ocular inflammatory complications of HZO from 50–60 % to 20–30 % [255–257]. Some experts favor intravenous acyclovir as initial therapy for selected patients (especially immunocompromised patients) with severe HZO. Systemic antiviral therapy has largely replaced topical antiviral preparations for treatment of the ocular complications of HZO [258, 259]. Referral to an experienced ophthalmologist to assist with management of HZO is recommended.

10.4.3 Herpes Zoster in Immunocompromised Patients

Patients with disorders of CMI are at increased risk for development of herpes zoster and zoster-related complications. Patients with the most significant immunosuppression (such as hematopoietic stem-cell transplant recipients or patients with lymphoproliferative malignancies) are at highest risk for VZV dissemination and visceral organ involvement. Clinical trials with intravenous acyclovir for treatment of herpes zoster in immunocompromised patients clearly demonstrated that treatment resulted in more rapid viral clearance, halted disease progression, and reduced the risk of disseminated infection. In addition, intravenous acyclovir is considered the drug of choice for treating VZV dissemination when it occurs, although efficacy data from prospective studies are limited. Patients with less severe immunosuppression (e.g., patients with solid tumor malignancies or HIV-infected individuals with preserved CD4+ T lymphocyte counts) can, in most cases, be safely and effectively treated with oral antiviral therapy.

10.4.4 VZV Retinal Necrosis

PORN occurs most frequently in immunocompromised patients, with the highest frequency in patients with AIDS. Responses to intravenous acyclovir or ganciclovir have been inconsistent and disappointing. Anecdotal evidence and expert opinion favor a combination of intravenous and intravitreal ganciclovir, with or without intravenous foscarnet [260, 261]. The optimal duration of induction therapy and options for long-term maintenance therapy for PORN in HIV-seropositive patients have not been established [262]. When ARN occurs in immunocompetent patients, the clinical outcome is clearly more favorable, with a good response seen to intravenous acyclovir therapy.

10.4.5 Management of Acyclovir-Resistant VZV Infections

Most cases of infection caused by acyclovir-resistant VZV have been described in AIDS patients with very low CD4+ T lymphocyte counts; most patients had previously been treated with acyclovir. The most common mutations causing acyclovir resistance result in absent or truncated expression of viral thymidine kinase [263]. With this mutation, the isolate will also be resistant to valacyclovir, famciclovir, penciclovir, and ganciclovir, all of which depend upon viral thymidine kinase for phosphorylation and activation. On the basis of anecdotal experience and expert opinion, the drug recommended for treatment of acyclovir-resistant VZV disease is foscarnet, a viral DNA polymerase inhibitor that is not dependent on viral thymidine kinase for activation. Most cases of disease caused by acyclovir-resistant VZV have been limited to cutaneous involvement, although a few instances of visceral infection have been documented. In addition, several reports emphasize that the appearance of the cutaneous lesions caused by acyclovir-resistant VZV may be atypical. In a situation where acyclovir-resistant VZV is suspected and foscarnet therapy is not a viable option, treatment with cidofovir would be considered the next alternative.

10.5 Prevention of Herpes Zoster by Vaccination

Immunologic studies have documented a positive correlation between the risk of herpes zoster and the declining frequency of circulating VZV-specific responder cells, occurring as a consequence of either natural aging or immunosuppressive disease or medical therapy. The hypothesis behind the zoster

vaccine was that administering VZV antigens via vaccination would boost VZV-specific immune responses, allowing the immune system to keep latent VZV in check and prevent reactivation [40, 264]. This would be the first vaccine licensed to prevent disease in patients already infected with the pathogen [265]. The zoster vaccine selected for testing contained live attenuated VZV_{oka}, the same attenuated VZV strain found in the varicella vaccine (although zoster vaccine has over 14-fold more plaque-forming units of virus per dose, thus the two vaccines are not interchangeable).

In a placebo-controlled clinical study of 38,546 subjects 60 years of age or older, the herpes zoster vaccine was shown to be effective by reducing the herpes zoster "burden of illness" (an index of disease severity and duration) by 61 % compared with placebo [40]. A total of 957 cases of herpes zoster were documented among 38,456 adult subjects, with 315 cases occurring in the vaccine group, compared with 642 cases among placebo recipients. Vaccine efficacy for reducing the incidence of herpes zoster was 51.3 % ($p<0.001$), with 5.42 versus 11.12 cases per 1,000 person-years seen in the vaccine and placebo groups, respectively (Fig. 42.5) [40]. The reduction of zoster incidence was significantly greater in the recipients aged 60–69 (63.9 %) than in those aged ≥70 years (37.6 %).

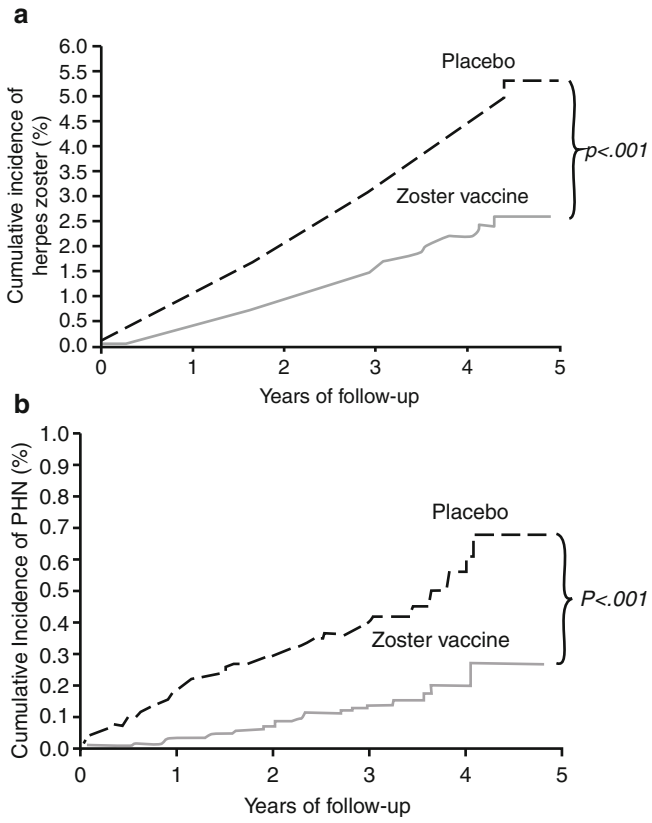

Fig. 42.5 Time-to-event estimates of the effect of the herpes zoster vaccine on the cumulative incidence of herpes zoster (**a**) and postherpetic neuralgia (**b**) [304]

Zoster vaccine resulted in a 66.5 % reduction in the incidence of PHN ($p<0.001$), largely through reduction in incident herpes zoster cases. The duration of protection of the zoster vaccine has not yet been firmly established, but follow-up studies to date have suggested that the duration of protection is at least 7 years [266]. Assuming vaccine efficacy of 63 % and sustained duration of protection, the number of 65-year-old individuals needed to vaccinate to prevent a case of herpes zoster, to prevent a case of PHN, or for a quality-adjusted life-year (QALY) is estimated to be 11, 43, and 165 individuals, respectively [267].

The most common adverse event observed in patients receiving the zoster vaccine was injection site reactogenicity (i.e., erythema, tenderness, and swelling) that occurred in 48 % of vaccine recipients versus 16 % of placebo recipients [268]. Inoculation site adverse events were more common in the 60–69-year-old population than in those 70 years of age and older. The zoster vaccine is equally well tolerated in patients who report a prior history of herpes zoster [269]. Post-licensure surveillance has not documented any significant safety concerns [270]. Neither the safety nor the efficacy of the herpes zoster vaccine is altered when it is coadministered with pneumococcal vaccine in older adults [271, 272]. A theoretical concern was raised that the zoster vaccine could induce cases of herpes zoster in patients with impaired immunity. In a review of a large Medicare database, receipt of zoster vaccine was not associated with any short-term increase in herpes zoster in a population of patients with autoimmune disorders [273]; indeed, vaccination was associated with a decreased incidence of herpes zoster over 2 years of follow-up.

A subsequent clinical trial of zoster vaccine in 22,439 individuals aged 50–59 years demonstrated an even higher degree of efficacy. In the vaccine cohort, 30 cases of zoster were observed for a zoster incidence of 1.99 per 1,000 person-years. In the placebo group, 99 cases of zoster were observed, yielding an incidence of 6.57 cases per 1,000 person-years [274]. Vaccine efficacy for preventing herpes zoster was 69.8 %. As noted in the previous study, a higher frequency of injection site adverse events was observed in vaccine recipients. The only commonly observed systemic adverse event was mild headache.

In prospectively followed subjects, VZV-specific CMI and antibody responses were shown to significantly increase 6 weeks after vaccine administration, with greater increases seen in persons aged 60–69 compared with those 70 years of age or older [230]. The CMI boost persisted over 3 years of follow-up, although the magnitude declined over time. In elderly adults <75 years of age, the zoster vaccine induced peak VZV-specific responder cell frequency higher than that induced by wild-type infection [87]. Vaccine virus has been recovered from saliva of patients following zoster vaccination, presumably reflecting active replication and

asymptomatic dissemination [275]. Other studies have examined potential benefits of a two-dose regimen. Compared with one injection, two doses of zoster vaccine were well tolerated, but did not result in any further significant increase in VZV-specific immune responses [276, 277].

The herpes zoster vaccine received FDA approval for administration to individuals 60 years of age and older in 2006 [278]. The vaccine is currently FDA approved for use in immunocompetent adults 50 years of age or older, although recommendations from advisory panels have maintained a recommended vaccination age of 60 years and older. Uptake of zoster vaccine among eligible older adults has been slow. In 2008, a National Health Interview Survey found that only 6.7 % of eligible adults had received zoster vaccine [279]. Surveys have suggested that barriers to uptake include vaccine cost, insurance reimbursement issues, and the requirement for a vaccine freezer in providers' offices [280–283]. Solutions to these issues will have to be implemented in order for the zoster vaccine to receive the appropriate level of coverage in the targeted population of adults 60 years of age and older.

As the zoster vaccine is a live virus vaccine, use is not generally recommended in immunocompromised patients. Zoster vaccine has been studied in HIV-seropositive adults with well-preserved CD4+ T lymphocyte counts. In small studies, herpes zoster vaccine was well tolerated, but measurements of VZV-specific CMI responses showed no significant increases [284].

It is estimated that the annual cost of providing medical care for herpes zoster patients in the USA is about $1.1 billion [285]. Healthcare expenditures are not limited to care of acute herpes zoster, but include management of chronic pain and hospitalization for zoster-related complications [155, 286]. Cost-effectiveness calculations are complex; variables include the age of vaccination, vaccine costs, herpes zoster incidence, incidence and duration of PHN, and duration of vaccine protection [287]. Overall, however, analyses have suggested that zoster vaccine will likely be cost-effective when given at age 65 if the duration of immune protection is >10 years [288, 289].

10.6 Herpes Zoster Prophylaxis

Controlled clinical trials in organ transplant recipients have clearly shown the benefit of prophylactic antiviral therapy for reducing the incidence of herpes zoster. Studies in HSCT patients have demonstrated that prophylactic oral therapy with acyclovir or valacyclovir can essentially eliminate herpes zoster occurrences in the posttransplant period [290–292]. Twelve months of acyclovir prophylaxis does not impair bone marrow engraftment or reconstitution of VZV-specific immune responses [290]. The frequency of herpes zoster increases after antiviral prophylaxis is discontinued, but the use of prophylaxis prevents herpes zoster during the early posttransplant period when patients are most severely immunosuppressed and are at highest risk for VZV-related complications [290, 293]. Three to six months of antiviral prophylaxis is the standard of care in most bone marrow transplant centers, with some centers extending prophylaxis to 12 months [294–296]. Because VZV reactivation occurs late and with lower frequency in solid organ transplant patients, many experts elect not to offer VZV-specific antiviral prophylaxis for this population [296]. However, prophylactic drug regimens designed to prevent HSV or CMV recurrences in SOT patients will also effectively prevent herpes zoster.

11 Unresolved Problems

While varicella vaccine coverage in the USA is very good, there are still substantial numbers of children (particularly immigrant children) who have not received vaccine. While varicella is not a top priority for international health authorities, efforts should continue to be made to find ways to make varicella vaccine available in countries where health resources are limited. It will not be possible to consider elimination of chickenpox in the foreseeable future, since most of the world's children remain unvaccinated. Even if effective universal childhood vaccination was accomplished worldwide, there will be continued exposure to VZV from naturally infected adults who subsequently develop herpes zoster.

It will be important to continue to monitor the epidemiology of both varicella and herpes zoster. Epidemiologists need more data to assess the impact of universal varicella vaccination as practiced in the USA on the incidence of herpes zoster, both in vaccinated children and naturally infected adults. It is also essential to monitor incidence rates to assess the duration of protection provided by each vaccine.

Means must be found to promote the broader use of the imperfect but still effective herpes zoster vaccine, which is currently greatly under-prescribed. Developing a laboratory marker that accurately predicts risk of herpes zoster in immunocompetent older adults could guide targeted vaccination. While the herpes zoster vaccine is certainly a major advance in our efforts to prevent shingles, the 50 % efficacy of the current vaccine for preventing incident herpes zoster is suboptimal. Improvements in the vaccine might include a modified vaccine virus, an inactivated vaccine containing immunogenic VZV proteins, a modified injection schedule, or use of adjuvants.

Treatment of herpes zoster also remains inadequate. Given that VZV reactivation causes extensive ganglionitis and nerve inflammation prior to appearance of skin lesions,

administering effective antiviral therapy early enough to make a real difference is a challenge. The currently available antiviral drugs are quite potent, but the natural history of herpes zoster dictates that drug therapy is usually started relatively late in the course of disease. Laboratory methods that provide much earlier diagnosis, such as PCR of blood or saliva, could be helpful. In addition, managing chronic neuropathic pain in patients who have had herpes zoster is a difficult and challenging problem. Improved therapeutic approaches to PHN represent a significant clinical need.

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Human Immunodeficiency Viruses Types 1 and 2

43

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1 Introduction

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that may produce an acute, usually self-limited mononucleosis-like syndrome but more inevitably causes a broad array of progressive clinical manifestations, widely known as the acquired immunodeficiency syndrome (AIDS). HIV type 2 (HIV-2) causes a similar but far less widespread infection and a generally more indolent chronic disease. This chapter emphasizes HIV-1 and selectively covers the more distinctive aspects of HIV-2.

For centuries pandemic influenza and smallpox have decimated the inhabitants of whole countries, often surging through in a matter of weeks or months, then virtually disappearing almost as fast. HIV/AIDS insinuated itself into human populations over years, and its less dramatic appearance and its chronicity have posed a different but no less formidable threat. Since its recognition in 1981, AIDS has had a profound impact not simply on the health of nearly all nations of the world but on their economic, social, ethical, legal, and political structures. During those three decades, more resources have probably been invested in research on and care of individuals with HIV-1 infection than for any other infectious disease in history. Cumulative expenditures on research alone exceed the equivalent of tens of billions of US dollars. In terms of the information generated, as a crude comparison, for the period from late 1981 through 2012, the National Center for Biotechnology Information bibliographic resource PubMed lists more than 250,000 publications under the terms "AIDS" or "HIV," compared with about 100,000 for "tuberculosis" and 55,000 for "influenza," the two infectious diseases probably commanding the next highest levels of interest and support for research.

In the years since the initial recognition of AIDS, teams of epidemiologists, virologists, immunologists, and scientists from many other disciplines have assembled an exquisitely detailed and comprehensive understanding of the two etiologic agents and their simian cousins, the complex biological and behavioral responses made by humans and primates and the factors in the physical, biological, and social environment that have driven this infection so relentlessly to pandemic proportions. Despite what has often seemed like painfully slow progress against the onslaught of HIV, multiple effective therapeutic agents have been developed, programs for treatment and prevention continue to be implemented on a global scale; and disappointments in the pursuit of an efficacious vaccine have not slowed efforts in that direction.

In retrospect, considering the unique nature of this infectious disease, the relatively modest insight into human retrovirology at the time the struggle began, and the depth of prejudice and anxiety about individual human sexual and other behavioral preferences, the accumulation of knowledge has enabled remarkable progress toward the goal of understanding and controlling the infection. What follows is an attempt to summarize that vast body of knowledge—documenting both the great distance traveled toward that goal as well as the critical shortfalls in the quest for effective prevention and treatment on a population scale. However, even this lengthy chapter cannot amply depict the viciously circular impact HIV/AIDS has had on the entire world—how 30 years of pandemic HIV-1 infection has profoundly altered

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fundamental socioeconomic and demographic characteristics of so many of the countries it has touched and vice versa.

2 Historical Background

During the three decades since the discovery of AIDS, for the first time in human history, the world witnessed a previously unknown infection leap from an obscure zoonosis into a full-scale pandemic in human populations around the globe. The virus was probably introduced into humans in the first half of the twentieth century [1, 2]. It became detectable at low frequency in stored samples of blood available from certain high-risk groups prior to 1980 [3]. From that time forward, one country after another experienced an oft-repeated pattern of silent introduction among a core transmission group followed by a usually insidious but occasionally more explosive spread of infection. The gradual but highly variable disease progression (with a median of 8–10 years from initial sexual transmission of infection to clinical diagnosis of AIDS) meant that the virus had usually penetrated deeply into a population before health-care providers and/or government officials grasped the extent of spread and its public health implications.

The earliest harbinger of the eventual worldwide pandemic consisted of a handful of cases of pneumonia among men who have sex with men (MSM) in Los Angeles, California, and New York City; this disease was due to *Pneumocystis carinii*, a relatively unusual agent now classified as the fungus *P. jirovecii*, which had not previously been known to occur in such a cluster [4]. Soon thereafter an equally unusual parallel epidemic of Kaposi's sarcoma (KS) was reported. Two things were common to these initial reports: both of these conditions had been seen sporadically in the past among immunosuppressed hosts in the developed world, and both were occurring simultaneously in MSM. From clinical and immunologic assessments of the earliest cases, it was clear that the common thread was cell-mediated immune deficiency characterized by profound reduction in the number of circulating T-helper lymphocytes.

Various alternative causal theories were proposed to account for the emerging pattern of the disease (e.g., immune exhaustion from constant stimulation and behavioral factors such as atypical sexual practices or the intense use of recreational drugs), but within less than a year, it seemed inescapable that an infectious agent was centrally involved. From the time these cases were first reported, less than 2 years elapsed before several groups of retrovirologists, through a series of elegant technical accomplishments, converged on the etiologic agent—the novel retrovirus that would come to be known as human immunodeficiency virus, type 1 (HIV-1).

Although the earliest work on RNA-based oncogenic retroviruses began in chickens more than a century ago [5], it was the discovery and elucidation of the properties of reverse transcriptase, the virally encoded enzyme-mediating synthesis of a proviral DNA from a viral RNA template in the 1970s, that led to the detection of the first human retrovirus, human T-lymphotropic virus type I (HTLV-I) in 1979 [6]. The close similarities between the known effects of HTLV-I (T-cell tropism, pathogenicity, and modes of transmission) and the new immunodeficiency identified in 1981 led laboratory scientists to postulate a novel retrovirus as its etiology [7]. The lymphadenopathy-associated virus (LAV) was discovered and reported as the likely cause of AIDS by Montagnier in 1983 [7]. In the following year, Gallo and his co-workers confirmed that the virus initially called LAV or HTLV-III but subsequently renamed HIV-1 was indeed the etiologic agent of AIDS [7–9].

After the initial recognition of AIDS, its definition rapidly expanded beyond the two sentinel conditions (*Pneumocystis* pneumonia and KS) to include a variety of other opportunistic infections [10]. Between 1981 and 1983, risk groups were defined for surveillance purposes in anticipation that an infectious agent was involved and transmitted by sexual routes, blood transfusion, and injection drug use and from mother to infant [11, 12]. Between 1983 and 1984, the etiologic agent was isolated and proven to be the cause of AIDS. Prospective studies in high-risk populations confirmed the causal temporal relationship between HIV-1 seroconversion and the development of AIDS as well as estimates of risk attributable to the various modes of viral transmission [13–15]. Over the next 5 years, rapid progress was made in developing an accurate blood screening test, characterizing the virus molecularly, defining certain aspects of pathogenesis, and determining the positive benefits of nucleotide analog antivirals and prophylactic therapies for prolonging the life of those with severe immunodeficiency and opportunistic infections.

Identification of the cause of AIDS opened the door to deeper inquiry into the origins and evolution of the virus and into the provenance of the infection in humans. The first documented case of AIDS occurred in a British seaman who died of *P. carinii* pneumonia in Manchester, England, in 1959 and was subsequently found to have had HIV-1 infection [16]. A very small proportion of serum samples taken in Zaire about that time contained antibodies suggestive of HIV-1 infection [17], and it has been postulated that UNESCO's exchange of educational technicians and perhaps military personnel between Zaire (Congo, at that time) and Haiti beginning in 1960 accounts for the early spread of the infection into the Caribbean [18].

Not long after HIV-1 was discovered, the related but less pathogenic HIV, type 2 (HIV-2), was linked to a milder form of the clinical syndrome occurring predominantly in West Africa [19, 20]. The earliest apparent cases of Africans with AIDS or pre-AIDs who were serologically positive for HIV-2 included a person who died in 1978 [21, 22] and a Portuguese family whose infections displayed several epidemiologic

features of HIV-2 infection: the propensity for heterosexual transmission, the low perinatal transmission rate, and the apparent long latency for disease. This virus was more closely related to a nonhuman primate virus, simian immunodeficiency virus (SIV) [23], and found in conjunction with AIDS-like illness in some primate species. Although there was fear that the spread of HIV-2 infection would lead to a parallel or overlapping epidemic of similar proportions, that concern was to prove largely unfounded.

In the decade after the discovery of HIV-1, great progress was made in understanding the origins of the virus itself. Phylogenetic relationships between human and simian immunodeficiency viruses raised the possibility of a nonhuman primate origin of HIV-1 [23]. Investigators isolated strains from chimpanzees, the only other species susceptible to HIV-1 infection, which appeared to bear a close phylogenetic relationship to the human virus [24], and to a new strain of HIV-2 from a West African that was more closely related to one found in sooty mangabeys (SIV_{SM}) than to prototype human isolates. This work suggested that HIV-2 has been transmitted from monkey to human and is a member of closely related primate virus family. The nonhuman primate-to-human hypothesis was further supported by reports of SIV infection in laboratory workers [25] and HIV-2 infection in women from Guinea-Bissau who prepared primate meat for human consumption (with potential blood exposure) [26].

Other observations in primates provided insight into viral evolution. SIV_{AGM} infection causes no disease in African green monkeys, its presumed natural host [27]. SIV_{SM} is nonpathogenic in the sooty mangabey, its apparent natural host, but may cause AIDS-like illness in other primate species [28]. SIV_{SM} was linked to epizootic AIDS-like disease resulting from cross-species infection of captive macaques (a nonnatural host) in US primate research centers [29]. These observations illustrate that slow adaptation between a virus and its host may have minimal pathologic consequences, but when the virus leaps to a new species, it often becomes much more pathogenic. In 1999 a group of researchers reported the discovery that a particular type of chimpanzee, once common in West Central Africa, was the source of HIV; they suggested that HIV-1 was introduced into the human population when hunters became exposed to an infected blood [30].

Throughout the latter half of the 1980s and early 1990s, as HIV/AIDS exploded into a pandemic, bad news generally outweighed the good. From 1993 onward, the CDC used an expanded definition of AIDS [31], but even before that, it had become increasingly clear that the numbers of people and places affected would continue to rise. By WHO estimates for 1992–1993, some 2.5 million cases of AIDS had occurred in adults and more than 14 million adults were infected. The single antiretroviral agent, the nucleoside reverse transcriptase inhibitor azidothymidine (AZT, now zidovudine), alone or in combination with other related drugs, offered the hope of interrupting the inexorable course of infection, but these agents turned out to be limited in the potency and duration of their effects. The following year, however, witnessed a major breakthrough with the success of AZT in reducing the rate of mother-to-child transmission by two-thirds [32]. At that point world health leaders began to realize that programs for prevention had been implemented in different countries with varying degrees of support and success, but many heavily affected countries still lacked a sense of unity and common purpose about the messages and strategies for reversing the trends. The United Nations announced the establishment of a new Joint United Nations Programme on AIDS (now called UNAIDS) to be cosponsored by a number of its components, with coordinated resources and forceful leadership [33]. By the end of 1995, it was estimated that 4.7 million new HIV infections occurred, 500,000 of them in newborn infants [34].

Advances in treatment during the next several years were encouraging. A second class of promising drugs (protease inhibitors) approved for clinical use was succeeded by a third, the nonnucleoside reverse transcriptase inhibitors (NNRTIs). One of these drugs, nevirapine, proved highly effective at preventing peripartum mother-to-child transmission (MTCT) [35], and although controversy over drug resistance and its longer term benefit arose, it has retained a prominent role in prevention of MTCT [36]. Trials of NNRTIs more broadly continued to demonstrate substantial and sustained decline in morbidity and mortality, and combinations of these drugs (termed "highly active antiretroviral therapy," HAART, and now "combination ART," cART) began to be used on a large scale in the USA and other countries where the regimens were more affordable.

This major advance was accompanied by another when the US Food and Drug Administration approved the introduction of a quantitative measure of burden of infection. Having undergone successive refinements since the original demonstration of its prognostic value [37], a test for the number of copies of viral RNA (i.e., viral load) has remained the single most critical clinical indicator of disease control.

As evidence for the effectiveness of cART continued to mount, a debate turned to whether, when, where, and how infected people should be treated. What was initially a cautious implementation of programs for treatment in less developed regions of Asia and Africa and elsewhere soon evolved into more ambitious plans and investments in drugs, infrastructure, monitoring, and evaluation on a global scale. Numerous clinical trials of derivatives of the approved classes of drugs were completed, and entirely new products were beginning to show promise. By 2002 a clinical trial of T-20 (later, enfuvirtide), a member of a new class of AIDS drugs called fusion inhibitors, had demonstrated its efficacy; in the next year it was approved for parenteral use in patients with drug-resistant HIV-1 infection. In the next few years, two other agents, maraviroc (a co-receptor antagonist) and raltegravir (an integrase inhibitor), also became available for

drug-resistant infection (see Sect. 9). However, the limited indications for their use and their higher cost precluded their incorporation into global treatment programs.

By the turn of the millennium, there were 34.3 million people infected with HIV worldwide, with 1.3 million under age 15. It was predicted that AIDS would cause early death in as many as half of the teenagers living in the hardest hit countries of southern Africa, causing population imbalances. In the midst of this raging epidemic 15 years after HIV-1 was established as the etiologic agent, South African President Thabo Mbeki was publicly doubting the viral origin of AIDS. For that entire period since AIDS first came to public attention, the superstition that surrounded it and the stigma haunting those who succumbed to it had proved to be as refractory an impediment to progress in preventing the infection as the novel viral biology itself. An entire parallel history of the social, cultural, and economic impact of HIV/AIDS has been documented in countless forms. The reader is referred [38] as one source of information on these historical aspects.

For approximately 15 years, from the mid-1990s until about 2010, as efforts to achieve universal access to treatment of HIV/AIDS accelerated, progress in prevention moved forward at a slower pace, reached fewer new milestones, and experienced some significant setbacks. Most frustrating of all has been the minimal success at producing an efficacious vaccine to prevent infection. Those who fully understand the virologic and immunologic obstacles to producing a new vaccine against *any* disease had no illusions about how quickly or easily those barriers could be surmounted. Indeed, experts at one extreme have considered the prospect nearly hopeless, while most others have scoured the information collected during the series of unsuccessful vaccine trials that have been conducted for any useful insight. The unfulfilled quest for a vaccine has not been the only disappointment in the area of prevention. Randomized controlled trials evaluating treatment of genital herpes and other genital infections have failed to demonstrate meaningful reductions in incidence of HIV-1 infection [39], despite every indication from studies of MSM, sex workers, and others that genital inflammatory conditions predisposed to new HIV-1 infection (see Sect. 5). It was only in 2010 that the first apparent success of topical prophylaxis with an antiretroviral agent was reported [40], and the full implications of this finding remains to be assessed.

On the more positive side, by 2004, teams in Uganda reported that reduction of sexual partners and other prevention measures in local communities was capable of lowering HIV-1 seroprevalence to half a million people compared with 1.5 million a decade earlier [41]. More recently, excitement was generated by the findings in clinical trials of circumcision that documented an approximately 50 % reduction of female-to-male heterosexual transmission (Sect. 9). The enthusiasm for promoting the practice more widely has been high, albeit tempered by the recognition that implementation on a wide scale faces a number of issues. Even more promising have been the clinical trials demonstrating the efficacy of preexposure prophylaxis and the proof of the principle of "treatment as prevention"—that early therapy of an infected individual can prevent transmission to the uninfected partner (Sect. 9). Finally, optimism has risen – some argue too high – with the apparent proof of principle that infection in one or more individuals might have been cured through bone marrow transplantation.

In 2007 for the first time in the history of HIV/AIDS, a report from UNAIDS announced the "stabilization of the global epidemic," estimating that 33 million people were living with HIV-1 worldwide (down from the 39.5 million estimate from 2006). Part of that reduction was attributed to better surveillance techniques in many countries, but another substantial part represented a real decline in HIV-1 prevalence in certain areas, including sub-Saharan Africa. The report also concluded that the decline in AIDS deaths from 2.2 million in 2005 to 2 million in 2007 reflected an increase in the number of people receiving antiretroviral drugs. In subsequent years, UNAIDS data have verified this "reversal" of the previous, ever-upward trends in the statistics that characterized the first 25 years of AIDS (Ref. [42] and Sect. 5).

During the past decade, medical and public health advances have been accompanied by significant advances in the commitment of social and economic resources to the battle against HIV/AIDS. In 2001 the United Nations Secretary-General called for a tenfold increase in spending on AIDS in developing countries—$7–10 billion to be spent under the auspices of a new entity called The Global Fund to Fight AIDS, Tuberculosis and Malaria [43]. The actual amount initially offered by donor countries was less than 25 % of that figure, and the Fund struggled during its early years. However, between 2001 and 2013 paid pledges to the Global Fund have actually totaled nearly $24 billion, and that did not include the numerous other public and private investments in treatment and prevention programs. Still, in 2011, even after 10 years of collaboration, the vulnerability of the global effort was again highlighted by the failure donors to honor pledges to the Fund. This prompted a promulgation of a new model of funding [44].

Clearly, chapters of the history of HIV/AIDS are still being actively updated. Only time will tell whether the insights thus far gained into host/agent molecular interactions and into individual and social behavior have been sufficient to overcome one of the greatest threats to humans ever posed by an infectious agent.

3 Methods of Epidemiologic Analysis

3.1 Sources of Mortality Data

3.1.1 HIV-1

USA

In the 15 years before effective therapy became widely available, the median time from initiation of infection (seroconversion) to clinically defined AIDS was about 9 years,

although it became clear that this interval varied greatly according to the mode of transmission of infection as well as several rather carefully documented host and viral determinants (Sect. 5). In contrast, the median time from onset of untreated AIDS to death was more predictably about 2 years, varying relatively little by specific clinical diagnosis [45]. In the USA the two primary sources of mortality data have been the vital statistics of all-cause mortality recorded by the National Center for Health Statistics (NCHS) and the National AIDS Surveillance. Both are managed by the Centers for Disease Control and Prevention (CDC) and depend on reporting of deaths due to AIDS by state health departments.

Even in such well-organized systems for gathering mortality statistics in the USA and other parts of the developed world, particularly in the early years of the epidemic, difficulties in attributing death to AIDS were due to diagnostic imprecision around a range of causes of immunodeficiency or to the absence of health services for disadvantaged populations. There was also often reluctance to diagnose and/or code socially stigmatizing AIDS-defining conditions [46]. Over time, as those barriers fell and the AIDS definition was refined, the attribution of cause of death evolved. By 1993 the definition had been expanded to require documentation of both HIV-1 infection and diminished CD4+ cell numbers [31], improving diagnostic sensitivity and specificity and thereby the accuracy of mortality data. In the years that followed, use of the Social Security Administration Death Master File has accounted for some additional improvement in ascertainment of AIDS deaths [47].

Global

As individual countries began to experience the impact of AIDS, they adapted surveillance systems for mortality and morbidity to their individual circumstances, which varied greatly with the resources available for diagnostic testing and delivery of health care. Efforts made by the UN Global Program on AIDS and its successor, UNAIDS, helped to centralize the documentation of deaths and death rates, but heterogeneity in the primary data continued to limit their usefulness. The heavy burden of coincident tuberculosis and HIV-1 infection and the difficulty of teasing apart their respective contributions to the cause of death are just two examples of the obstacles faced more by resource-poor regions. AIDS mortality statistics have also been less consistently reliable from country to country as a result of the different points in time when HIV-1 was introduced, the varying trajectories with which it spread, the use of prophylaxis for opportunistic infections, and the penetration of ART. The difficulty in arriving at a definitive mortality estimates is illustrated in the steadily changing proportions surviving in the years after the introduction of HAART in the USA (Fig. 43.1).

Mortality has also been less informative because it has represented a lagging indicator of the actual burden of HIV-1 infection in a population [49].

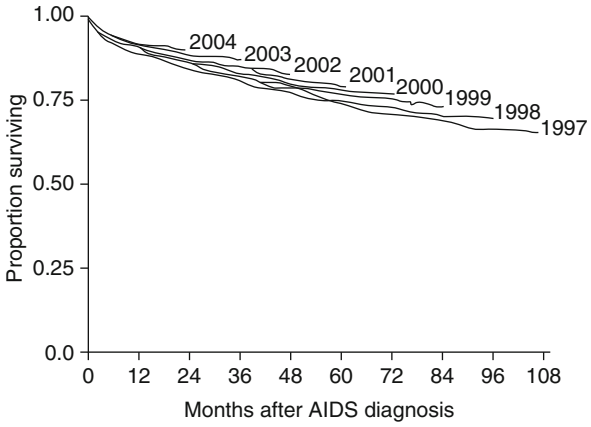


Fig. 43.1 Cumulative proportion of patients surviving by months after the first diagnosis of an AIDS-defining opportunistic infection 1997–2004 (Reproduced from CDC [48])

3.1.2 HIV-2

HIV-2 presumably originated in West Africa and has remained largely confined to residents of that region, to travelers or immigrants from there, and to their contacts. Accordingly, the main sources of epidemiologic information are the uneven descriptive studies in former colonies of Portugal (e.g., Guinea-Bissau, Cape Verde, Goa, and Brazil) or other neighboring West African countries (e.g., Senegal and the Gambia).

Mortality experience with HIV-2 has not been well documented because of the strong geographic influence on its occurrence as well as its frequent dual occurrence with HIV-1 and its lower pathogenicity than that of HIV-1. The largest and most reliable estimates of mortality probably come from a study of deaths due to serologically confirmed HIV-1, HIV-2, and HTLV-I infections among more than 5,000 residents of a rural area in northwestern Guinea-Bissau [50]. The study spanned the 18-year period before ART became available in 2007. Although there are concentrations of immigrants from different West African countries throughout the USA and other regions, systematic studies of HIV-2 mortality in these places have not been published.

3.2 Sources of Morbidity Data

3.2.1 HIV-1

USA

CDC established a nationwide surveillance for AIDS soon after the initial cases were reported. In a matter of months, insights emerging from rapidly accumulating cases in the different risk groups supported an infectious etiology. In 1983 CDC adopted a surveillance definition of AIDS based on a collection of clinical conditions reflecting an underlying immunodeficiency unexplained by known causes [51]. By 1987 when the viral etiology had been discovered and

serologic testing for HIV-1 antibody had revealed that AIDS represented an even wider spectrum of sequelae related to immunodeficiency, the CDC revised the definition [52]. Beginning in 1993 the agency made a major revision that substantially broadened the criteria [31] and then several successive minor changes to arrive at the current (2008) AIDS definition and HIV-1 classification system for adults and adolescents. In addition to the medical conditions defined as AIDS in 1993 (Table 43.1), the 2008 definition established three stages of infection based on CD4 cell measurements (Table 43.2), with stage 3 (AIDS) defined as the occurrence of HIV-1 infection accompanied by a CD4 count <200 cells per μL or a percentage of CD4 lymphocytes <14 [54]. The definition of AIDS in infants and children was also updated in 2008.

The earlier modifications of the AIDS case definition produced significant increases in case numbers [55]. By including cases based on a laboratory marker of an outcome (CD4 cell count) preceding end-stage clinical immunodeficiency, the 1993 revision accounted for a 111 % increase in reported AIDS cases, most notably among women, minorities, and those infected through heterosexual contact. In the 20 years since this last major revision, fluctuations of disease occurrence in the USA and other developed countries have diminished, but opportunities for misclassification still occur. For example, persons experiencing a transiently high viremia and low CD4 cell count during acute HIV-1 infection (see Sect. 5) could be erroneously classified as having reached a later stage of infection than would be revealed once viral load set point has been reached. In the USA, a parallel scheme for surveillance by source of HIV-1 infection classifies patients hierarchically according to their membership in a risk group [54]. However, misclassification can occur in this system too because a case with multiple risk factors can only be classified as belonging to a single (the highest) risk group. Thus, in areas where injecting drug use is common, preferential assignment to that risk group could exaggerate its role as the primary risk factor among women also highly likely to acquire HIV-1 through heterosexual transmission.

Table 43.1 1993 Revised classification system for HIV infection and expanded AIDS surveillance case definition for adolescents and adults

	Clinical categories		
$CD4^+$ T-cell categories	(A) Asymptomatic, acute (primary) HIV or PGL	(B) Symptomatic, not (A) or (C) conditions	(C) AIDS-indicator conditions
1. ≥500/μL 2. 200–499/μL 3. <200/μL AIDS-	Asymptomatic HIV infection; persistent generalized lymphadenopathy (PGL); acute (primary) HIV infection with accompanying illness or history of acute HIV infection	Bacillary angiomatosis; candidiasis, oropharyngeal (inrush); candidiasis (thrush); candidiasis, vulvovaginal; persistent, frequent, or poorly responsive to therapy; cervical dysplasia (moderate or severe)/ cervical carcinoma in situ; constitutional symptoms, such as fever (38.5 °C) or diarrhea lasting >1 month; hairy leukoplakia; oral herpes zoster (shingles), involving at least two distinct episodes or more than one dermatome; idiopathic thrombocytopenic purpura; listeriosis; pelvic inflammatory disease, particularly if complicated by tubo-ovarian abscess; peripheral neuropathy	Candidiasis of the bronchi, trachea, or lungs; candidiasis, esophageal; cervical cancer, invasive; coccidioidomycosis, disseminated or extrapulmonary; cryptococcosis, extrapulmonary; cryptosporidiosis, chronic intestinal (>1-month duration); cytomegalovirus disease (other than the liver, spleen, or nodes); cytomegalovirus retinitis (with loss of vision); encephalopathy, HIV related; herpes simplex, chronic ulcer(s) (>1-month duration); bronchitis, pneumonitis, or esophagitis; histoplasmosis, disseminated or extrapulmonary; isosporiasis, chronic intestinal (>1-month duration); Kaposi's sarcoma; lymphoma, Burkitt's (or equivalent term); lymphoma, immunoblastic (or equivalent term); lymphoma, primary, of the brain; *Mycobacterium avium* complex or *M. kansasii*, disseminated (or extrapulmonary); *Mycobacterium*, other species or unidentified species, disseminated or extrapulmonary; *Pneumocystis carinii* pneumonia; pneumonia, recurrent; progressive multifocal leukoencephalopathy; salmonella septicemia, recurrent; toxoplasmosis of the brain; wasting syndrome due to HIV

Reproduced from Centers for Disease Control [31]

Table 43.2 Surveillance case definition for human immunodeficiency virus (HIV) infection among adults and adolescents (aged ≥13 years)—USA, 2008

Stage	Laboratory evidence	Clinical evidence
Stage 1	Laboratory confirmation of HIV infection *and* CD4+T-lymphocyte count of ≥500 cells/μL *or* CD4+ T-lymphocyte percentage of ≥ 29	None required (but no AIDS-defining condition)
Stage 2	Laboratory confirmation of HIV infection *and* CD4+ T-lymphocyte count of 200–499cells/μL *or* CD4+ T-lymphocyte percentage of 14–28	None required (but no AIDS-defining condition)
Stage 3 (AIDS)	Laboratory confirmation of HIV infection *and* CD4+ T-lymphocyte count of < 200cells/μL *or* CD4+ T-lymphocyte percentage of < 14	*or* documentation of an AIDS-defining condition (with laboratory confirmation of HIV infection)
Stage unknown	Laboratory confirmation of HIV infection *and* no information on CD4+ T-lymphocyte count or percentage	*and* no information on presence of AIDS-defining conditions

Adapted from Refs. [31, 54]

During the early years of the epidemic in the USA and other advanced countries, underreporting in the numbers and distribution of AIDS was suspected due to fear of the deadly consequences of the disease that led those affected to deny it and to the stigma attached to it that drove those affected and their caretakers to conceal it. Even health authorities found themselves uncomfortably joining groups who resisted standard surveillance approaches and demanded anonymity with screening for HIV-1 because of concerns about confidentiality and discrimination. Those counterproductive responses were gradually converted to more constructive ones, especially once counseling, serologic testing, and effective antiretroviral therapy became more widely available.

With regard to AIDS in children, CDC established a pediatric surveillance case definition in 1987 and updated it periodically thereafter its current, comprehensive 2008 revision [54]. It covers both children aged 18 months to 13 years and children <18 months old. In the younger group, the latest laboratory criteria for an AIDS diagnosis are exacting; and only those babies with a compatible clinical condition who were born to an HIV-infected mother meet the case definition in the absence of a positive serologic test.

Global

In the decade that followed the recognition of AIDS, the World Health Organization (WHO) assumed major responsibility for monitoring the pandemic. Although its surveillance activities and definitions have been modeled broadly on those of the CDC and other advanced countries, the approach has accommodated the inadequacy of resources to diagnose infection or disease in many other areas. As UNAIDS became operational in the mid-1990s and financial support began to increase significantly, the surveillance systems in the developing world improved dramatically. In 2007 UNAIDS added laboratory confirmation as a criterion for the surveillance case definition for HIV infection. By 2012 there were 186 countries maintaining and reporting AIDS statistics and, in turn, submitting the data summarized in a remarkably comprehensive annual report [56]. These sources of data along with the methods used to curate, report, and analyze them have been refined by a reference group of epidemiologists, demographers, and other experts on HIV and described in a series of articles [57–59]. Using these and other resources, UNAIDS has provided comprehensive guidance to national AIDS programs and other groups on the application of core indicators for reporting their data, their national response, and their progress toward 2015 global HIV/AIDS targets [60].

Despite this immensely successful global effort to increase awareness of HIV/AIDS and document epidemiologically important features of its occurrence, of the risk factors for acquiring it, and of the benefits of treatment, some countries have remained unable to counter entirely the forces of poverty, ignorance, and stigma that have prevented them from gathering and reporting the data needed to characterize their national epidemic.

3.2.2 HIV-2

As with mortality, systematic studies of the nature, frequency, and course of disease manifestations of HIV-2 are sparse. In addition to the population-based cohort in Guinea-Bissau described above, sources include Senegalese sex workers [61] and another cohort in the Gambia [62].

3.3 Serologic Surveys: Sources of Prevalence and Incidence Data

3.3.1 HIV-1

Prevalence

Soon after the virus was discovered, the first generation of serologic test systems was created for biological research and epidemiologic and clinical purposes. These and successive generations have utilized the enzyme immunoassay (EIA) technique to detect antibody to HIV proteins or peptides encoded by the conserved regions of the genomes of HIV-1 and HIV-2. The assays include combinations of viral

core, polymerase and envelope proteins; technical details of their composition, performance, and application are discussed in Sect. 3.4. Because both viral replication and antibody production at some level are considered lifelong in humans, the extremely sensitive and specific assays developed immediately after the initial isolation of the virus are highly reliable at documenting past or present infection in population surveys [3.463, 64].

In the USA and other developed areas, serologic testing increased steadily since the assays first became available. In recent years, with the increased emphasis on earlier intervention both for its own sake and as part of prevention strategy aimed at infected persons, many health-care systems around the world have implemented testing programs. In more socioeconomically advanced countries, testing is now increasingly a routine [65]. In 2009, for example, the US Department of Veterans Affairs adopted recommendation that an HIV-1 test be performed on all patients under care in its health-care facilities at least once during their lifetime [66], and the data on testing in that population are updated annually as part of the Federal government comprehensive update of the status of testing. Numerous prevalence surveys have been conducted in the subpopulations at higher behavioral or demographic risk—hemophiliacs, blood transfusion recipients, prostitutes, injection drug users (IDUs), patients with sexually transmitted infections (STI) and tuberculosis, Latino and black patients in communities with high seroprevalence, and those attending prenatal and mental health clinics. Estimates of seroprevalence in lower risk populations still rely on testing of anonymized samples from blood donors, hospital patients without HIV-associated conditions, pregnant women, applicants for military service, and other special populations (e.g., prison inmates).

Incidence

In contrast to prevalence, which can be measured more straightforwardly, incidence of HIV-1 infection has been more difficult to document for several reasons. The earliest estimates of incidence relied on indirect techniques of back calculation from statistics on AIDS mortality and morbidity [67]. Because rates of infection by definition can change more rapidly than rates of disease, estimates across population subgroups were subject to substantial variation in accuracy because of differential access to care and intensity of surveillance. Although more direct observation of incident infection was possible in certain fixed cohort studies during in the years soon after follow-up began [68–70], estimates in those cohorts inevitably grew more biased as behavior changed; screening and other measures protected blood product supplies; early seroconversions removed the most vulnerable members from the pool of susceptibles; and effective treatment became widely available [71]. Increasingly effective prevention and treatment brought an end to large-scale unbiased longitudinal surveillance in these and other samples of individuals who were representative of their high-risk target populations.72

In Africa and Asia current estimates of incident HIV-1 infection have been available from various cohorts of subjects assembled for the purposes of studying various prevention measures—behavioral interventions, vaccines, male circumcision, and antimicrobial treatment of predisposing STIs. Examples of these sources include serodiscordant couples receiving counseling and serologic testing in Zambia and participants in a vaccine preparedness study in Tanzania [72].

The dearth of opportunities to measure incidence directly led to the development of approach involving detection of biological markers (e.g., p24 antigen) to gauge the timing or maturity (i.e., duration) of a prevalent infection [73]. However, the brevity of antigenemia and the cost of testing large number of specimens rendered this approach less suitable than originally hoped.

Another alternative strategy for incidence estimation was also devised in the late 1990s; it is known as the serologic testing algorithm for recent HIV seroconversion (STARHS) [74]. This approach depended on measurement of differential titer of HIV-1 antibodies between recent and long-term infection. Once initial shortcomings were overcome, the algorithm was more widely adopted for incidence estimation in conjunction with a simple enzyme immunoassay (EIA) that could quantify a gradual increase of HIV-1-specific IgG against immunodominant sequences of gp41 from HIV-1 B, E, and D. Evaluation in longitudinal specimens taken from 139 incident infections in the USA and Thailand documented the improvement in performance [75]. Further refinements culminated in the first direct estimates of HIV-1 incidence in the USA in representative serum samples from newly diagnosed HIV-positive patients in 22 states who were >13 years of age in 2006 [76]. This BED HIV-1 capture EIA classified recent or long-standing infection, with statistical adjustment for testing frequency and extrapolation to the entire country.

The accuracy of incidence estimates based on this assay continues to be evaluated [77], and still other approaches, for example, based on antigen avidity maturation are under investigation [78]. However, once the STAHRS and BED methods were judged conceptually valid tools for wider application, CDC proceeded to establish and fund efforts at HIV incidence surveillance in conjunction with the National HIV Surveillance System (NHSS), in which state and local jurisdictions with moderate-to-high morbidity conduct surveillance using those methods to identify incident HIV-1 infection. Details on the strategy for estimating incidence can be found at reference [79].

Lately, strategies have been proposed for estimating HIV incidence using multiple biomarkers measured in samples obtained from cross-sectional surveys. By combining data from BED, antibody avidity, viral load, and CD4 assays, algorithms can be optimized for accuracy, feasibility, and

cost individually and jointly to support surveillance and research in a variety of settings [80, 81].

Another strategy, especially in regions where laboratory-based approaches were infeasible, has been to model HIV-1 incidence by inferring it from prevalence estimates, under assumptions about average survival of and effects of ART of infected individuals [82]. Despite the uncertainty and instability of current HIV-1 incidence estimates, the fervor around the potential preventive impact of early ART has led statistical modelers to begin exploring the magnitude of the reduction in *future* incidence that might be projected under different intervention scenarios [83]. Such projections in suboptimally resourced countries must obviously depend on often dubious inferences about numbers of infected individuals, their stage of infection, their likelihood of receiving effective treatment, the transmissibility of their infection, and other features.

Unfortunately, the lingering stigma attached to the infection as well as AIDS itself remains another barrier to HIV testing and reporting of new or newly recognized infection. In some places this may still account for a significant discrepancy between the estimates of actual occurrence and reported new infections.

3.3.2 HIV-2

Estimates of HIV-2 seropositivity have also come mainly from the countries mentioned above, although more deliberate efforts to detect infection with this virus as well as HIV-1 have been made in places where risk groups are concentrated—the New York City vicinity, other parts of the northeastern USA, and certain other countries where appreciable numbers of residents have West African connections.

3.4 Laboratory Diagnosis of HIV-1 and HIV-2

3.4.1 Virus Isolation

Historically, a major challenge in the detection and isolation of HIV-1 was its propensity to lyse the target CD4 cell. Unlike HTLV-I, which promotes the growth of CD4 cells, HIV cell cultures result in the death of these cells. Initial laboratory techniques developed to isolate the virus involved serial propagation in tissue culture by continually coculturing with normal peripheral blood lymphocytes or cord blood lymphocytes [84]. Detection of virus relied on assays for virally associated reverse transcriptase in culture media, detection of viral antigen, and electron microscopy, as shown in Fig. 43.2.

A major breakthrough occurred when Popovic and colleagues grew the virus in continuous mature T-cell lines derived from leukemic patients [86]. Virus grown in these lysis-resistant cell lines could be purified and its antigens studied as the basis for subsequent development of serologic assays. It is noteworthy that in this first paper describing the growth characteristics of the virus, there was marked variation from patient to patient and from cell line subclone to subclone in virus growth and cytopathic effects [86]. Since these early breakthroughs, refinements in culture techniques have provided insights about the protean variables that define virus growth characteristics and cell trophism. While cellular trophism of HIV-1 has been classified on the basis of growth in CD4/T cells versus macrophages, these characteristics are not absolute; most strains of HIV-1 will grow in either cell type but manifest a strong predilection for one or the other. The methods used for HIV-2 viral isolation are essentially the same as for HIV-1.

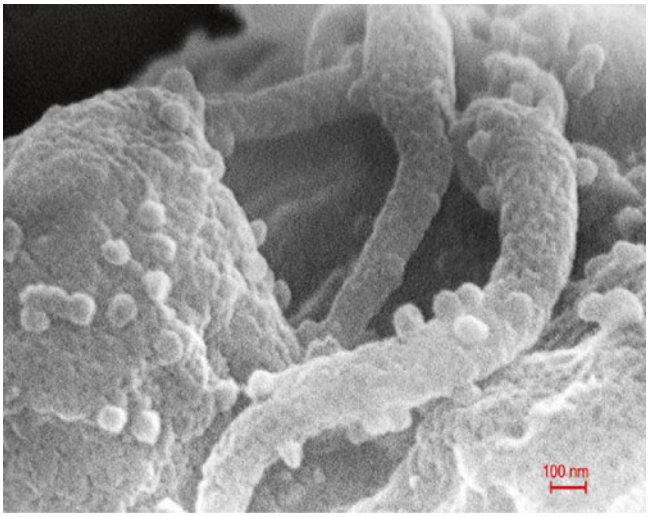


Fig. 43.2 Electron micrograph of HIV-1 virions budding off the membrane of an infected cell (Electron microscopic image courtesy of Cynthia Goldsmith, Centers for Disease Control and Prevention (CDC) Goldsmith et al. [85].

3.4.2 Serologic Diagnostic Methods

During primary infection with HIV-1, the initial buildup of virus in the plasma is followed by the appearance of HIV-1 antibodies. Concomitant with this early phase of infection, there is a decline in CD4 cells that can be modest or quite dramatic; immune activation markers such as neopterin and β_2-microglobulin also increase with HIV-1 infection. Measurements of all of these markers [viral antigen (p24 antigen), viral antibody, lymphocyte subset count (especially CD4 cells and other markers of immune activation)] have been used in epidemiologic studies to detect infection and define predictors of endpoints such as AIDS and death [87–90].

The HIV Western blot identifies antibodies to individual HIV-1 proteins (Fig. 43.3) and is considered more specific

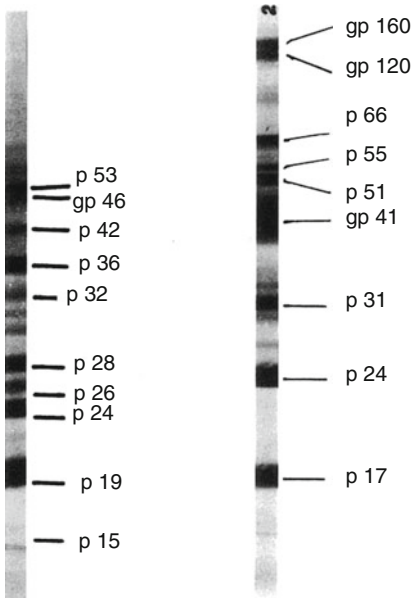


Fig. 43.3 Western blot of major antigenic proteins of HIV-1 (Photograph courtesy of Dr. Steven Alexander)

than EIA for diagnosis. It is therefore widely used to confirm the diagnosis of HIV-1 when the EIA is repeatedly positive. The abbreviations for the Western blot proteins include "p" for protein or "gp" for glycoprotein followed by the number indicating its molecular weight. The major bands used to diagnose HIV-1 include envelope protein (gp160, gp120, gp41), the gag core proteins (p55, p24, p17), and polymerase proteins (p66, p51, p31). Other smaller HIV-1 protein bands may also be seen but are not used for diagnosis. There are several criteria for a diagnosis of HIV-1 by Western blot, but the WHO one includes two envelope bands with or without gag or pol proteins [91]. The algorithm developed for testing requires the presence of a repeatedly reactive EIA and a positive Western blot test for confirmation (Fig. 43.3).

The mainstay of HIV-1 detection is testing for antibody to the virus. HIV-1 antibody testing is highly predictive of infection [92] except when antibody is passively transferred at birth [93–95] or lost during late-stage infection [96]. In view of the short time from initial propagation of the virus in continuous T-cell lines to assay licensure, first-generation HIV-1 whole virus lysate tests achieved remarkable sensitivity and specificity [63, 64]. Second- and third-generation enzyme immunoassays (EIA) have replaced viral lysates with recombinant proteins or peptides from conserved regions in HIV-1 and HIV-2. Many have also included modifications to the detect outlier (O) group of HIV-1. While these modifications have increased the sensitivity and specificity of the assay, there are still situations where false-positive and false-negative tests occur. False-positive tests can occur as part of procedural mistakes or as a result of hypergammaglobulinemia, autoimmunity, or acute or chronic inflammatory states [97–99]. False-negative tests can also be due to procedural mistakes but are more commonly due to early infection when the antibody response has yet to develop to detectable levels [100, 101]. Fourth-generation EIA assays are now including p24 antigen detection to decrease the number of false positives [102, 103]. HIV-1 viral antigenemia may occur early in infection or at the end stage of disease, and serologic assays can detect the 24,000 molecular weight, p24, viral nucleocapsid antigen. The antigen-capture assay is relatively insensitive compared to techniques such as RNA PCR assays; sensitivity can be enhanced by acid dissociation, which frees the p24 antigen from its complexing antibody [104].

Because HIV-1 and HIV-2 differ antigenically, HIV-1 screening tests are unreliable for detecting HIV-2. The methods for serologic diagnosis for HIV-2 infection are complicated because HIV-1 and HIV-2 have a nucleotide sequence homology of about 60 % for the *gag* and *pol* glycoproteins and about 40 % for *env* [105, 106]. The amount of cross-reactivity varies between assays and between areas. It also decreases concurrently with the level of immunosuppression, so that HIV-2-associated AIDS cases are less likely to be detected by an HIV-1 assay than are healthy carriers. HIV-2 screening tests have been developed and now include combination tests with antigens representing both virus types. Confirmatory testing for HIV-2 also requires HIV-2-specific assays to distinguish the two infections, since HIV-2-infected persons may test positive, indeterminant, or negative by HIV-1 Western blot. Although many early reports of apparent dual infection with HIV-1 and HIV-2 simply represent cross-reactivity, true dual infection confirmed by PCR and viral culture does occur [107].

In the USA blood donors have been screened for HIV-2 antibodies since June 1992 with licensed combination enzyme immunoassays (EIAs). Persons from HIV-2-endemic areas have also been asked to refrain from donating blood. These actions have reduced the probability of transfusion-acquired HIV-2 infection to essentially zero [106]. Although routine screening outside of blood centers is not recommended, according to CDC guidelines, HIV-2 testing should be included for individuals who are specifically at risk of that infection, either by nationality, by sexual exposure to a person from an endemic area, or by birth from an infected mother [105]. The reported performance of the HIV-1/HIV-2 combination assays licensed by the US Food and Drug Administration (FDA) for detection of HIV-2 infection is quite high, with >99 % sensitivity.

In recent years, the FDA has approved several rapid HIV antibody tests, by providing results within an hour of sample collection. Depending on the rapid test, analysis can be performed on the whole blood, serum, plasma, or even oral fluids or urine. Most of these rapid tests achieve the sensitivity and specificity that are equivalent to those of standard

EIA and Western blot assays; however, all rapid tests should have a confirmatory testing via Western blot regardless of the EIA result [108]. Rapid tests are now routinely used for initial diagnosis of HIV in many emergency departments in all patients who have not had recent testing. These tests are also useful in situations where decisions on using combination antiretroviral therapy (cART) for HIV-1 prevention are urgently needed—in the setting of needle exposure or at the time of delivery in pregnant women who had not had routine HIV testing as part of their maternal care.

3.4.3 Nucleic Acid Amplification Diagnostic Methods

A variety of advanced molecular techniques involving amplification of HIV-1 viral RNA or proviral DNA have been developed. The most widely used is PCR, but approaches such as ligase chain reaction [109] and branch chain amplification are also available [110]. During the past 15 years, advances in viral RNA detection and quantification by PCR have significantly improved their sensitivity and specificity. Both quantitative and qualitative tests are commercially available. The current version of the quantitative PCR assay has a lower limit of detection of 20 copies of viral RNA per ml of plasma and is routinely used for assessing prognosis, guiding decisions about initiation of cART, and monitoring patients after therapy has begun (see Sect. 9.3) [111, 112]. Quantitative viral RNA by PCR is also routinely used to diagnose primary HIV-1 infection because serologic methods are insufficient to detect the presence of virus during this "window" period before HIV-1 antibodies have had time to develop [98, 111, 113, 114]. Plasma viral RNA measurements during primary HIV infection are generally very high (10^4–10^7 copies/ml) prior to the appearance of antibodies [112, 114, 115].

Qualitative plasma RNA or peripheral blood mononuclear cell (PBMC) DNA detection methods using PCR are routinely used for diagnosis in infants following mother to child transmission [116–122]. In this situation antibody testing would yield false-positive results due to the presence of maternal HIV-1 antibodies passively transferred during gestation. Qualitative RNA/DNA PCR analysis is also used for blood donor screening in addition to the previously described serologic methods [123–125]. Because viral RNA or DNA assessment by PCR is associated with false-positive results (especially when copy numbers are low), all positive results should be confirmed by eventual HIV-1 seroconversion for a definitive diagnosis.

3.4.4 Markers of Immunologic Status

Both HIV-1 and HIV-2 are trophic for the CD4 cells, and perturbation of the CD4 lymphocyte count is a sine qua non of infection. Early in the AIDS epidemic before the discovery of the etiologic agent, the CD4 count was used as a surrogate for infection [126]. Since the discovery of HIV-1, the CD4 count has been widely used clinically as an intermediate marker of disease and epidemiologically as a staging tool [127, 128]. In epidemiologic studies, CD4 count has been used to estimate the duration of disease following seroconversion [129–131] and to predict AIDS onset [13]. The number of CD4 cells is also used for defining AIDS in the 1993 CDC AIDS definition [31]. Since the use of cART has become a standard practice, the CD4 count is routinely used to measure immune reconstitution during administration of the drugs. Clinical decisions regarding the discontinuation of prevention and treatment medications for opportunistic infections are also based on CD4 count measurements following cART administration.

The CD4 lymphocyte count is measured most reliably on a fluorescence-activated cell counter that uses a laser to activate chemiluminescent-tagged antibodies specific for cell surface markers such as CD4 (T helper) or CD8 (T suppressor). Because of its critical value in clinical and research settings, considerable attention has been paid to issues of quality control of these measurements [132]. Over the years, various alternative parameters related to CD4 cell numbers (e.g., the % of total lymphocyte count, logarithmic transformation) have been proposed [133], but none has been deemed to improve upon the clinical utility of the count itself enough to gain acceptance as a staging measure or as a prognostic indicator. Other immunologic markers (e.g., soluble CD4 measurements and markers of immune activation, such as neopterin, β_2-microglobulin, and interferon [89, 132, 134–136]) also appeared to have some value in predicting disease outcome; however, as with the alternatives to CD4 cell number, none has been accepted as clinically advantageous, either on its own or as an adjunct to CD4 count.

3.4.5 HIV Diagnostic Testing Recommendations

In 2006, the CDC revised their recommendations on individuals who should receive HIV testing [137]. All individuals aged 13–64 should receive an HIV test unless the patient declines testing. Patients at high risk for HIV should be tested at least once per year. Pregnant women should be tested as part of routine prenatal screening, and repeat screening is considered in certain areas with elevated rates of HIV infection among pregnant women. Ideally, screening should be part of the general consent for medical care and should not require a separate written consent. These changes were motivated by several factors including the concern about the estimated 250,000 individuals in the USA who are unaware of their HIV diagnosis, the importance of early and effective treatment, the potential reduction in behaviors associated with HIV transmission, and the possible reduction in stigma associated with HIV testing.

In the past few years, the availability of reliable rapid HIV testing procedures has increased interest in testing at the point of contact (POC) and providing results to the patient at the time of the initial evaluation. The algorithms recom-

mended by the Association of Public Health Laboratories and CDC for screening for HIV-1, for HIV-2, and for both can be found in a joint report [138]. In brief, they involve the use of combinations of standard EIA tests, Western blot assays, and supplementary nucleic acid amplification tests, with the choices depending on whether the testing is performed in a laboratory or at the POC and which of the two viruses testing is aimed at detecting.

4 Biological Characteristics of HIV

Much of the basic HIV structure and its replicatory processes were elucidated during the first 15 years after the discovery of HIV. More recent research has expanded our knowledge of the interactions of the virus with various host elements deployed with varying success to control the infection. Three classes of human retroviruses—oncornaviruses, lentiviruses, and spumaviruses—are enveloped RNA viruses. HIV-1 and HIV-2 are lentiviruses with a single-stranded RNA genome that replicates through a double-stranded DNA proviral intermediate (cDNA) (Fig. 43.4) [139, 140]. Reverse transcriptase, a viral enzyme, catalyzes the unique life cycle of these viruses by converting genomic RNA into cDNA; viral integrase incorporates cDNA into the host genome [140]. Integration of retroviruses into the human genome facilitates lifelong infection and may explain the propensity of human retroviruses to cause diseases of long latency [139].

Morphologically, HIV-1 is approximately 110 nm in diameter with a thin electron-dense outer envelope and an electron-dense, roughly cone-shaped cylindrical core (Fig. 43.5). In the production of new HIV particles, retroviral genes are expressed as large overlapping polyproteins that are later processed into functional peptide products by the viral-encoded and cellular proteases [140].

HIV-1 long terminal repeats (LTR) at the 5′ and 3′ ends of the genome contain regulatory elements that promote virion production [141]. The genomic structure of HIV-1 and HIV-2 (Fig. 43.6) contains the following genes: *gag* (group-specific antigen), whose products form the skeleton for the virion; *pol* (polymerase/integrase), whose products are involved in enzymatic functions of the virus; *env* (envelope), which codes for the external and transmembrane outer envelope

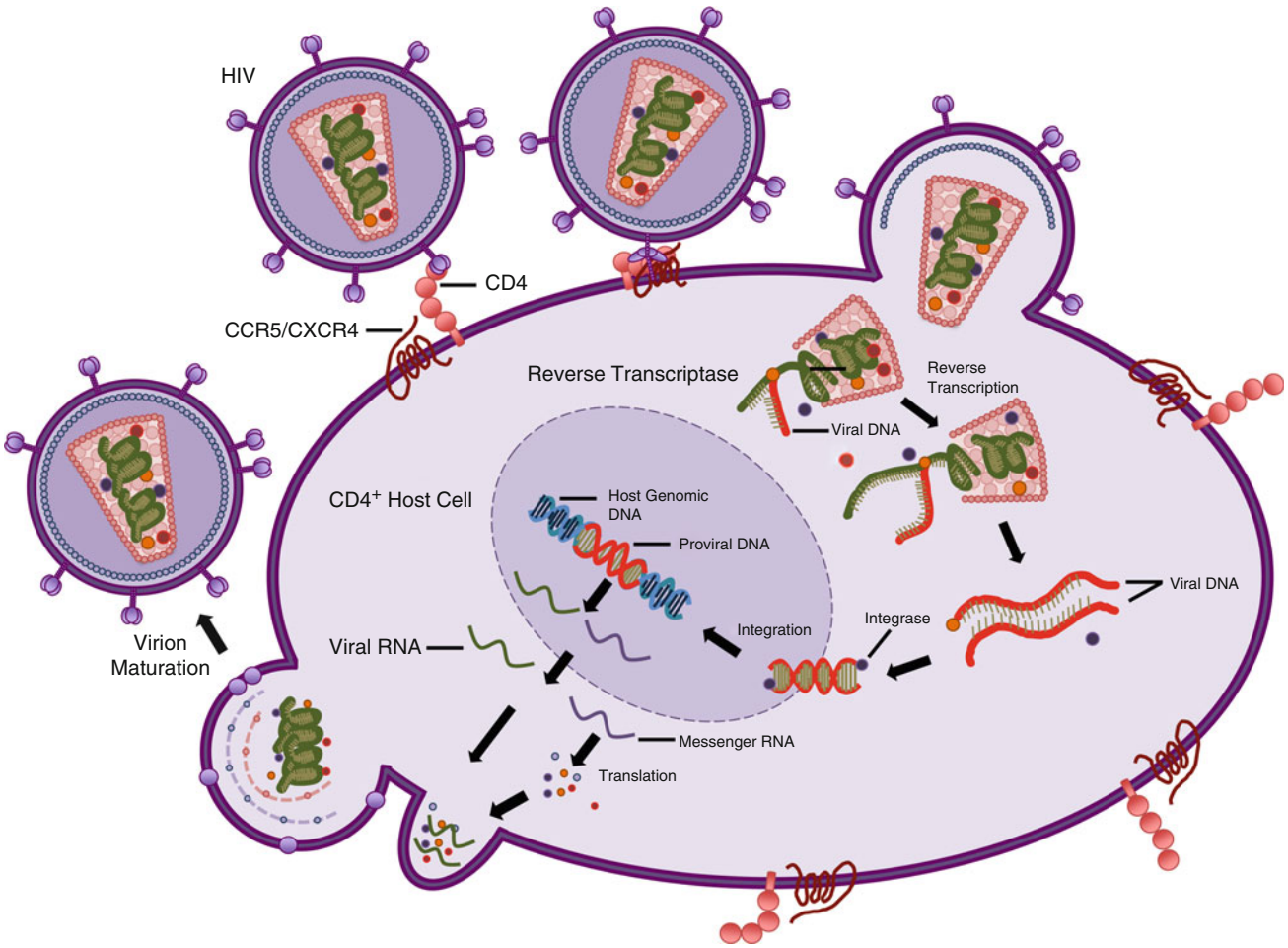


Fig. 43.4 HIV-1 replication cycle (Diagram, courtesy of Nilesh Amatya)

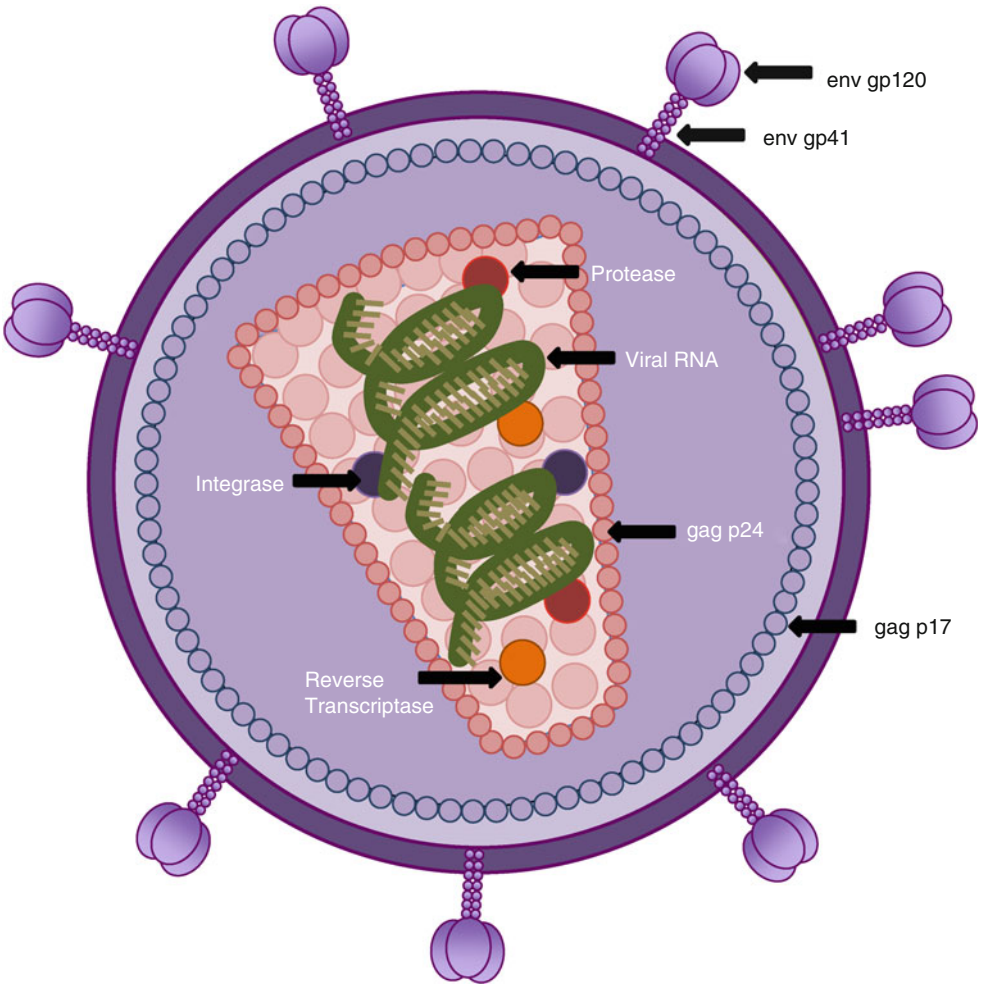


Fig. 43.5 HIV-1 virion structure (Diagram, courtesy of Nilesh Amatya)

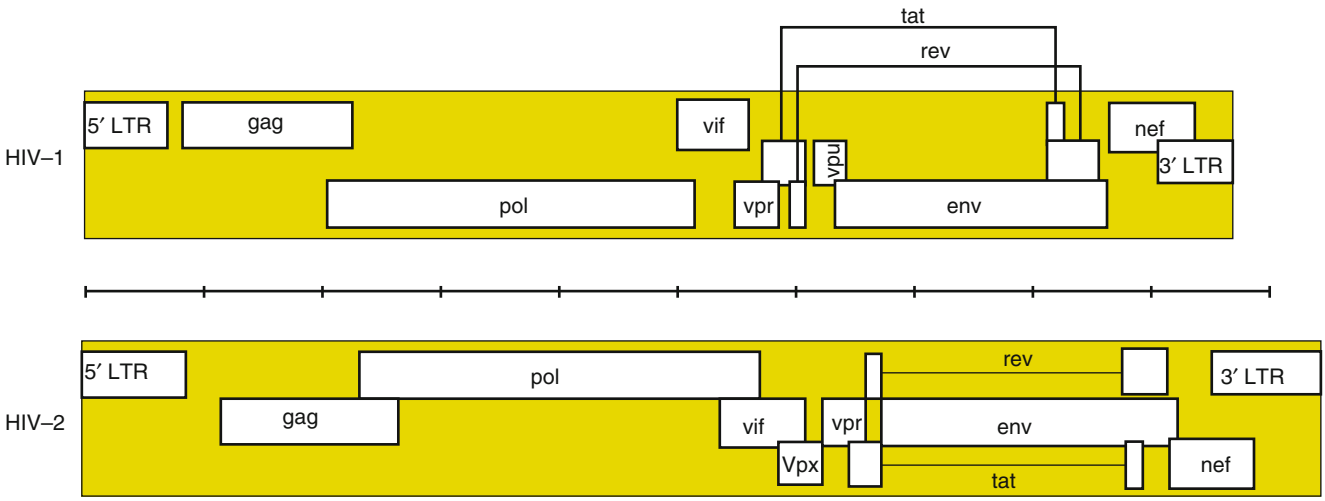


Fig. 43.6 Genomic organization of HIV-1 and HIV-2. In their approximately 10,000 base-pair sequence, HIV particles have a complex array of structural and regulatory genes: *gag* (coding for the viral capsid proteins), *pol* (coding for *reverse transcriptase*), *env* (coding for HIV's envelope-associated proteins). Regulatory genes: *tat*, *rev*, *nef*, *vif*, *vpr*, *vpu* (not in HIV-2), *vpx* (not in HIV-1) (Adapted from Ref. [142, 143])

elements involved in virus binding; and a series of accessory genes that regulate virus expression [7, 139, 144]. The *gag* gene products are synthesized from a single message, and the functional proteins are formed through enzymatic cleavage using the viral-encoded protease [145]. These structural elements are the matrix protein of 17,000 Da (p17), the capsid of 24,000 Da (p24), the nucleocapsid of 9,000 Da (p9), and the nucleic acid-binding protein of

6,000 Da (p6) [146]. On Western blot these proteins (except for p6) are strongly antigenic and appear as distinct bands with additional bands representing precursor intermediates. The *pol* gene codes for several enzymes: reverse transcriptase (involved in RNA to DNA transcription), endonuclease (ribonuclease-H), and integrase, which functions for viral integration [147]. The *env* gene encodes the major components of the viral coat: the surface glycoprotein of 120,000 Da (gp120), the transmembrane component of 41,000 Da (gp41), and the precursor called gp160 [148].

HIV-1 virion production and propagation involves a complex process controlled by genes encoding a series of multiple spliced mRNAs encoding a sequence of early and late regulatory proteins. The early regulatory genes are *tat*, *rev*, and *nef*, and the late regulatory genes are *vif* and (for HIV-1 only) *vpu*; accessory proteins are encoded by additional genes, *vpr* and (for HIV-2 only) *vpx*, which influence replication efficiency [149, 150]. HIV *tat* is responsible for enhanced transcription of viral gene products and also has a transactivating repressor function for some genes in the major histocompatibility complex [150, 151]. Free *tat* molecules released by the infected cell are able to activate uninfected CD4 T cells [152, 153]. Rev (regulator for HIV) modulates the complex transport of virion components and the translation of viral messages containing the *rev* responsive element (RRE); Rev thereby switches the production of regulatory proteins dominant early in the replication cycle to mainly structural proteins [151, 154]. Nef modulates CD4 receptor and human leukocyte antigen A and B expression and facilitates in vivo viral replication and pathogenesis [155–157]. Nef also plays a role in CD4 T-cell depletion by inducing apoptosis of uninfected bystander cells [158, 159]. Additionally, Nef activates resting T cells and has been shown to promote AIDS in transgenic mice even without the expression of any other HIV-1 gene [160, 161]. Vif prevents the inactivation of HIV-1 by the host protein, APOBEC3G, which is an innate immunity factor that resists HIV-1 through cytidine deamination of proviral DNA [162, 163]. Vpu influences virion maturation and release by antagonizing the action of tetherin, a molecule that prevents the release of virions from the infected cell [164–166]. Vpr induces cell cycle arrest in G2, enhances replication in macrophages, binds cyclophilin A, and contributes to apoptosis [167–169].

The life cycle of HIV involves an infection phase (including viral attachment, entry, reverse transcription, and proviral integration) and an expression phase (including transcription, translation, assembly, and budding of the virion) (Fig. 43.4) [140]. The initial stage of infection involves the binding of either HIV-1 or HIV-2 to cell surface structures that determine viral trophism. Many of the pathogenic effects of the virus result from immune dysfunction, which compromises the ability of the immune system to perform its myriad surveillance and regulatory processes. The HIV-1 envelope has a high-affinity binding site specific for the CD4 molecule. The CD4 molecule occurs on mature T-helper lymphocytes and on other cells of the immune system, particularly circulating and fixed tissue (dendritic) monocytes, macrophages, and microglia [86, 170–172]. The virus also attaches to other cells: follicular dendritic cells in the lymph nodes, M cells on the Peyers patches, and galactosylcerebroside-positive cells in the brain and gut.

A second co-receptor is also necessary for viral infection, and for this purpose HIV-1 uses either of the two chemokine receptors, C-C chemokine receptor 5 (CCR5) or C-X-C chemokine receptor 4 (CXCR4) [173–175]. Both receptors are important for chemotactic activity in lymphocytes and are generally expressed on cells that also express CD4. HIV-1 viral isolates vary in their use of the co-receptor, but the majority of viral strains establishing acute infection use CCR5, while a greater proportion of viral isolates from chronically infected individuals use CXCR4 [176, 177]. Although most HIV-1 isolates bind to one or the other co-receptor, so-called dual tropic viruses can utilize both [178].

Following attachment, a fusogenic domain on the gp41 appears to be involved in the uncoating and release of virion genetic material into the cell. The viral reverse transcriptase synthesizes a cDNA copy of the virus that is transported to the nucleus; viral integrase is then involved in proviral integration. Integration of HIV-1 appears to be random but may be influenced by host factors [179]. Once integrated into the host chromosome, the virus is replicated like a cellular gene and incorporated into the progeny cells. The proviral genome is the template for new virus production, and the expression of new virus production is controlled by viral and host cellular regulatory elements. The first elements produced are the regulatory elements, which then modulate the production of genomic RNA and mRNA necessary for production of virion structural proteins. The process is orchestrated by the regulatory elements discussed above, with the *rev* protein playing a key role in the switch to virion production.

The morphology, life cycle, and genomic structure of HIV-2 are quite similar to that of the HIV-1, but the regulatory genes of HIV-2 differ from those of HIV-1: in the former *vpx* appears in place of *vpu*, as noted above. The *env* gene encodes a gp120 membrane and a gp32–40 transmembrane glycoproteins. The *gag* gene encodes a p55 precursor to p24–26 and p15. The *pol* gene products include the p64 and the p53 of reverse transcriptase, p34 of integrase, and p11 of protease [180].

5 Descriptive Epidemiology of HIV/AIDS

HIV-1 has propagated from one risk group to another in different places and times, evolving into a pandemic with myriad patterns of occurrence, transmission, and modulating influences that stretch beyond the scope of this section. The statistics

in this section very briefly depict the force of mortality, morbidity, and infection caused by the virus. Where differences seem informative, the picture is presented first in global terms; then in the more developed world as represented by the USA and other countries in North America, Europe, or Australia; and then in regions lacking robust epidemiologic capabilities, most notably southern Africa or Southeast and East Asia. The section next addresses the contagiousness of the infection and the distinctive modes and patterns of its transmission. Finally, it summarizes the various behavioral and biological factors believed to modify the rate of disease progression. Except for host genetic factors, several of which have been implicated in acquisition of HIV-1 infection as well as disease progression, factors that more directly alter the risk of transmission or acquisition of infection are reviewed in Sect. 6.

5.1 Mortality

5.1.1 Global

The series of illustrations below provide the most current mortality estimates for the pandemic. The estimated annual numbers of deaths from AIDS peaked during the first few years of the new millennium and declined steadily since then to 1.7 million in 2011 (Table 43.3). An estimated 86 % of deaths were in adults.

5.1.2 USA and Other Regions of the Developed World

Rates of mortality due to AIDS in the USA climbed steadily during the first decade of the epidemic and peaked in the early 1990s. By 2010 it is estimated that 619,000 persons had died of AIDS since the epidemic began. In 1992 HIV-related conditions were the leading cause of death in men between 25 and 44 years of age and the fourth leading cause in women of that age (Fig. 43.7). Their place among the most frequent causes of death then steadily declined after the introduction of HAART, and by 2010, AIDS was the seventh and tenth leading cause in 25–44-year-old men and women, respectively. In 2011 only 28,000 deaths occurred in all of North America and Western and Central Europe (Table 43.3).

After 1996 striking changes began to occur in most places where HAART became available. In one analysis of nearly 10,000 patients seen in 70 European centers, in just the 8-year interval (1994–2002) spanning the pre-, early post-, and later post-HAART periods, deaths among the most immunodeficient declined by half from the early to the later HAART period [181]. A similar conclusion was drawn by the HIV-CAUSAL Collaboration in a recent analysis of nearly 63,000 individuals with HIV-1 infection who were followed for an average of 3.3 years with or without treatment. Compared with untreated patients, those who initiated combined ART

Table 43.3 Regional HIV and AIDS statistics and features 2012

	Adults and children living with HIV	Adults and children newly Infected with HIV	Adult prevalence (15–49) [%]	Adult and child deaths due to AIDS
Sub-Saharan Africa	25.0 million	1.6 million	4.7 %	1.2 million
	[23.5–26.6 million]	[1.4–1.8 million]	[4.4–5.0 %]	[1.1–1.3 million]
Middle East and North Africa	260,000	32,000	0.1 %	17,000
	[200,000–380,000]	[22,000–47,000]	[0.1–0.2 %]	[12,000–26,000]
South and South-East Asia	3.9 million	270,000	0.3 %	220,000
	[2.9–5.2 million]	[160,000–440,000]	[0.2–0.4 %]	[190,000–340,000]
East Asia	880,000	81,000	0.1 %	41,000
	[650,000–1.2 million]	[34,000–150,000]	[<0.1–0.1 %]	[25,000–64,000]
Latin America	1.5 million	86,000	0.4 %	52,000
	[1.2–1.9 million]	[57,000–150,000]	[0.3–0.5 %]	[32,000–81,000]
Caribbean	250,000	12,000	1.0 %	11,000
	[220,000–280,000]	[9,400–14,000]	[0.9–1.1 %]	[9,400–14,000]
Eastern Europe and Central Asia	1.3 million	130,000	0.7 %	91,000
	[1.0–1.7 million]	[89,000–190,000]	[0.6–1.0 %]	[66,000–120,000]
Western and Central Europe	860,000	29,000	0.2 %	7,600
	[800,000–930,000]	[25,000–35,000]	[0.2–0.3 %]	[6,900–8,300]
North America	1.3 million	48,000	0.5 %	20,000
	[980,000–1.9 million]	[15,000–100,000]	[0.4–0.8 %]	[16,000–27,000]
Oceania	53,000	2,100	0.2 %	1,200
	[43,000–59,000]	[1,500–2,700]	[0.2–0.3 %]	[<1,000–1,800]
Total	35.3 million	2.3 million	0.8 %	1.6 million
	[32.2–38.8 million]	[1.9–2.7 million]	[0.7–0.9 %]	[1.4–1.9 million]

Source: UNAIDS Global Report, 2012
The ranges around the estimates in this table define the boundaries within which the actual numbers lies, best available information

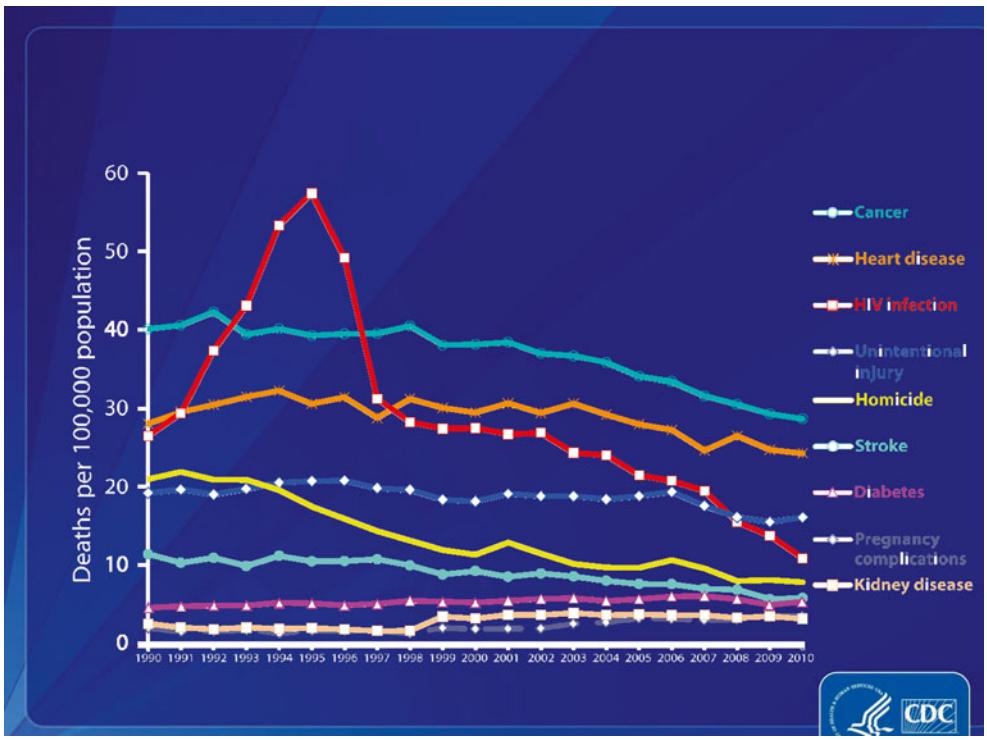


Fig. 43.7 Trends in annual rates of deaths due to the 9 leading causes among persons 25–44 years old, USA, 1987–2010. Data were compiled from death certificates from all 50 states and the District of Columbia by the National Center for Health Statistics (NCHS) (Reproduced from Centers for Disease Control and Prevention, http://www.cdc.gov/hiv/pdf/statistics_surveillance_HIV_mortality.pdf)

had a 0.48 mortality hazard ratio, with those whose prognosis was worse at the beginning of the follow-up period showing the greater reduction in mortality [182].

Even where treatment is readily accessible, however, it has not had uniformly dramatic results or an equal impact in reducing deaths from all of the AIDS-defining conditions. For example, a study of 30,000 European and North American patients treated with ART reported that the adjusted hazard ratio for death from high-mortality conditions like non-Hodgkin's lymphoma and progressive multifocal leukoencephalopathy (7.3) is higher than for such complications as cryptococcosis, cerebral toxoplasmosis, AIDS dementia complex, and disseminated *Mycobacterium avium* complex (2.4) and other milder conditions (1.5) [183].

5.1.3 Regions of the Developing World

Of the 1.7 million deaths from AIDS worldwide in 2011, approximately 70 % occurred in sub-Saharan Africa, and another 15 % in South and Southeast Asia (Table 43.3). However, programs for distributing effective and affordable treatment have had significant impact on mortality in many of the poorer countries of the world, and in half-dozen years between 2005 and 2011, gains in those places have been almost as impressive as those where far greater resources have been committed [56].

In sub-Saharan Africa deaths dropped 32 % overall and 50–70 % in various other African countries. Cambodia and Thailand experienced 77 and 49 % reductions in mortality, respectively. One of the most frequent causes of death in HIV-1-infected persons in many poorer parts of the world is tuberculosis (TB), and the expansion of treatment for HIV-1 during the past few years has led to a reduction in annual AIDS-related TB deaths from 600,000 to 450,000 (25 %). As encouraging as these gains are, they represent relative reductions from very high mortality rates, and reaching levels comparable to those achievable under optimal treatment conditions will require sustained aggressive effort.

5.2 Morbidity

5.2.1 Acute Retroviral Syndrome

The initial weeks of infection are frequently characterized by an acute syndrome, which in US and other populations in the developed world closely resembles that of the Epstein–Barr virus-induced mononucleosis (see Sect. 8) [184]. Data on the overall frequency of this syndrome have not been systematically collected in large populations of prospectively identified seroconverters. However, only a minority of seroconverting individuals experience these acute symptoms, and it may differ by host characteristics in different settings [185]. The syndrome may be associated with higher levels of viremia, but neither the mode of transmission nor various

other factors examined appear to explain why or when the syndrome is likely to occur.

5.2.2 Pre-AIDS

Beginning a few months after the acute seroconversion phase, the vast majority of recent HIV-1 infections quiesce for months or years before classic AIDS-defining conditions develop. During that long variable period, an intermediate stage may be punctuated with one or more minor conditions (e.g., localized zoster, hairy leukoplakia, and other dermatologic lesions) (Table 43.1), but the frequency, patterns of occurrence, and determinants of those conditions have not been well studied.

5.2.3 AIDS

From the time the outbreak began and for the years prior to the introduction of HAART, the primary metric of morbidity and mortality was the annual or cumulative number of AIDS cases—OIs and cancers in the absence of known causes of natural or iatrogenic immunodeficiency. However, ironically, as a result of the huge impact of treatment, in recent years in the USA, simple total or annual numbers of AIDS cases are now seldom reported as such. Rather, disease is staged, and according to CDC figures for 2010, the number of persons with stage 3 disease (AIDS) at the time of diagnosis of HIV-1 infection (11,898) represented 27.7 % of all persons with newly diagnosed HIV infection (42,901) [186]. The reason for reporting the data in this form is that a concerted effort to reduce the number of infections diagnosed at this late stage is a goal of the 2010 National HIV/AIDS Strategy for the United States (NHAS) (see Sect. 9.1).

Data on the individual AIDS-defining conditions are even more difficult to find and interpret. Their frequencies have varied according the place, time, risk group, and other features, as well as the diagnostic capabilities in the specific populations under surveillance. For example, from the inception of the epidemic, the striking excess of Kaposi's sarcoma before the advent of HAART was inexplicably confined almost exclusively to MSM. In contrast, IDUs were more frequently affected by bacterial and other agents known to contaminate drug injection paraphernalia. Other pathogen distributions have usually mirrored their distinctive prevalence in different geographic areas. Thus, in a review of multiple observational studies and trials conducted in southern Africa just before the advent of treatment, OIs included not only those seen commonly in the populations of North America, Europe, and Australia but also those more common with immunodeficiency in less developed areas: *Streptococcus pneumoniae*, *Salmonella* species, *Cryptococcus neoformans*, *Cryptosporidium parvum*, *Microsporidium* species, and *Isospora belli* [187].

As with mortality, in the past 15 years, the incidence of these late complications has steadily diminished. In the developed world, the OIs and AIDS-defining cancers that were formerly frequent occurrences in the severely immunodeficient have declined sharply; among carefully treated individuals, some have almost disappeared. Still, current overall morbidity due to HIV-1 is by no means negligible. For instance, rates of hospitalization during the HAART era in Australia were 50–300 % higher in a cohort of treated individuals who were comparable in age and sex to the general population, and the rates were higher for those who were older, had been infected longer, or had been treated with more antiretroviral drugs [188].

5.2.4 Non-AIDS-Defining Comorbidity

Among those HIV-1-infected people living longer as a result of treatment, such non-AIDS diseases as other neoplasms (skin, anus, liver, etc.) appeared to occur at incidence rates above background [189]. However, two other major chronic infectious diseases (tuberculosis and hepatitis C) have flourished disproportionally where prevalence of coinfection with these agents is high. In 2011, of the estimated 8.7 million people who developed TB worldwide, people living with HIV-1 accounted for 1.1 million (13 %); nearly one-quarter of people with TB who were tested were positive for HIV-1 [190].

Comorbidity with HIV-1 and hepatitis C virus (HCV) coinfection is also significant. Some four to five million persons worldwide are infected with both viruses; the prevalence of HCV is 72–95 % in IDUs, 1–12 % in MSM, and 9–27 % in heterosexuals who are HIV-1 positive in the USA and Western Europe [191]. A meta-analysis covering 30 studies and more than 100,000 patients coinfected with HCV documented increased overall mortality, but from non-AIDS-defining conditions rather than AIDS itself [192]. A number of studies of dually infected patients have shown 2–3-fold higher rates of progression to chronic hepatitis, severe fibrosis, cirrhosis, or death in IDUs and other groups compared with HCV-monoinfected patients [193].

5.3 Prevalence

5.3.1 Global

As noted earlier, uneven surveillance efforts as well as actual variation in the pattern of occurrence of HIV-1 infection from place to place account for substantial differences in reported prevalence around the world. Data collected and assembled relatively uniformly by the United Nations between 1990 and 2012 document a steady rise in prevalence until about 2000, an inflection in the trajectory, followed by

a slower rise. By 2012 the number of adults and children totaled 35 million, broken down by continent and major subdivision in Fig. 43.8.

Sub-Saharan Africa again accounts for about 70 % of the total, and South and Southeast Asia for about 12 %. Of course, these figures include infection present at any stage in living individuals, treated or untreated.

5.3.2 USA

Based on back calculation from the latest available state- and area-level HIV-1 and AIDS data [186], in the USA at the end of 2009, an estimated 1,148,200 persons aged 13 and older were living with HIV-1 infection. That figure represented approximately 0.45 % of the adolescent and adult population, with clear heterogeneity by age, sex, and race. Overall about 18 % of these infections are estimated to remain undiagnosed. In Western and Central European countries, the overall 0.2 % prevalence is lower than in the USA.

5.3.3 Regions of the Developing World

Eastern Europe and Central Asia experienced more rapid increases in prevalence in the 1990s, with the most recent overall figure reaching 0.8 % [42]. Elsewhere the figures range from 0.1 % in East Asia to 1.0 % in the Caribbean; however, prevalence remains exceptionally high (4.9 %) in sub-Saharan Africa.

5.4 Incidence

5.4.1 Global

Worldwide, 2.3 million infections (7 % of the total) are estimated to be new or incident (Fig. 43.9). This aggregate figure belies the striking heterogeneity by age, sex, race, geography, risk category, and other factors whose roles are examined in greater detail below. Encouragingly, estimates from UNAIDS have shown a steady decline in global incidence rates from a peak of about three million per year in the mid-1990s to about 2.5 million in 2012 [56].

5.4.2 USA and Other Developed Regions

Incidence estimates have been based on increasingly more sophisticated back calculation coupled with other estimation techniques described in Sect. 3. Estimates have been extrapolated from a study of 6,864 diagnostic specimens taken in 2006 [76]. In 2133 (31 %) results were indicative of recent infection. These data translate to 56,300 new infections or an incidence rate of 22.8 per 100,000 in the entire USA in that year. The incidence was compared closely with incidence estimates for 2003–2006 based on back calculation from HIV diagnoses and AIDS incidence. More than half were among men who have sex with men, and nearly half were among black individuals. The overall incidence increased in the mid-1990s, declined slightly after 1999, then stabilized.

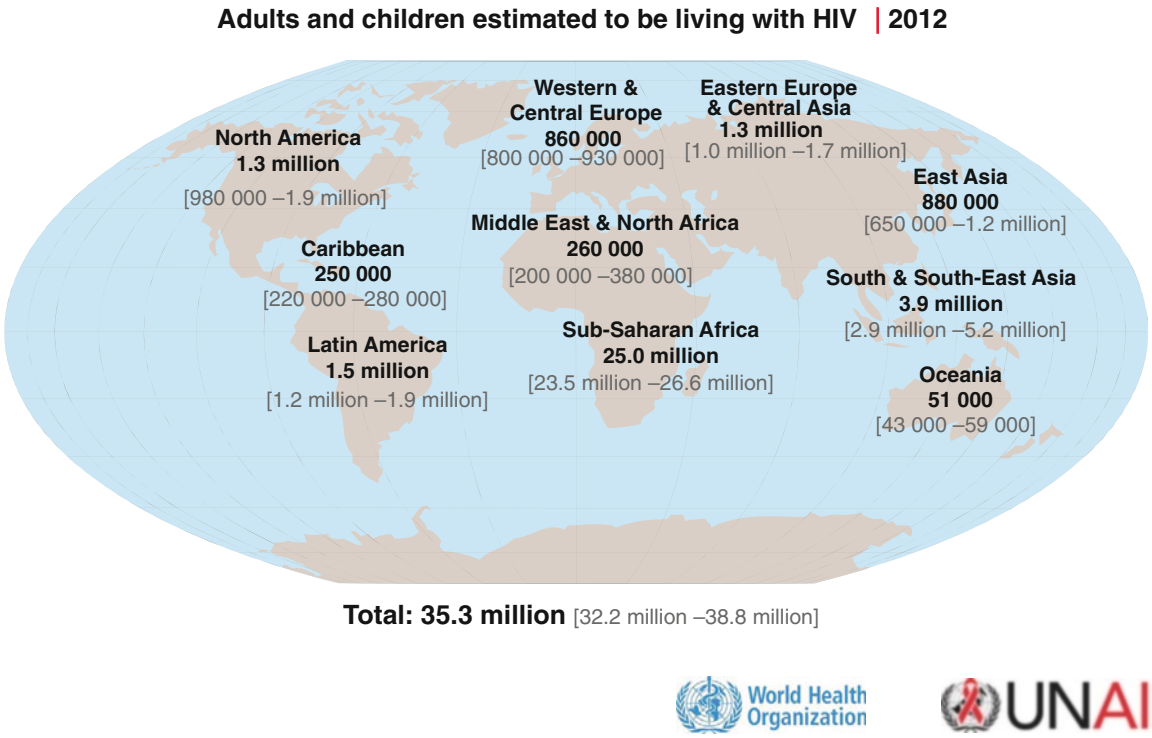


Fig. 43.8 Adults and children estimated to be living with AIDS, 2012 {UNAIDS, September 2013}, http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2013/gr2013/201309_epi_core_en.pdf

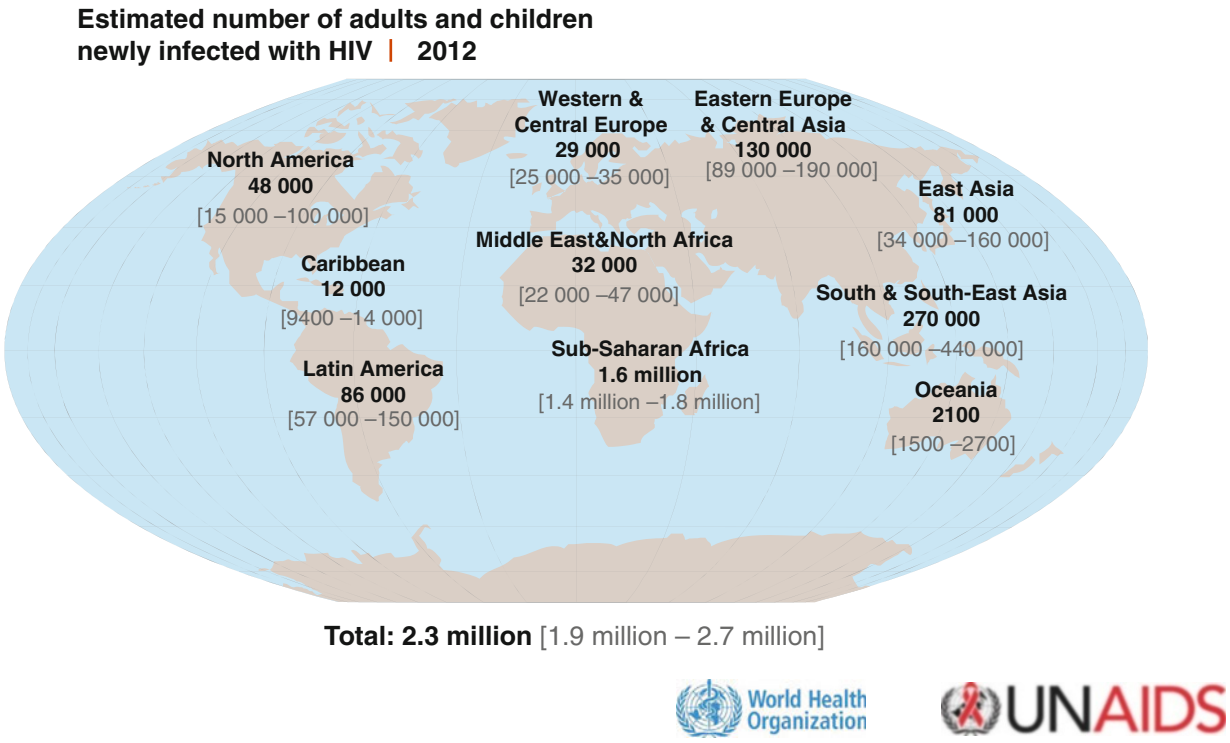


Fig. 43.9 Estimated number of adults and children newly infected with HIV, 2012 {UNAIDS, September 2013}, http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2013/gr2013/201309_epi_core_en.pdf

National HIV Surveillance System figures have confirmed the overall stability in incidence rates after 2006, but they have also reemphasized the concern about disproportional increases among young MSM in general (accounting for 61 % of new infections) and young African–American MSM in particular, who accounted for one-quarter of all new infections in 2010 (Figs. 43.10 and 43.11) [196].

5.5 Epidemic Behavior and Contagiousness

In the 30 years since HIV-1 was first discovered, one of its most prominent characteristics has been its capacity for epidemic spread. However, to summarize the nature and extent of this epidemic behavior would be to recapitulate the Introduction, Historical Background, and much of the material in Sect. 6. The focus here is on contagiousness, which despite the relatively low intrinsic capability of the virus has proved high enough to enable it to propagate from person to person in epidemic fashion.

HIV-1 replicates efficiently in certain human lymphoid cells and far less so in most other human cell types. Contagiousness depends mostly on the quantity and quality of free virus passing from those infected cells into the bodily fluids and tissues that can, in turn, make direct physical contact with the appropriate mucosal surface or blood of a new susceptible host.

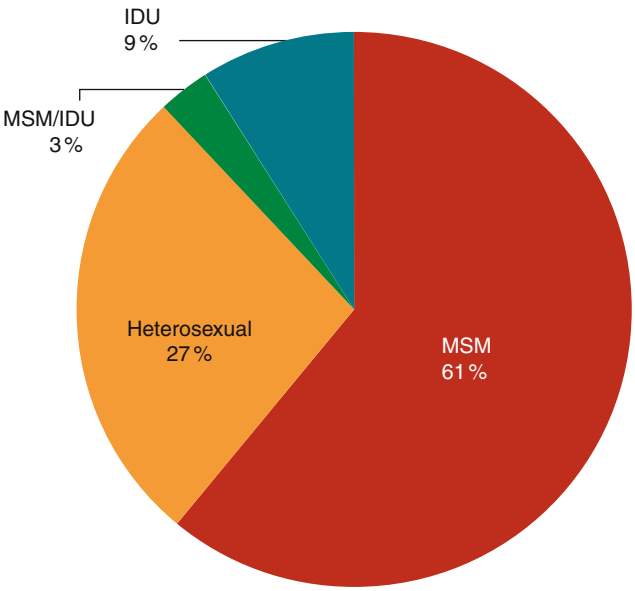


Fig. 43.10 Estimates of new HIV infections in the USA, 2009, by transmission category (Reproduced from Ref. [194] as adapted from Prejean et al. [195])

The likelihood of that sequence of events is determined by both the transmissibility of the virus from the infected host and the susceptibility of the uninfected host. The level of virus in the plasma or genital secretions of the infected host appears to be the strongest determinant of transmissibility.

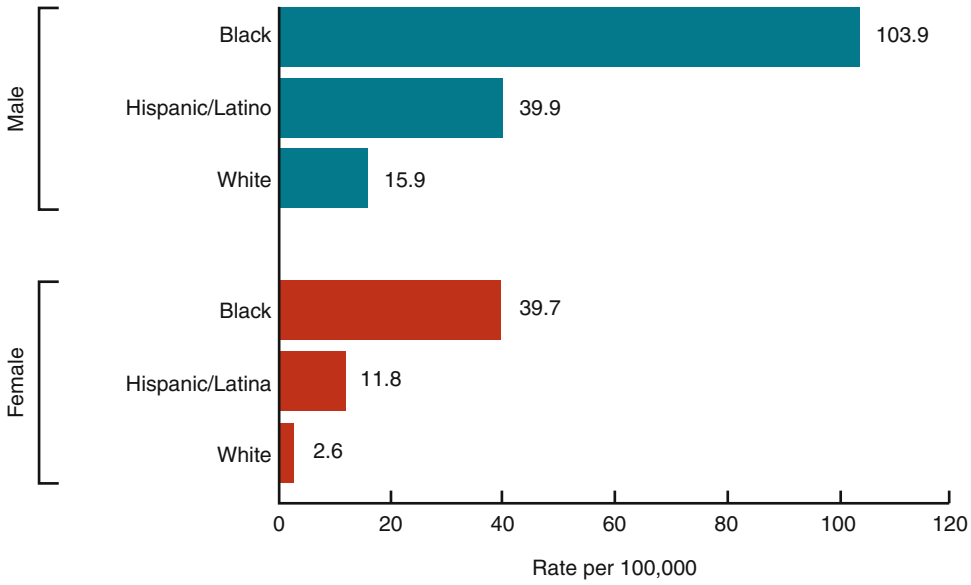


Fig. 43.11 Estimated Rate of New HIV Infections, 2009, by Gender and Race/Ethnicity (Reproduced from Ref. [194] as adapted from Prejean et al. [195])

Table 43.4 Estimates of infectivity due to sexual and parenteral exposure

Transmission probability from a single contact with an infected partner	
Heterosexual transmission	
Male to female (California Partner Study)	0.001–0.03
Male to female (Transfusion Partner Study)	0.001–0.189
Female to male (STD patients, Kenya)	0.082
Female to male (Thailand military recruits)	0.025–0.075
Homosexual transmission	0.008–0.032
Per partnership transmission probability from a relationship with an infected partner	
Heterosexual transmission	
Male to female	0.10, 0.18, 0.20, 0.22, 0.27, 0.28
Female to male	0.01–0.12
Homosexual transmission	
San Francisco Men's Health Study	0.10
Transmission probability from single contact in nonsexual setting	
Blood transfusion	0.60–0.72
Needle stick, health-care worker	0.0046–0.009
Laboratory worker	0.0048–0.023
Perinatal transmission	0.12–0.30

Adapted from Brookmeyer and Gail [200]

Other modifying characteristics of the source virus are its lack of ability to induce syncytium formation and its trophism for cells of the monocyte–macrophage lineage [197]. Contagiousness may also be higher at the earliest and latest stages of the disease when viral load (VL) tends to be higher and when the number and duration of partnerships at the time of acute infection is greater [198, 199]. As for susceptibility to HIV-1 infection, in addition to source virus characteristics, a number of host factors have been well documented to increase or reduce the probability of acquiring infection, while other associations remain unproved. They are described in the broader context of cofactors for transmission (Sect. 6). The remainder of this section summarizes the approaches that have been taken to estimating contagiousness, based on transmission rates irrespective of viral and host determinants.

Early partner and cohort studies attempted to quantify the contagiousness of HIV-1 from a single sexual or parenteral exposure (Table 43.4) before the advent of ART. Estimates of the frequency of transmission following a single sexual or parenteral exposure depend on such variables as the type of exposure, the gender of the infected partner, and the viral and host factors described elsewhere.

High probabilities of infection occurred with transfusion, where follow-back studies indicated that two-thirds or more of recipients who received a positive unit became seropositive [201–203]. In one study, 200,000 screened samples

yielded 124 transfusion recipients with no other risk factor and 111 (89 %) were seropositive [202]. HIV-1 can be efficiently transmitted in whole blood, lymphocytes, packed red cells, platelets, and plasma. None of the products purified from donated blood (e.g., albumin or immune globulin) have proved infectious for HIV-1.

Transmission from HIV-1-positive mothers to their infants occurred in approximately 10–30 % of their pregnancies in the absence of prophylactic intervention [12, 204].

The estimated risk of acquiring HIV-1 as an IDU is about 7 per 1,000 per event, as reported earlier in the epidemic and in a recent study in Thailand [205]. HIV-1 transmission between IDUs occurs by injection with a needle that is contaminated by residual virus as a consequence of sharing contaminated injection equipment [206]. In many cross-sectional studies, duration of drug use and older age are the predominant risk factors, probably reflecting cumulative risk over time [207–210]. In incidence-based studies, in contrast, younger age appears to predispose to HIV-1 infection, possibly because the risk for acquiring new infection may be highest at the onset of drug use when experimentation with new networks may occur and patterns of self-protective behaviors, such as accessing equipment exchange programs, have not yet formed [206]. The risk for infection from a single injection will vary not only by individual injection practices but also by the average HIV-1 viral load in the community network of needle/syringe users. The per-contact risk is obviously high in settings such as "shooting galleries," where exposure to contaminated equipment is likely. Individual practices may further amplify the likelihood of exposure to higher levels of HIV viral particles. Repeated flushing of the syringe ("booting") and other practices also increase the risk by increasing the quantity of blood exchanged between individuals. Although once promoted as decreasing risk, bleach treatment for disinfecting injection equipment has not been fully effective in prevention HIV transmission [211, 212].

Sexual transmission of HIV-1 may also be an important independent source of HIV-1 infection among drug users. In one prospective analysis, subjects with a history of sexual exposure to an HIV-1-infected partner had a fourfold increased risk of HIV infection, independent of needle or syringe sharing [206]. Similar to other studies of heterosexual transmission of HIV, this risk was higher for women than for men.

In the early work on sexual transmission, although risks from sexual contact overall were comparatively low, the risk per contact of male-to-female transmission was similar to the risk for male to male but higher than for female to male. Based on more recent direct observations in prospective studies of untreated HIV-1-discordant couples in Africa, heterosexual transmission in either direction on a per single contact or per act basis now appears closer to the lower ends of those shown in Table 43.4 [213]. MTF and FTM risks unadjusted for age, prior infection with HSV-2, or source partner VL were 0.0019 and 0.001, respectively, but converged to a relative risk of nearly unity after adjustment. As noted above, lower VL in women explained much of their apparently lower transmissibility. Other comparable studies have differed in their estimates, possibly due in part to inclusion or exclusion of virologically linked seroconverting partners [198, 214, 215].

Those with needle-stick injuries or accidents in clinical settings or in laboratories where exposure was known to occur have had lower risks, similar to those following sexual contact (see Sect. 6) [216]. As for casual skin or mucous membrane exposure, the risk has been estimated to be extremely low [217].

Because most of the transmission has involved direct contact, the epidemic has often tended to propagate primarily within and more haphazardly between groups. For example, female sex workers in urban areas of Africa and Asia represented core transmission groups who passed the virus to customers such as itinerant laborers or truck drivers traveling a distance from their rural homes. High prevalence in the sex workers meant high rates of transmission to their male customers and, in turn, high levels of secondary spread when those men returned to their spouses and other sexual partners. In Southeast Asia and India, injection drug use and sex work combined to augment secondary spread into the community. In Thailand parallel epidemics were produced by two distinct viral subtypes, a clade B-related virus spread largely among IDUs in Bangkok [218, 219] and a clade E virus spread heterosexually among a network of female sex workers, their male military clients, and the sexual partners of those men.

The growing body of information about the virus and host characteristics influencing transmission has stimulated a number of efforts to construct models of HIV-1 contagiousness and individual evolving epidemic. One example, a multi-compartmental model (Fig. 43.12) depicts the interrelationships of the compartmentalized risk groups and the likelihood of sexual and parenteral exposure. Other more theoretical and quantitative models ultimately rely on the rather sparse data and wide-ranging parameter estimates for each of the included variables.

As details of viral dynamics have become clearer, so has a more empirically based insight into the contagiousness and evolution of microepidemics in the main risk groups. For example, in a study of the epidemic among heterosexuals in the United Kingdom, sequencing of regions of the viral protease and reverse transcriptase in a large number of non-subtype B viruses isolated from more than 11,000 individuals, phylogenetic analyses revealed a much lower degree of clustering of heterosexuals than previously estimated for MSM [220]. These analyses also suggested a longer maximum transmission interval for heterosexuals (median—27 months) than for MSM (median—14 months). Likewise in an outbreak in IDUs in Sweden in 2006, investigators capitalized on

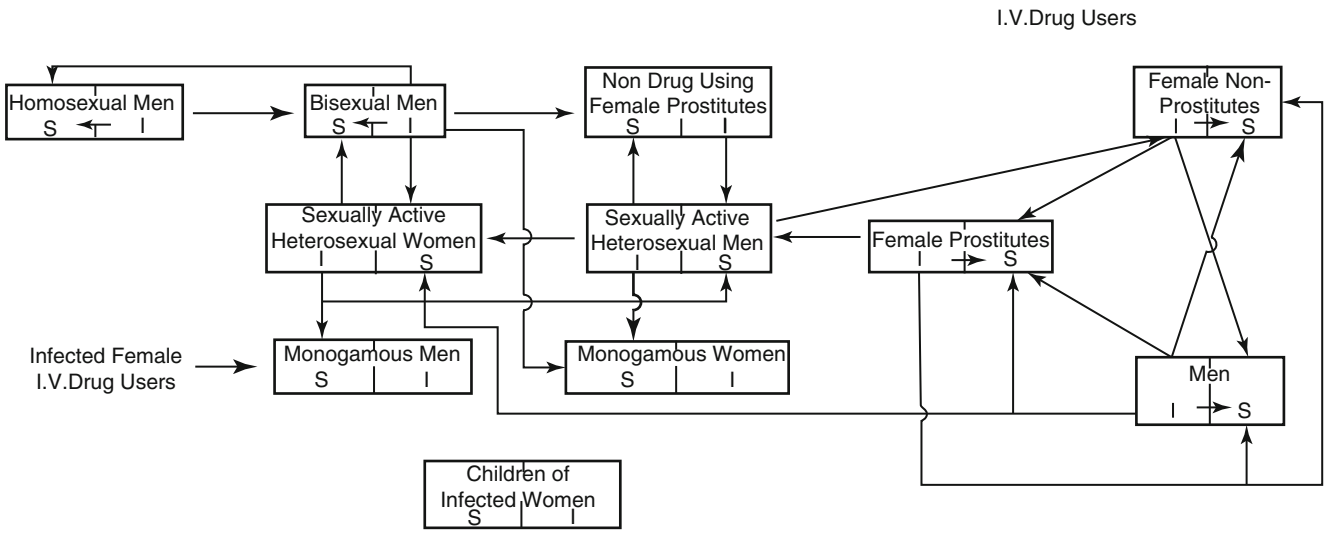


Fig. 43.12 Compartmental model for spread of AIDS (Reproduced with permission from Brookmeyer and Gail [200])

epidemiologic and phylogenetic data on the highly variable sequence of the gene for the V3 domain of the HIV-1 envelope [221]. The new viral strain, imported into Sweden from Finland, did not appear more virulent or more transmissible than the form already circulating; rather the work suggested that the availability of a network of uninfected IDUs led to an abrupt 12-fold increased incidence once the new virus was introduced. Other approaches to modeling of the contagiousness and transmission of HIV-1 are summarized and assessed elsewhere [222]. Finally, effective and especially early treatment of infected individuals has begun to show impressive effects on viral transmissibility, as described in Sect. 9.

5.6 Geographic Distribution

The broad patterns of occurrence throughout the world have been addressed in Sect. 5.1. covering the magnitude of the current pandemic. As apparent from that summary, the occurrence in any particular place has usually been most strongly determined by its peculiar mix of high-risk subpopulations. At the global level, the disproportional intensity of HIV-1 infection and disease at the epicenter in sub-Saharan Africa cannot be overstated. But peculiarities of the southern African geography do not explain this phenomenon. Rather, it appears to have been the combination of multiple sexual partnerships and practices coupled with ineffective early responses to the impending epidemic that fueled its rapid expansion from one place to the next. In comparison, the concentration of commercial sex workers, risk-prone heterosexual men, and IDUs in Thailand and other parts of Southeast Asia in the early 1990s led to projections that region would soon surpass all others in the world in incidence. Indeed, the numbers of new infections in Thailand were then increasing steeply. However, concerted efforts to reverse that rise began to pay off soon thereafter, and although the incidence rate did not decline as sharply as it rose, an epidemic equal to or worse than the one in South Africa did not materialize.

At the other extreme, areas reporting the lowest prevalence estimates have usually been predictable based on their sociobehavioral characteristics. For example, although data from the Middle East and North Africa are not equally reliable for every nation, the burden of HIV-1 infection in those regions is not nearly as onerous as in others (Fig. 43.8), ostensibly because the high-risk activities leading to rapid spread of infection elsewhere have been less common in the more traditionally conservative cultural and religious environments.

Even within a single country or city, variations in behavior have often accounted for large differences among its subpopulations. The epidemic in the USA began in east and west coast cities and quickly spread to other metropolitan areas in the USA, while it simultaneously disseminated in the cities of Western Europe, reflecting the concentrations of MSM. However, the geographic distribution has evolved considerably in the ensuing years, and HIV-1 infection shows similar concentration in many parts of the southeastern USA (Fig. 43.13), reflecting more intense spread across the major transmission risk groups within the African–American community.

HIV-1 in IDUs and commercial sex workers in the USA initially followed the same coastal pattern, but elsewhere in the world, the broader distribution of those risk groups and their connections with each other and with other risk groups in many locations facilitated global spread of HIV-1 among them. As an especially compelling example of how personal behavior has been the primary determinant of the geography of HIV-1 infection, prevalence of infection in numerous places around the world rose rapidly in locations along routes

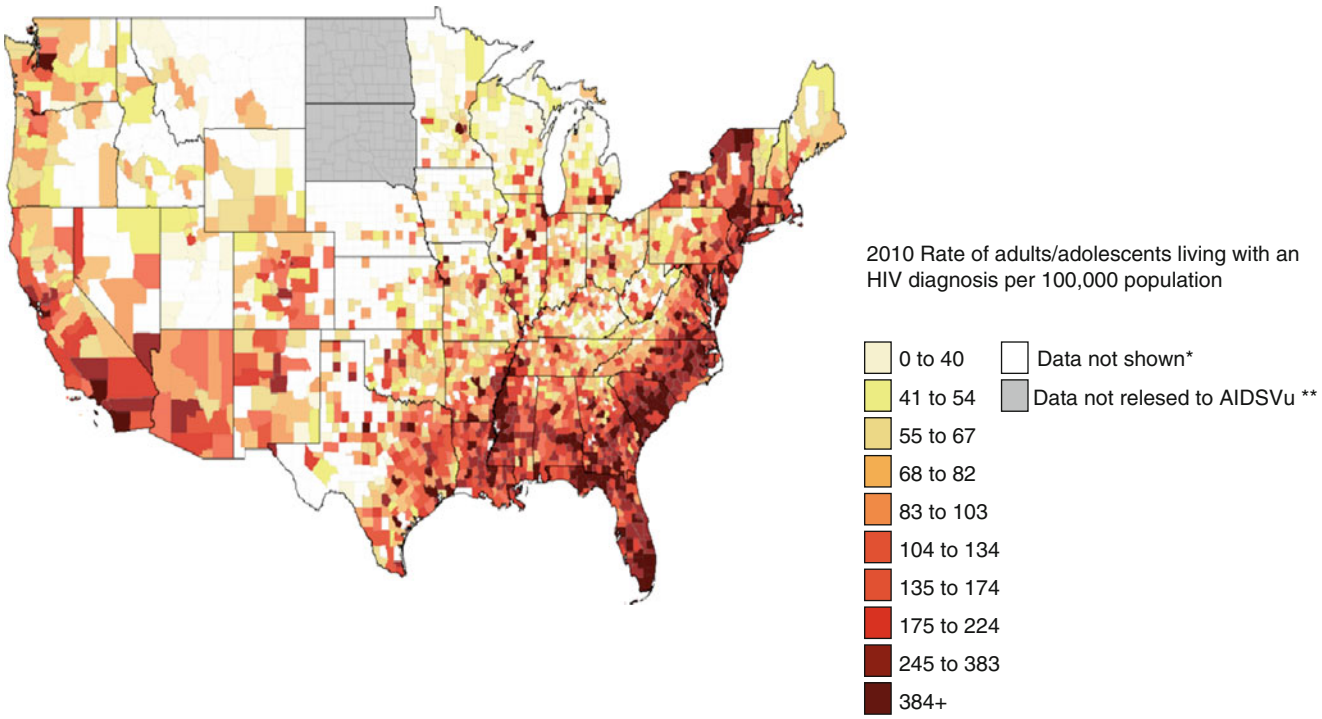


Fig. 43.13 Rates of persons living with an HIV diagnosis, by county, 2010 (Reproduced from Source: AIDSVu [223]; #1798}). This map shows the estimated county-level rates (per 100,000 population) of adults and adolescents living with an HIV diagnosis at the end of 2010. Data include adults and adolescents living with a diagnosis of HIV infection, regardless of the stage of the disease at diagnosis, and have been statistically adjusted to account for reporting delays and missing risk-factor information, but not for incomplete reporting. All displayed data are estimates based upon actual data reported to CDC through June 2012. Persons living with an HIV diagnosis are classified as adult or adolescent based on age at end of 2010

where long-distance truck drivers traveled and where other migrant workers congregated; this distribution was soon accompanied by secondary spread to the rural areas when these workers, upon returning home, infected their spouses and other sexual partners.

5.7 Temporal Distribution

Unlike many acute viral infections, HIV-1 does not display predictable seasonality, nor has it sporadically surged and subsided in the form of an epidemic of weeks or months. Unlike other chronic viral infections and their sequelae, HIV/AIDS did not become endemic throughout much of the world long before it was discovered. Rather, HIV-1 infection has proved unique in its temporality in that, except for perhaps a few dozen occurrences during the several decades before its discovery, it produced a pandemic that evolved almost entirely in plain view of clinicians, public health officials, biological and social scientists, politicians, and the rest of humankind (Sect. 2). Therefore, unlike any other viral infection, many facets of its occurrence, diagnosis, treatment, and prevention have changed so radically in the years since its discovery that the temporal distribution of HIV/AIDS cannot be easily examined without reference to those changes. Rather than reiterating here how each of those various developments has affected its temporality, the reader is referred to the sections above and below that cover them.

5.8 Age

Age enters the epidemiologic picture of HIV/AIDS in various ways. With regard to morbidity and mortality in the early years of the epidemic, it was clear that AIDS incidence (and death rates) showed an age distribution pattern typical of infections transmitted by the sexual and blood-borne routes. Cases clustered primarily among the most sexually active groups (20–40 years of age). This primary mode in the age distribution of AIDS was apparent for homosexual men and heterosexually active populations throughout the world [224, 225, 92, 226] It also characterized IDUs, in whom a peak of AIDS at the lower end of that age range probably reflected the shorter latency between infection on disease onset due to their more intense exposure (i.e., higher inoculum size).

Among military recruits, blood donors, NHANES, and other relatively representative populations in the USA, the highest prevalence of seropositivity has been found in the

group 20–30 years of age (Fig. 43.14) [224, 227–230]. On a global basis, the pattern of HIV-1 prevalence in Uganda typifies the age-specific patterns of most developing countries with a peak among men 20–30 years old and among women 5–10 years younger [20, 231]. For the most part this pattern probably reflects the usual age differential in male–female partnerships; it has also been observed for the incidence of infection in studies of transmission among HIV-1-discordant heterosexual couples [232].

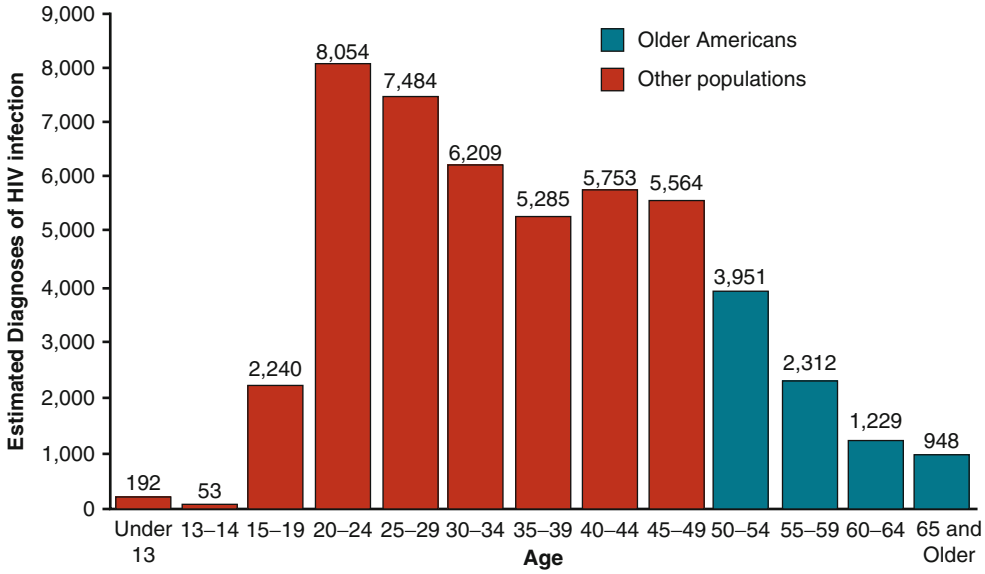
During the first years of the epidemic, a minor mode in the age distribution was formed by pediatric AIDS cases. About 20 % of infants with MTCT of HIV-1 infection became symptomatic in the first year of life, most likely due to intrauterine transmission. In the remainder the incidence of AIDS was about 8 % per year [233]. This longer interval from infection to AIDS more closely resembles the latency period in adults and suggests transmission during the trauma of birth or thereafter through breastfeeding [234].

More recently, this bimodal distribution has changed for several reasons, most dramatically in the developed world. Drug treatment of primary HIV-1 infection and prophylaxis against opportunistic infections have significantly prolonged the AIDS-free period after initial infection. In places where these medications are readily available, adults and children infected at or after birth now live much longer. In countries providing optimal perinatal care, the original pediatric age mode has all but disappeared.

Besides its influence on the peak periods of risk for infection, age exerts its biological effects independently on morbidity and mortality rates as it does with most other chronic diseases. Not long after AIDS was recognized, it became clear that when the time to AIDS and death among older children and adults was stratified by age, older individuals showed progressively shorter intervals from infection to AIDS and from AIDS to death. The strong age effect was especially apparent in hemophiliacs, virtually all of whom received contaminated blood products within a very short period of time but succumbed at rates that differed substantially with their age at the time of infection. Many other studies have confirmed this pattern.

5.9 Sex

As the epidemic first unfolded, MSM, IDUs, and hemophiliacs were the groups most heavily affected. Soon the infection had spread to female commercial sex workers, sex partners of those in the groups initially affected, and transfusion recipients; within just a few years, among the subset of patients who immigrated to Europe from sub-Saharan Africa, proportions of men and women with AIDS were nearly equal [225]. Men with AIDS continued to outnumber the women in a gradually declining ratio, but the sex ratio of new infections in groups other than MSM approached unity. Thus, despite the evidence that male to female is more efficient than female-to-male transmission on a per sex act basis (Sect. 6), such contributing factors as the type and number of partners, coinfection with genital tract pathogen,



Source: CDC. Diagnoses of HIV infection in the United States and dependent areas, 2011. *HIV Surveillance Report* 2013; vol.23.

Fig. 43.14 Estimated diagnoses of HIV infection, by Age, 2011, United States [227]

absence of circumcision of a susceptible ultimately seem to offset that differential risk in determining sex-specific incidence rates.

One well-established difference between men and women is the lower level of viremia among women in the early stages of HIV-1 infection. The approximately one-log lower concentration of virus in copies/ml plasma has been consistently documented in various populations but remains unexplained [235]. Intriguingly, the levels of viremia in untreated men and women gradually converge over the course of infection; and with comparable follow-up, the two sexes experience overall similar disease courses and ultimately equal hazards of AIDS and death [236].

5.10 Race and Ethnic Group

Once the AIDS epidemic had emerged and spread beyond MSM, the racial and ethnic disparities in cases grew increasingly apparent. By the early 1990s, African–Americans in the USA already accounted for 36 % of all AIDS cases and Hispanics for 17 % compared with their proportions of the US population, 12 and 6 %, respectively. The particular vulnerability of heterosexual minority adolescents and young men was also becoming apparent [237]. In very large serosurveys among the prospectively followed military cohorts, the incidence of new infections was 3–4 times higher in African–Americans and Hispanics compared with troops of European ancestry [238]. This ominous trend in black men has continued [239].

As the pandemic expanded into populations of different ancestry and from developed to less developed areas of the world, it became clear that higher rates of disease in one or another race or ethnic group reflected the higher rate of infection rather than the underlying host differences between the groups in their ability to control the disease. As uncertain as international comparisons may be, at least in adults the course of HIV-1 infection appears to be roughly comparable across the major racial groups. What racial and ethnic differences have been observed in disease progression have been modest and largely attributable to factors other than race or ethnicity. In African settings where more rapid development of AIDS has been observed, it has usually been attributed to poor nutrition or other immunocompromising conditions rather than race per se [240, 241]. Still, although the data are thin, some direct racial comparisons suggest that real differences may exist. In an earlier study of the natural history of the infection, including MSM of different racial and ethnic backgrounds, and in more recent analyses of European cohorts, individuals of African descent have appeared to be at a slight advantage as measured by slower decline in CD4 cell count or lower viral load [242–244]. No similar direct comparisons including Asians have been made.

5.11 Genetics

A number of genetic influences on progression of HIV-1 infection have been proposed, but relatively few have been confirmed [245]. For the several gene systems discussed here, the epidemiologic and experimental evidence is more consistent and compelling. The first heritable effect on the course of disease to be convincingly documented was that of the gene, now known as *CCR5*, which encodes the major co-receptor for HIV-1 on the surface of target cells [246]. About 1–2 % of persons of European ancestry are homozygous for a loss-of-function deletion in that gene that renders their cells incapable of being infected by the form of the virus that is most often transmitted. Elegant laboratory experiments along with numerous population studies have confirmed that virtually all of these individuals are completely protected from acquiring the infection [247]. Furthermore, heterozygotes, whose risk of HIV-1 infection is little if any lower than those carrying two copies of the fully functional gene, show modestly prolonged AIDS-free times and lower viral load set points [248]. Other polymorphisms in the coding and promoter regions, and its neighbor, *CCR2*, appear to confer different degrees of risk on their carriers [247, 249]. In addition, research on the loci encoding several chemokines, the natural ligands of this co-receptor, has generated more controversial evidence that polymorphic alteration in their gene structure or their copy number affects both the risk and course of HIV-1 infection. For *CCL3L1* in particular, despite elaborate epidemiologic and biological studies as well as a meta-analysis indicating that relatively low numbers of copies of this gene confer a slightly higher risk and less favorable outcome of infection, methodologic concerns and inconsistency in the results have left uncertainty about its role [250–252].

A second major gene system also exerts considerable influence on the course of infection and very likely on its acquisition as well. The major histocompatibility complex, known in humans as the human leukocyte antigen (HLA) system, is a cluster of genes encoding proteins with clefts that bind small antigenic peptides derived from the host or foreign biological material including viruses. Two principal classes of HLA molecules (HLA-I and HLA-II), complexed with their bound peptides, migrate to the surface of antigen-presenting cells, where they may be recognized and destroyed by effector (CD4 or CD8 T lymphocytes). The HLA genes are extraordinarily polymorphic, and some of the innumerable allelic variants encoded by class I genes are more or less efficient than others at stimulating a strong CD8 cytolytic T-lymphocyte (CTL) response. Certain HLA-I alleles, most notably HLA-B*57 but others as well, have been conclusively shown to alter the rate of disease progression differentially, and the preponderance of evidence indicates that they do so by exercising variable control of viremia beginning

soon after infection occurs [253, 254–256]. Each of at least a half-dozen HLA-I variants has been associated with up to a 0.5 log difference in the virus copies/ml of plasma, and they may operate additively [256, 257]. The involvement of HLA-I polymorphisms in the control and evolution of HIV-1 infection has been extensively corroborated in comprehensive population studies and in experimental work demonstrating highly HLA-I sequence-specific cytotoxicity for epitopes in regions of the virus that tend to be more essential to its fitness for replication [258–260].*257*

Further studies of polymorphisms in HLA-I and in HLA-II genes have suggested, albeit not yet so persuasively as for the HLA-I effects described above, that they exert other influences on susceptiblility to or control of infection [261–263].

Strong evidence has been emerging for involvement of a third, even more complex system of genes encoding natural killer cell receptors—the killer cell immunoglobulin-like receptor (KIR) loci. Different combinations of KIR genes are present or absent in each individual, and the genes themselves show considerable sequence polymorphism. In aggregate they may enhance or diminish the overall effects of the natural killer cells. KIR gene polymorphism may have its own independent effect, but HLA-I molecules also serve as ligands for KIRs, and the two systems likely act in close concert. Both population and functional studies have suggested that at least one such gene combination (*KIR3DS1*/*KIR3DL1*), and even more likely certain products of the latter gene, in conjunction with particular HLA-I alleles, confers a more favorable disease prognosis [264]. The intricate HLA and KIR systems and their interactions have just begun to be explored in the depth that will be required to unravel their full contributions to HIV-1 infection and disease control.

Epidemiologic associations of DNA sequence polymorphisms in a number of other candidate genes have been reported with HIV-1 viremia or disease progression. However, these associations generally lack definitive replication at the population level along with unambiguous evidence that the variations lead to meaningful differences in gene function. It is also worth mentioning that, even for the firmly established genetic relationships with disease course, their prognostic value has not been considered high enough to justify the addition of genotyping to the measurements already being used (i.e., viral load and CD4 cell count) for management of patients in clinical practice or as stratification variables in context of treatment trials.

At the dawn of the genomics era, high-throughput genotyping technology with assays for hundreds of thousands of human single nucleotide polymorphisms (SNPs) to cohorts of HIV-1-susceptible and HIV-1-infected individuals held early promise of detecting previously unrecognized key genetic determinants. However, these genome-wide association studies (GWASs) have been surprisingly unenlightening [245]. Few if any new variants beyond those already discovered in and near the HLA genes have withstood rigorous confirmatory analysis, probably for any of several reasons. To date GWASs have incorporated neither the sample sizes needed to detect key but infrequent variants nor the technology needed to detect key structural differences nor the bioinformatics needed to recognize transcription factor binding or mRNA splice sites. Deeper insights into genetic influences on HIV-1 infection may have to await the application of newer, even more powerful study designs, laboratory methods, and analytical approaches.

5.12 Coinfection

A large body of research has addressed the role of coinfecting agents in altering the course of the disease among HIV-1-infected individuals. It was natural to suspect that other retroviruses (HIV-2 and HTLV-I and HTLV-II) would enhance the pathogenetic processes initiated by HIV-1; however, evidence is meager and potentially conflicting. In some studies, those coinfected with HTLV-I have experienced poorer clinical outcomes, especially neurologic complications [265–267], while another study demonstrated a survival benefit with HIV-1/HTLV-II coinfection [268].

In West African countries impacted by HIV-2, up to 1 % of HIV-2-infected persons may be infected by HIV-1 as well. In these dually infected patients, the evidence for their mutual impact has been somewhat inconsistent. Similar rates of mortality from dual and HIV-1 monoinfection corroborate the greater virulence of HIV-1 as a pathogen [269, 270]. However, a report from a cohort in Guinea-Bissau whose participants had known times of HIV-1 acquisition suggested that the small number who had antecedent HIV-2 infection had a slower rate of disease progression than those with HIV-1 infection alone [271].

Because HIV-2 represents a less virulent pathogen, there has been great interest in investigating whether HIV-2 can either protect against HIV-1 acquisition or attenuate the natural history of HIV-1 progression in dually infected individuals, thus providing lessons for design of either preventive or therapeutic vaccines. However, these questions have been difficult to answer conclusively through epidemiologic studies. In the case of protection against HIV-1 acquisition, it is difficult to ascertain or control for level of sexual exposure to HIV-1 and investigators have reported mixed findings [272]. With regard to the question of whether HIV-2 attenuates the pathogenicity of HIV-1 by retarding disease progression, it is often difficult to ascertain which virus was acquired first in dually infected individuals through serologic surveys alone. Furthermore, the availability of effective antiretroviral therapy in West African countries obligates researchers to

provide access to care and treatment at the time of HIV-1 diagnosis, thus altering the natural history of dual infection.

The devastating impact of infection with HIV-1 and *Mycobacterium tuberculosis* on individuals and whole populations—a so-called syndemic in regions with high dual prevalence—has generated intense concern [56, 273, 274]. By every epidemiologic, clinical and experimental measure, HIV-1 and TB are a deadly combination. By the end of the first year of HIV-1 infection, the risk of active TB doubles. The case–fatality ratio for incident TB in HIV-1-positive people appears to be more than twice the ratio in HIV-1 negatives—due to a faulty immune response to mycobacteria, delayed diagnosis of either HIV-1 or TB, and delayed or ineffective treatment of one or both of the infections. On the other hand, the effect of latent or clinically apparent TB on the course of HIV-1 infection is far less certain. Carefully analyzed observational population studies are quite sparse, and reports that TB alters isolated immunologic or virologic responses relevant to the control of HIV-1 have been difficult to interpret or remain unconfirmed. Furthermore, treatment of active TB in 111 coinfected and prospectively followed South African patients did not significantly improve their viral loads or CD4 counts [275].

For coinfection with hepatitis C virus (HCV), the experience is much the same as for TB. Dually infected individuals with immune deficiency due to HIV-1 have intractably higher and more sustained HCV viremia (even with ordinarily more effective HCV treatment [276, 277]), and they follow an accelerated course of hepatocellular destruction and death. This rapid decline may be due to the adverse effects of microbial translocation on hepatic inflammation and fibrosis [278, 279]. In contrast, although certain studies have suggested that HCV coinfection hastens the development of AIDS or is associated with less robust response to cART [280], other work including a meta-analysis casts doubt on those findings [281].

The most noteworthy if not yet conclusive evidence for an effect of coinfection on the outcome of HIV-1 infection comes from a series of reports about the favorable influence of a virus called GBV-C—a flavivirus (formerly known as hepatitis G virus) that replicates primarily in lymphocytes but is not known to cause disease in humans. In 1998 the first of a number of investigations documented a protective effect among HIV-1-positive subjects, among whom up to 40 % of some subgroups have been coinfected with GBV-C [282, 283]. A meta-analysis has indicated that rates of progression were about 2-fold slower in dually infected individuals compared with those without GBV-C infection in the period beginning 2 years after the onset of HIV-1 infection [284]. Numerous experiments have suggested several mechanisms by which GBV-C may interfere with fusion and entry of HIV-1 into target cells or suppress T-cell activation required for HIV-1 replication. Findings in different populations have not been uniformly positive, and laboratory experiments have not yielded definitive biological explanation of the protective effect, but supportive evidence continues to accumulate [285, 286].

Investigation into the potential impact of various other coinfecting agents (e.g., herpesviruses, hepatitis B virus, HTLV-II, and agents of malaria) on the pathogenesis and course of HIV-1 infection has been less convincing.

5.13 Recreational Drugs, Alcohol, and Tobacco

Many of the substances used for recreational (i.e., nonmedicinal) purposes were implicated as risk factors for sexually transmitted infections (STIs) even before HIV/AIDS appeared. In light of their documented capability of stimulating or disinhibiting high-risk behavior, it has not been necessary or even possible to invoke additional biological activity to explain their associations with high rates of STIs.

In contrast, there have been many efforts to link the use of various chemicals to an atypical (usually a more rapid) course of HIV-1 infection or to other complications. For any of several reasons, these efforts have generally fallen short of their goal. The research has shown inconsistent or insignificant effects; the observed associations could be relatively easily explained by biased selection of subjects (e.g., individuals who may have changed their pattern substance use after developing HIV-1 infection or symptoms of advancing disease) or experimental work has failed to confer biological plausibility on the observed effects. Considerable attention has also been devoted to how these substances might disrupt immunobiological pathways involved in resistance to the pathogens causing AIDS-defining OIs [287]. Here the data are somewhat more persuasive, but still inconclusive.

Results of studies on alcohol use and disease progression typify the findings relating substance use to HIV-1 infection. Population and clinical studies have more often than not yielded equivocal or no evidence of an association, whereas experiments on animal or human biological material have suggested perturbation of some features of the immune system [288]. Work on associations of amphetamines, opiates, and other classes of recreational drugs with HIV-1 disease progression itself has likewise been inconclusive; on the other hand, experimental and clinical studies of these substances specifically on the pathogenesis of neurologic manifestations of the infection are more noteworthy. With regard to tobacco use, although smoking has long been known to predispose to respiratory tract infection (e.g., *Pneumocystis* pneumonia) and may alter certain immune system functions along with lung microanatomy, those effects have not translated into acceleration of HIV-1 disease at the clinical level.

5.14 Nutrition

Vitamins and micronutrients have deservedly received considerable attention because dietary supplementation has previously been credited with success in reducing morbidity and mortality from various childhood infections, and it is relatively simple and an inexpensive intervention to provide. While observational studies have found that low vitamin A levels are associated with increased MTCT, randomized trials have actually demonstrated increased risk with vitamin A supplementation [289]. Differences in stage of maternal HIV-1 disease may account in part for these contradictory observations. Fortunately, consistency in the salutary effect of vitamin A on disease progression in infants and children has been more reassuring.

Multivitamin supplementation (specifically with vitamins B, C, and E) has also been widely promoted as protective, although the evidence is uneven. Overall nutritional status has received much attention in the underdeveloped regions of Africa and Asia. In these impoverished areas, close concurrence of malnutrition with generally poorer health as well as other potential cofactors (e.g., vitamin deficiency, TB, malaria) and with systematic problems of diagnosis and management of HIV-1 infection has made it difficult to isolate the independent contribution of nutritional deficiency to a more ominous disease course.

5.15 Sex Hormones

The role of sex hormones, particularly those used by women for contraception, has also received attention. In brief, there is evidence that they prevent MTCT, but in a carefully analyzed prospective study in African women, neither oral nor injectable contraceptive hormones accelerated HIV-1 disease progression [290]. Sparse findings with male hormones as used by MSM have not suggested that they confer any excess risk.

5.16 Occupational, Physical, and Sociobehavioral Factors

Factors such as occupational and environmental exposures, physical exertion and trauma, psychological stress, and others have also been proposed as determinants of HIV-1 infection or disease progression. As already discussed, workers in the commercial sex and health-care industries are at higher risk of acquiring infection than those in most other occupations for obvious reasons. By the same token, it is difficult to envision how a factor as nonspecific as stress could be dissociated from behaviors that increase the risk of HIV-1 infection well enough to establish its independent causal relationship in an observational study. Likewise with regard to disease progression, the associations reported almost invariably have more plausible explanations. At best, the supporting data are usually equivocal; more generally the findings remain confounded, unconfirmed, and/or unaccompanied by strong biological plausibility.

5.17 HIV-2

5.17.1 Mortality

As suggested above (Sect. 3), the most reliable mortality statistics probably come from Guinea-Bissau, where median survival with HIV-2 infection during the 18-year period before ART became available in 2007 was significantly decreased compared with survival of uninfected individuals but approximately twice as long as in individuals with HIV-1 infection [50]. More on the reciprocal influences can be found in Sect. 5.12.

5.17.2 Morbidity

As implied by the longer survival among persons infected with HIV-2 than those infected with HIV-1, the course of infection with the former is more indolent. The distribution of clinical complications would also be expected to be more characteristic of the opportunistic infections seen in West African populations.

5.17.3 Prevalence and Incidence

Seroprevalence surveys conducted in the mid-1980s documented the presence of a second distinct retroviral infection (HTLV-IV, subsequently renamed HIV-2) in a cluster of cases identified in Senegalese prostitutes in 1987. Serosurveys conducted in other African countries documented that HIV-2 was highly concentrated in West African countries, including Guinea-Bissau, the Gambia, Cape Verde, Mali, and Sierra Leone, with cases few or nonexistent in countries of central, eastern, or southern Africa.

Although initial fears that the epidemic of HIV-2 could quickly expand to the rest of the continent, these fears were somewhat allayed by several early reports from suggesting that HIV-2 may be less transmissible compared to HIV-1. The first observation came from a community-based prevalence survey in Guinea-Bissau showing that the highest prevalence of HIV-2 infection occurred in the oldest (>40 years) age stratum, evidence that either HIV-2 exposure or transmission efficiency in that community might have been decreasing [291]. The overall prevalence in adults was 8.9 %. A follow-up population-based study in the same region documented an incidence rate of 1 per 100 person-years [292]. Another report from the West African region focused on a dynamic cohort of high-risk group, Senegalese female sex

workers, and followed them for an average of 3.2 years during the period from 1985 through 1992. The overall incidence rate for both HIV-2 and HIV-1 infections was the same (1.1 per 100 person-years). However, the HIV-1 incidence rate showed an increasing trend, while for HIV-2 it remained stable during that study period [[293]. From all of these data together, it was reasonable to infer that HIV-2 had been present in the Senegalese population for a relatively long time and that the HIV-2 epidemic was either reaching a steady state condition or waning. These reports further suggested that the level of infectiousness of HIV-1 may have been greater, because its incidence was increasing despite its lower prevalence relative to HIV-2.

HIV-2 has been detected in many places outside of West Africa, although the infection can usually be connected to that region in some way. From a summary of nationwide surveillance statistics for the period 2000–2009, newly recognized instances of HIV-2 infection in the USA have averaged 12 per year [294]. Over 80 % of those have a connection to West Africa, and about half of them were reported from New York City. The total number reported is likely to be an underestimate because nearly 60 % were initially misclassified as HIV-1 by the Western blot, and other undoubtedly go unrecognized.

5.17.4 Epidemic Behavior and Contagiousness of HIV-2

Epidemiologic data have demonstrated that the modes of transmission of HIV-2 infection are the same as those of HIV-1 (sexual, maternal to child, and blood borne), although lower rates of transmission for HIV-2 suggest reduced capacity for spread [293, 295, 296]. This reduced risk of transmission for HIV-2 for a given mode likely reflects the presence of fewer viral particles of HIV-2 than HIV-1 at comparable stages of infection [297]. While of HIV-1 has moved rapidly throughout sub-Saharan Africa to cause devastating generalized epidemics in much of the continent, epidemics of HIV-2 have remained focused in West Africa near the epicenter of its initial introduction into the human population or among groups who have migrated directly from those locations. Some reports have indicated either a plateau in prevalence of HIV-2 or modest waning of the epidemic, but simultaneously increasing prevalence of HIV-1. That increase in HIV-1 prevalence raises doubts that implementation of public health strategies to prevent HIV-2 transmission is the principal reason for its decline.

5.17.5 Geographic Distribution

As noted, several decades after the initial reports of this distinct retrovirus, HIV-2 has remained relatively confined to West African countries with only limited spread even to countries that have historic ties to this region and therefore migration. Clusters of infection with HIV-2 have been reported in Portugal (historically linked to Cape Verde), in North America among immigrants from West Africa, and in regions of India linked to Portugal during its colonial era [297]. This relatively focused epidemic contrasts with the HIV-1 pandemic, which spread rapidly throughout the African continent during the same time frame.

5.17.6 Temporal Distribution

Epidemiologic reports that have focused on HIV-1 and HIV-2 surveillance in relatively consistent populations over time have documented either stable or waning prevalence. In an STI clinic in the Gambia, the prevalence of HIV-2 decreased from 7 to 4 % with each sequential serosurvey performed between 1988 and 2003. In contrast, HIV-1 prevalence rose significantly from 4.2 to 17.5 % with each successive wave of sampling, despite relatively constant average CD4 cell counts at time of diagnosis [298]. These reports are consistent with very early predictions of epidemic trends discussed in Sect. 5.2.3.

5.17.7 Other Factors Influencing Acquisition and Control of Infection

In view of the unequivocal influence of HLA-I polymorphism on the control and course of HIV-1 infection (see Sect. 5.11), it would be surprising if it were not involved in the control of HIV-2 infection. However, given the exquisite specificity in the peptide-binding properties of HLA molecules, the class I alleles implicated in HIV-2 infection have, predictably, been distinct from those consistently implicated in control of HIV-1 [299]. Indeed, differences between HIV-1 and HIV-2, not only in their interaction with HLA molecules and KIR ligands but in other gene systems as well, may help explain the more benign course of the latter infection.

6 Mechanisms and Routes of Transmission

HIV-1 is transmitted by blood and other biological fluid, especially genital secretions, which may be cell-free or contain infected cells. Major routes of transmission involve sexual (anal, vaginal, or oral) contact with an infected person, sharing of needles or other injection paraphernalia with an infected person, or perinatal (transplacental, intrapartum, or postpartum from breastfeeding). Transfusion of blood products from infected individuals in locations where those products are not properly screened and removed from the supply is now, fortunately, a relatively uncommon to rare mode of transmission throughout most of the world. Although the virus can be found in saliva, the circumstances under which it may be transmitted by the oral route remain controversial. Despite sparsely documented claims to the contrary in the early years of the epidemic, the virus does not naturally reproduce outside of humans. It does not spread by the airborne, waterborne, or animal vectorborne routes; and transmission is not known to occur through an insect bite, saliva,

tears, sweat, casual contact (e.g., shaking hands or sharing tooth brushes, razors, or dishes), or closed-mouth or social kissing [299–302].

6.1 Men Who Have Sex with Men (MSM)

AIDS was first recognized in MSM, and they accounted for the intense transmission that spread the infection through the urban areas of the USA and both Europe and Caribbean (e.g., Haiti) during the early stage of the epidemic [303–305]. In the first dozen years, this risk group dominated as the source of cases in the USA as well as in other more developed countries of Latin America, Northern and Western Europe, Australia, and New Zealand.*302*

By back calculation from cases and deaths occurring in the 1990s, the incidence of HIV-1 infection in MSM peaked a decade earlier [45]. Then, presumably because of the catastrophic increase in the numbers of deaths and modification of high-risk behaviors, infection rates gradually began to fall. They were followed by a decline in AIDS diagnoses and deaths with a lag corresponding to the 8–10-year median latency period before the advent of effective ART.

Although morbidity and mortality has declined in all groups with access to HAART (Sect. 5), unfortunately, the earlier downward trajectory of new infections in MSM has not been sustained. Prevalence in MSM remains at 10–30 % in parts of the world with both lower and higher incomes; for example, in 2008 estimates in six West African countries ranged from 10 to 25 % [306]. In the USA annual incidence in this risk group has reached a plateau of 2–3 %, and MSM represented nearly two-thirds of the 47,500 new infections in 2010 (Fig. 43.10). As an example of how refractory this group is to prevention efforts even in the most advanced countries, as recently as 2008 nearly one-quarter of the 20–29-year-old African–American MSM around the USA were infected, yet far too few were aware of it [307]. Concern now focuses on overcoming the particular barriers to enrolling MSM, in both advantaged and disadvantaged populations, into health systems where they can receive treatment effective at both controlling the disease and preventing the infection [308].

6.2 Heterosexuals

Transmission between men and women is now the dominant mode in most regions and countries of the world. However, the intensity and pattern of heterosexual transmission differs greatly from one place to another depending on the number and nature of partnerships to which infected individuals tend to belong, the intensity of prevention efforts, access to ART, and other factors.

6.2.1 Global

Although connections between cases in the USA and Haiti at the onset of the epidemic involved predominantly MSM, bisexual and heterosexual spread in Haiti was also documented soon after AIDS was recognized [309]. Almost simultaneously it appeared in heterosexuals living in and emigrating from francophone countries of central and eastern Africa. Over the next decade, rampant spread, particularly concentrated in the less developed areas of the world, transformed the numerous separate introductions into a pandemic of 20 million infected, with a sizable majority of those infections occurring in heterosexual men and women in nearly equal proportions. The multiplicity of partnerships and certain distinctive sexual practices in regions of sub-Saharan Africa intensified transmission there, in contrast to the more circumscribed occurrence in Asia, where relatively fewer infections were transmitted heterosexually. The intensity of exposure among commercial sex workers and their high risk is well documented [310].

Paradoxically, there is a subset of sex workers in Kenya and other heavily exposed sex partners who have remained seronegative for inordinately long intervals [311, 312]. The mechanism(s) of their protection would be especially important to discover and has been the focus of attention. However, it remains controversial whether these sex workers and other exposed seronegatives were protected by effective T-cell-mediated immune responses typical of long-term controllers or by some as yet unknown form of immunity; careful study of CTL and humoral responses in different groups of these exceptional individuals have yielded inconsistent results [313, 314].

From the statistics cited in Sect. 5, there are clearly whole countries where, until implementation of massive intervention programs began, the health impact and socioeconomic consequences of heterosexual HIV-1 transmission represented an existential crisis. Lately, the delivery of critical health services under the auspices of large-scale enterprises like The Global Fund for AIDS, Tuberculosis and Malaria and the President's Emergency Plan for AIDS Relief has reversed some of the more ominous trends in heterosexual transmission (see Sect. 9). Nevertheless, the existing burden of morbidity and prevalent infection and the socioeconomic obstacles to effective delivery of resources are still formidable.

6.2.2 USA and Other Developed Regions

In the initial stages of the epidemic in the USA, relationships between women and MSM or IDUs accounted for most of the heterosexual transmissions. Not unlike the situation in urban Africa and Asia, commercial sex work served as another pathway for movement of the virus into the general population—in much the same way as it did in those regions (see Sect. 5). In the ethnic and other subcultures where

"drugs for sex" was a common nexus, it has also been a particularly powerful amplifier of heterosexual transmission. Expansion of HIV/AIDS into the heterosexual populations in the more developed regions of North and South America, Europe, Australia, and New Zealand proceeded at a pace that was less alarming than some observers predicted. Nevertheless, the burden for the health and social systems of these populations remains serious. In 2010 one-quarter of all new infections in the USA were heterosexually transmitted (Fig. 43.10).

6.3 Parenterally Exposed

6.3.1 Injection Drug Use

Accurate population-based estimates of occurrence of HIV/AIDS in IDUs have always been difficult to obtain because of the uncertain numbers at risk and because injection as the mode of transmission may be confounded by the extensive drug use among members of high-risk sexual networks. It was nevertheless clear as soon as epidemics took hold in individual countries of the developed world that, relative to the general populations of most countries, IDUs would be the most severely affected by HIV-1 infection.

As noted above cross-sectional studies during the first 15 years of the epidemic established relationships of HIV-1 risk with a variety of injection-related activities including older age, duration of drug use, and geographic proximity to a high-transmission area, frequency of syringe sharing, and use of "shooting galleries" (places to buy drugs and rent injection equipment) [206–208, 210]. Many of these predisposing factors were and still are intertwined with high-risk sexual practices (e.g., sex in anonymous venues, transactional sex) and involve multiple partners. In a prospective analysis, subjects reporting sexual exposure to an HIV-1-infected partner had a fourfold increased risk of infection, independent of needle or syringe sharing [206]. Similar to other studies of heterosexual transmission of HIV-1, this risk was higher for women than for men.

Prevalence in IDUs rose rapidly during the 1980s, and new infections continued to occur throughout the 1990s particularly in Spain, Portugal, and Italy, followed by countries in Eastern Europe and states in the western part of the former Soviet Union at the beginning of the new millennium. In some of these countries, increases have continued at an alarming pace (Fig. 43.15). Thus, the pattern of HIV-1 infection in IDUs worldwide now varies markedly, depending on the prevalence of injection drug use in a given region as well as the uptake and penetration of public health countermeasures to minimize transmission risk (Sect. 9).

Beginning in the late 1980s, there was an explosive rise in new HIV-1 infections among IDUs in South and Southeast Asia. In Thailand within a matter of 5–7 years, prevalence reached at least 40 % in some communities. Following efforts to implement more aggressive prevention policies for all risk groups, the rapid increase infection ceased rather abruptly. Even so, the experience in Thailand illustrates the difficulty of uniformly sustaining or reversing established trends in every risk group and every location. Despite a decline in new IDU-associated infections in the northern

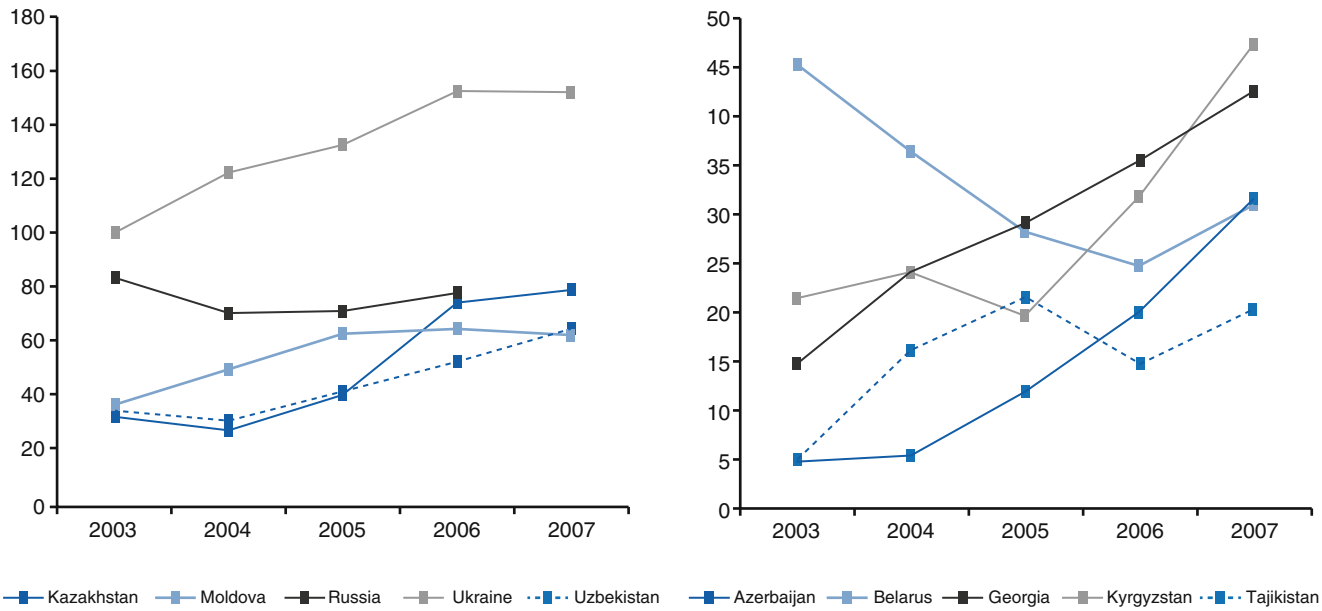


Fig. 43.15 Newly diagnosed reported HIV infections among injecting drug users, rate per million population, no EU/EFTA countries in the eastern part of the WHO European region, 2003–2007 (Reproduced with permission from Wiessing et al. [315])

Thai provinces, overall prevalence remained high because that improvement was offset by increases in the central region and Bangkok. Thus, overall prevalence peaked in the 1999–2001 period and has fluctuated in the 25–50 % range in more recent surveys [226]. Moreover, those consistently high proportions contrast sharply with the remarkable reversal of trends in other risk groups, where the 2008 prevalence figures of 2.5–5 % represent two- to fivefold reductions during the preceding 13 years.

In 2007 the number of people worldwide who inject drugs was estimated at 16 million [316], of whom approximately three million were infected with HIV-1. The largest numbers of IDUs living with HIV-1 were in Eastern Europe, East and Southeast Asia, and Latin America. In most of the 49 countries reporting 2012 data to UNAIDS, the prevalence of infection in IDUs was above 2 %, and in 11 of them it was at least 50-fold higher than in the general population [56]. Further perspective on this particularly refractory population can be found in Reference [308].

6.3.2 Contaminated Blood Products

The hazard associated with continual exposure to the factor concentrate between 1981 and 1984 was extremely high. Soon after testing for HIV-1 antibodies became available, 20–90 % of hemophiliacs who had received commercial factor VIII concentrate preparations in the USA between 1978 and 1984 were discovered to be seropositive (Fig. 43.16). Prevalence depended on severity of the disease and amount of concentrate used [317]. It also depended on the source of the concentrate. Hemophilia A patients for whom commercially available products were used had HIV-1 prevalence of 60–90 %; in contrast, hemophiliacs in countries that made their own products from non-risk donor populations had much lower prevalence [318].

At the peak of transfusion transmission, before the implementation of donor deferral procedures, as many as 1 % of all donations in San Francisco were HIV-1 contaminated [203]. From 1984 onward rigorous screening of the donated blood products and exclusion of contaminated units from the supply in most countries where testing was readily affordable, the prevalence and incidence in hemophiliacs, and other less susceptible transfusion recipients declined precipitously. Lately, recombinant DNA technology has been used to produce clotting factors for treatment of coagulation deficiencies, thus eliminating the risk of blood-borne pathogen exposure altogether.

Further precautions in the form of nucleic acid testing (NAT) added to standard serology were introduced beginning in 2000 in the USA, Europe, and many other countries. An example of the impact can be seen in the report from 93 Italian transfusion centers where NAT screening of more than ten million units of blood between 2001 and 2006 eliminated the already rare event of an HIV RNA-positive but still

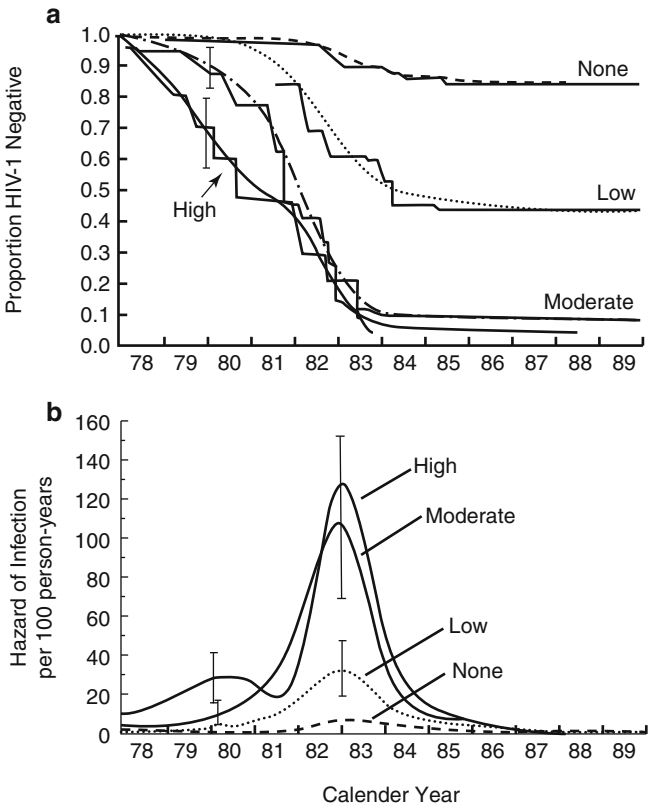


Fig. 43.16 Hazard of HIV prevalence in hemophiliacs. Estimates of (**a**) the HIV-1-free survival curve and (**b**) the corresponding hazard rate for members of the five-center cohort with type A hemophilia, grouped by mean annual dose of factor VIII concentrate used between 1978 and 1984 (Reproduced with permission from Kroner et al. [317])

seronegative donor. NAT excluded an additional 1.8 RNA-positive units per million units screened seronegative [319].

In economically challenged areas, introduction of screening of blood products and precautions for eliminating iatrogenic and related transmission by reused infusion equipment have been far more gradual and variable. A particularly egregious disregard of hygienic practices occurred between 1990 and 1994 in at least one province of China (Henan) and has probably occurred elsewhere. Pooling of plasma from multiple donors during collection, re-infusion of red blood cells, and reuse of tubing at innumerable commercial plasmapheresis centers were responsible for thousands of HIV-1 infections before public health authorities intervened [320].

6.3.3 Contaminated Injection Equipment

HIV-infected blood on needles, syringes, and other medical equipment can survive for up to 2 h outside of the body. WHO recently estimated the overall worldwide probability of acquiring HIV-1 infection by a medical injection at 1.2 %; other estimates have varied from 0.1 to 6.9 % [321]. The absence of training, new equipment, and sterilization resources in developing countries has accounted for gross hygienic

failures reported from such places as Kazakhstan [322] and Romania, where both contaminated injections and unscreened blood transfusions accounted for thousands of new infections. Recent efforts to eliminate such unsafe practices have been more successful when support has come from the international aid groups focusing on this issue (see Sect. 9).

6.3.4 Needle-Stick Injury

At the initial stages of the epidemic, the potential for transmission from relatively casual exposure to biological fluids raised fear among those caring for AIDS patients in the health-care systems around the world and in laboratories handling infected biological materials [216]. Early estimates of risk per contact partially relieved the fear (Sect. 5). However, percutaneous injuries from hollow-bore needles that had penetrated an artery or vein, shown blood on the device or contacted an AIDS patient, continued to pose appreciable risk of transmission [323]. In successive publications, most recently in 2005, CDC reported attempts to quantify the risk of occupational transmission of HIV in the USA (approximately 0.3 % after percutaneous exposure to HIV-infected blood and 0.09 % after mucous membrane exposure) and to update recommendations for postexposure prophylaxis (PEP) in health-care settings [324]. This latest guidance also reiterated that the risk for transmission after non-intact skin or mucous membrane exposure or from fluids or tissues other than HIV-infected blood is unknown but probably considerably lower than for blood exposures. In developed countries, incidence of infection has dropped as the prevalence of unrecognized and untreated infection has declined and guidelines for PEP have evolved, although its acceptance by health-care workers and first responders has varied greatly not simply by the degree of risk but also by occupational, setting, gender, and temporal factors [325, 326]. Current figures for transmission by needle stick in high prevalence, poorly resourced areas like sub-Saharan Africa and Asia are difficult to obtain, although rates of infection are undoubtedly higher than in developed countries.

Fortunately, in the USA and other developed countries, despite their close care of HIV/AIDS patients and/or high frequency of documented needle-stick, surgical exposures or other accidents, health-care and laboratory workers have experienced low seroconversion rates, which declined further after the widespread adoption of blood/secretions precautions [327].

6.4 Perinatally Exposed

Vertical or mother-to-child transmission (MTCT) of HIV-1 infection may occur antepartum (in utero), intrapartum (at delivery), or postpartum (via breastfeeding). The first probable cases of perinatal transmission of HIV-1 infection in the USA were reported within months after AIDS was recognized [328]. During the first decade of the epidemic in the USA, case numbers rose steadily, peaking at nearly 1,000 in 1992, and then declined rapidly following implementation of testing and preventive interventions (Fig. 43.17). By 2002 CDC estimated that 144–236 HIV-infected infants were born

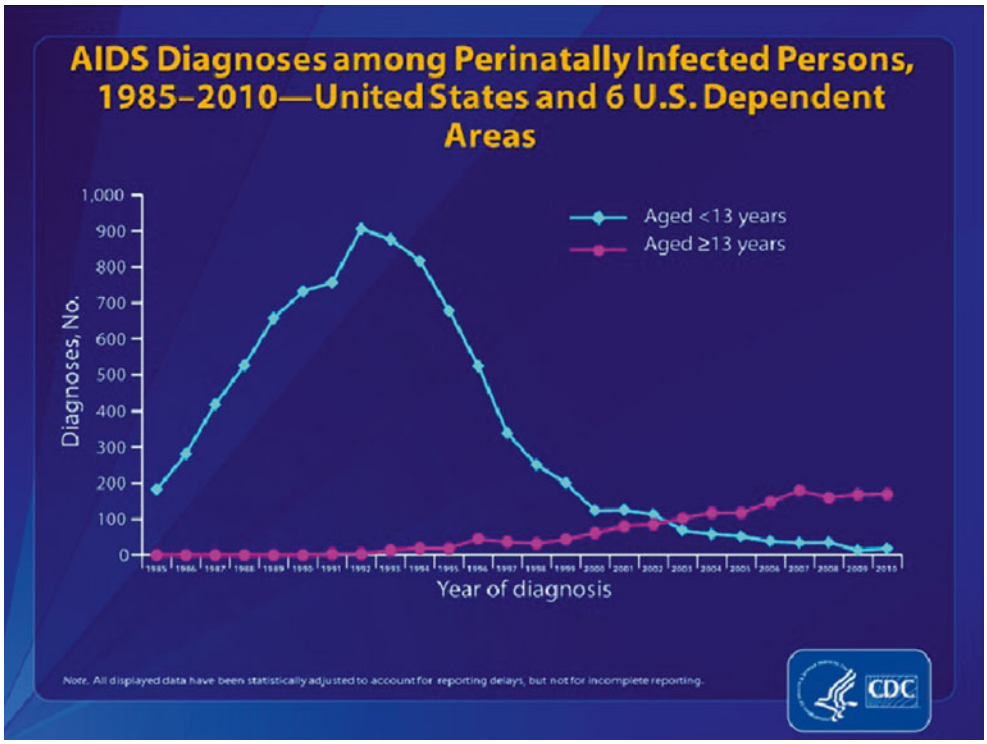


Fig. 43.17 AIDS diagnoses among perinatally infected person, 1985–2010 – United States and 6 U.S. Dependent Areas [329]. Data adjusted for reporting delays and for estimated proportional redistribution of cases in persons reported without an identified risk factor

in the USA, and the numbers continued to fall to a very few each year. The USA has implemented detailed recommendations for interventions in pregnant women to prevent all but a small number of MTCTs, and comparable approaches have led to similar success in most other developed countries [330, 331].

At the international level, the progress has been less favorable. Unfortunately, overall no more than half of pregnant women and probably considerably fewer have been receiving appropriate treatment for their own health, and even in the southern regions of sub-Saharan Africa, where prevention programs have been promoted aggressively, perinatal transmission has only dropped from 35 to 20 %. In economically marginalized regions, MTCT has occurred almost 2.5 times more frequently than in the general population.

Several factors modulate the likelihood of vertical transmission. Maternal plasma viral load is probably the most important determinant of whether transmission will occur, but other factors also play a role [332, 333]. Advanced maternal immunodeficiency, concomitant genital infection, and chorioamnionitis may be independent risk factors. Delivery by elective Caesarian section has been shown to reduce intrapartum transmission, although the availability of that option has been limited in resource-poor regions.

Breastfeeding increases the risk of postpartum transmission and accounts for perhaps one-third of all perinatal HIV-1 transmission. However, because breastfeeding is widely accepted as improving overall health of the infant, recommendations against its use in places lacking access to effective prophylactic drug regimens have been one of the biggest challenges. In 2006 WHO recommended that infected mothers be counseled individually and decide between options including breastfeeding or not. Soon thereafter, several ART trials of breastfeeding mothers or their infants demonstrated the capacity of treatment to reduce total MTCT rates to 1–5 % at age 6 months. As a result in 2010 WHO began emphasizing either breastfeeding with an established ART regimen or none at all.

6.5 Other

There are rather rare anecdotes of cutaneous or mucous membrane exposure that may have led to HIV-1 infection in home settings [334, 335]. More systematic household studies covering more than 1,700 person-years of follow-up revealed no infections (95 % confidence interval: 0–0.2 infections per 100 person-years) [336, 337].

6.6 Host Cofactors for Transmission or Acquisition

Much work on contagiousness has been done in the context of sexual transmission. Early epidemiologic studies demonstrated that ulcerative or other inflammatory genital infection coincided with or antedated HIV-1 infection among MSM and heterosexuals [338–341]. Whether these relationships with transmission and/or acquisition of HIV-1 infection represent predispositions due entirely to biological properties of the coinfecting etiologic agents themselves, to the mucosal disruption or attraction of inflammatory cells, or to other coincident host characteristics has not been definitively established for all of these agents.

Most of the evidence favors the second mechanism. Loss of integrity of the genital tract lining or skin due to ulcerative genital infections [e.g., syphilis, herpes simplex virus type 2 (HSV-2), or chancroid] provides a clear path whereby HIV-1 can reach its subepithelial target cells. In addition to the agents causing genital ulcers, chronic infection with other organisms responsible for disrupting the genital mucosal surfaces (e.g., *Trichomonas vaginalis* and bacterial vaginosis) has been associated with acquisition of HIV-1 infection [342, 343]. Alternatively, the concentration of HIV-1 in semen is much higher in men who have both HIV-1 infection and an STI (e.g., with *N. gonorrhoeae* or herpes simplex virus) than in men with HIV-1 alone [344]. Inflammatory genital infections may lead to increased risk of HIV-1 transmission by raising the concentrations of those target cells in genital fluid [345, 346].

Trials of antimicrobial agents known to suppress or cure these infections (e.g., HSV-2, *Trichomonas*) have achieved some suppression of target pathogens and may decrease genital shedding of HIV-1. Nevertheless, treatments for HSV-2 and other STIs have disappointingly failed to reduce the incidence of HIV-1 infection [347].

The evidence for relative protection of circumcised men (but not their female partners) has come largely from clinical trials in Africa. The reductions in acquisition achieved in those trials have been the basis for the inclusion of circumcision in prevention strategies described in Sect. 9.

The host genetic factors known or thought most likely to modify HIV-1 transmission from an infected host or its acquisition by a susceptible host are discussed in Sect. 5.

HIV-2 is spread by the same routes as HIV-1, although in the case of heterosexual transmission, probably at a lower rate. This lower level of contagiousness is likely due to the lower level of free HIV-2 virions in the plasma compartment for a given disease stage. Because HIV-2 is less easily transmitted and has been largely confined to West Africans and

those in contact with them, epidemiologic research on HIV-2 transmission between discordant partners has not received the same attention accorded HIV-1. As a result, event-level risk estimates of transmission and investigation into cofactors have not been as systematic or refined. In a report from rural Guinea-Bissau, the level of provirus in HIV-2-infected male partners was associated with concordant serology in wives, and older women appeared to be more susceptible [348]. In cross-sectional analysis, ulcerative genital disease has also been associated with an elevated risk of HIV-2 infection, as observed with HIV-1 transmission [349].

7 Pathogenesis and Immunity

7.1 HIV-1

Our understanding of exactly how HIV-1 induces AIDS remains incomplete despite substantial insights gained over 30 years of intense genetic, molecular biological, and immunologic research. The schematic portrayal (Fig. 43.18) of the interrelationship between expression of HIV-1 and the decline in CD4 lymphocytes oversimplifies the broad interpersonal variability of the process [128, 350–352]. In vitro HIV-1 mimics, in a crude way, the in vivo natural history of the virus in that HIV-1 targets and infects the CD4 cell and causes cell lysis [139]. AIDS pathogenesis involves complex interactions of the virus with an organ whose very core functions that would ordinarily defend against that virus are subverted to promote virus replication. Sophisticated molecular approaches often coupled to epidemiologically well-defined populations have gradually expanded our understanding of the pathogenic process from infection to end-stage failure of the immune system [353–358].

HIV-1 exists and can be transmitted as cell-free and cell-associated virus in the semen, vaginal fluids, blood, and plasma [359, 360]. HIV-1 cannot survive outside of the bloodstream or lymphatic system and quickly dies when outside of this environment. As such HIV-1 is only transmitted in situations where infected blood or secretions

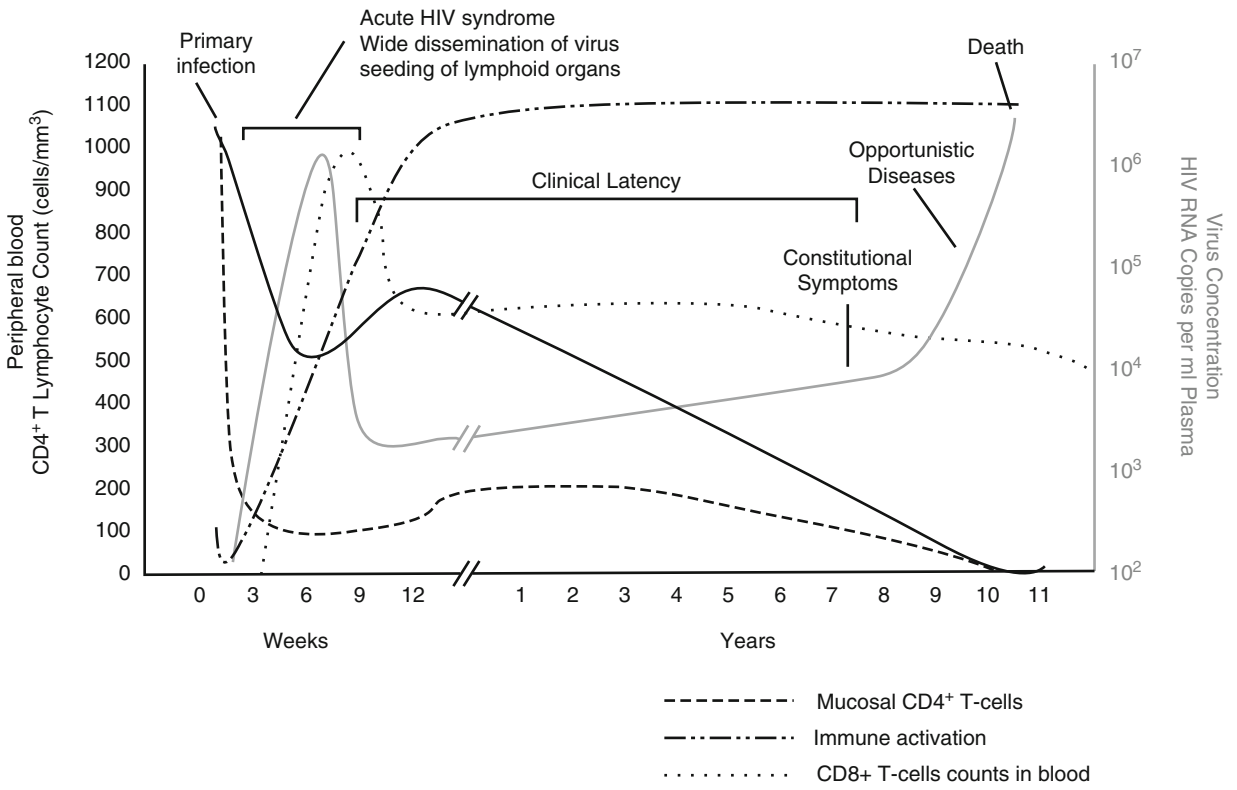


Fig. 43.18 Typical course of HIV infection. During the early period after primary infection, there is widespread dissemination of virus and a sharp decrease in the number of CD4 T cells in peripheral blood. An adaptive immune response to HIV ensues with the induction of antibodies and cell-mediated immunity. This stage is followed by a decrease in detectable viremia and a prolonged period of clinical latency. Immune activation that is both HIV-1 specific and nonspecific develops following acute infection and persists thereafter. The CD4 T-cell count continues to decrease with varying speed during these years, until it reaches a critical level below which there is a substantial risk of opportunistic infections and neoplasms typical of AIDS (Diagram is courtesy of Nilesh Amatya and LaTonya Williams)

come into contact with mucosal tissue or damaged skin (e.g., via abrasions from needles or sexual intercourse). Depending on the route of infection, the initial target of infection is either the tissue dendritic cell, the CD4 lymphocyte, or the monocyte–macrophage [361]. Recent data suggest that HIV-1 replication first occurs in a small number of resting CD4 T cells in the genital mucosa [362]. No matter the route of infection, the vast majority of transmitted HIV-1 viral isolates that have been characterized use CCR5 rather than CXCR4 as their co-receptor [363]. As noted earlier, the 1–2 % of persons of European ancestry who lack expression of CCR5 are highly resistant to HIV-1 infection; if they acquire infection, it is with a virus using CXCR4 [364, 365]. In different circumstances the differential trophism of the virus for either lymphocytes or macrophage-related cells may be important because the number and availability of different target cells may vary; however, much of the evidence suggests that CD4 T cells represent the dominantly infected cell type early during infection [362, 366]. As indicated earlier, the virus load in a potential transmitter may determine the success of an infection as well; epidemiologic studies have consistently associated the presence of antigenemia and high viral loads with increased risk for infection [256, 367, 368]. Also, ulcerative genital infections may increase the risk of infection by disrupting normal protective barriers and enhancing inflammatory cell exudation, thus increasing numbers of susceptible target cells.

HIV-1 diversity in a population and even in a single patient with chronic infection can be an enormous, yet remarkably, establishment of infection following heterosexual transmission usually occurs with a single viral strain [363, 369]. Even in transmission in MSM and IDUs, the median number of viral strains establishing infection is relatively low [370, 371]. Within 72 h of establishment of viral infection, HIV-1 infects the site of infection and the draining lymph nodes [372]. Within the first week, the infection becomes systemic and the virus disseminates to other lymph node compartments in addition to infecting a number of different tissues including the brain and kidney; however, most of the early replication takes place in the lymph nodes [373, 374]. Virus is generally detectable in the blood by day 8 following infection and demonstrates a doubling time of about 0.3 day during the first 2–3 weeks of infection [113, 372–379].

Since the GI tract has the largest concentrations of lymph nodes, early infection is characterized by extensive viral replication in this tissue [355, 380]. Furthermore, data in both humans and rhesus macaques point to the rapid depletion of CD4 T cells in the gut as a major factor in disease pathogenesis [355, 380–382]. Because CD4 T cells are rapidly depleted in the gut following acute HIV-1 infection, the host is left with a relatively impaired immune system unable to properly contain gut pathogens from undergoing persistent microbial translocation [383]. This process in turn likely leads to chronic immune activation contributing to immune pathogenesis from the onset of infection and continuing even when viral replication is controlled with cART [353, 384]. In fact, major research effort in the era of cART has been devoted to understanding the causes and treatment of persistent immune activation that is likely contributing to early immunosenescence and cardiovascular, kidney, and liver disease in addition to higher rates of diabetes in patients whose HIV-1 is controlled on treatment [385, 386]. As disease becomes more severe and CD4 cells become increasingly depleted, there is increasing disruption of lymph node architecture and destruction of the follicles. Thus, the pathogenesis of HIV-1 disease involves not only destruction of large numbers of circulating lymphocytes, especially CD4 cells, but also destruction of the lymphoid organ itself, i.e., stromal tissues and those with specific functional responsibilities for regeneration and immunologic diversity [387].

Figure 43.18 represents a typical disease course for the vast majority of those infected with HIV-1. Following acute HIV-1 infection, rapidly replicating virus achieves very high copy numbers within 2–3 weeks following infection, with viral load usually above one million copies/ml. It is during this viremic phase that the acute seroconversion syndrome described below occurs [388]. Concomitantly, CD4 T cells in the peripheral blood also decline, with the rate and level of decline varying markedly between individuals [387, 389]. It is interesting that the decline of mucosal CD4 T cells is more profound initially and remains so during the entire disease course. As discussed previously, this gut depletion of CD4 T cells likely contributes to the onset of immune activation, which persists throughout infection [353, 354, 380]. During the first month of infection, long-lived viral reservoirs become established [390] with large numbers of viral particles being sequestered in follicular dendritic cells, where HIV-1 is protected from destruction [391]. In smaller numbers, HIV-1 integrates into resting CD4 T cells that serve as a latently infected viral reservoir [392, 393] with a long life span (about 44 month half-life). The latter process is one of the main reasons that current cART is not likely to achieve a cure [390, 391].

The viral load begins to decline within 4–6 weeks following infection associated with an increase in the host responses of CD8 T cells and antibodies [113, 373, 379, 394–402]. Neutralizing antibody responses generally takes longer to appear (8–24 weeks) but likely plays a role, along with other arms of the immune system, in establishing the viral load set

point that takes place during this time period [403, 404]. At this point the CD4 T cells partially recover but never achieve their preinfection levels. In most untreated individuals, a period of clinical latency ensues during which no symptoms attributable to HIV-1 infection occur. This latent period lasts around 8–10 years on average but varies greatly [405, 406]. During this long latency, however, while there is persistent viral replication and CD4 T-cell turnover, the host is able to maintain at least some level of viral control [407, 408]. Nevertheless, in the vast majority of situations, sooner or later the host immune system fails, and untreated patients develop AIDS and die.

The process by which HIV-1 causes the progressive destruction of CD4 T cells and the immune system is not entirely known but is likely multifaceted. Mechanisms include the direct lytic destruction of cells by the virus, immune killing of virally infected cells, cellular apoptosis, innocent bystander killing, and possibly destruction of the stem cells [354, 382, 409–415]. The principal markers that have been used to predict disease progression include viral loads, CD4 T-cell counts, and measures of immune activation [37, 416–419]. There is some evidence that CD8 T-cell immune activation is the best predictor of disease progression [420], although this measurement has not been adopted for clinical use. In general terms, viral load measurements are used to predict progression to AIDS with the higher loads correlating directly with disease progression. Absolute CD4 T-cell counts are more often used to determine the stage of disease but also correlate with outcome. Disease progression is also reflected in increasing virus load in lymph nodes and the disruption of lymph node cytoarchitecture, particularly the destruction of the follicular dendritic cell network [350, 357].

The determinants of virus control are poorly understood but certainly involve host genetic determinants of immune response, especially those in the human leukocyte antigen type I (HLA-I) system [253, 255], as well as characteristics of the virus phenotype [421]. As noted in Sect. 5, genetic epidemiologic investigations including genome-wide association studies of clinical outcomes or with viral load set point have consistently demonstrated that certain HLA-I alleles, most notably HLA-B*57, are associated with a favorable response [253, 255, 422]. This particular HLA allele occurs at high frequency in patients who maintain an undetectable plasma viral load far longer than others in the absence of cART (so-called elite controllers). The data on these protective effects of HLA-I suggest that CD8 T cells play a vital role in HIV-1 control since HLA-I molecules present epitopes to these cells. Additionally, CD8 T cells force extensive mutation in the virus as it attempts to escape recognition [394, 423–426]. The evidence from humans along with nonhuman primate models of HIV-1 infection [402, 427] all points to an essential role for CD8 T cells in viral control. It is equally clear that innate host factors are playing a role in HIV-1 control; among the important factors recognized to date are CCR5 (the viral co-receptor) [246, 247], TRIM5α (blocks HIV-1 capsid uncoating) [428–430], APOBEC3G (cytosine deaminase countered by HIV-1 vif) [431], and cyclophilin A (proline isomerase important for HIV-1 infection) [432]. Indeed, nearly every major arm of the immune system studied now appears to be working in concert to control HIV-1.

For disease progression of HIV-1 acquired neonatally, a more complex pattern reflects factors associated with timing of infection, e.g., parturient versus prenatal. Children who progress to AIDS in weeks to months (median, 4.1 months) are thought to have acquired infection in utero [433, 434]. For the remainder (median, approximately 6.1 years [435]), infection appears to have occurred later in the perinatal period. Besides timing of infection, additional determinants presumably include viral load and viral strain [435]. Among children and adolescents infected after birth, the natural history of infection also differs from that seen in adults. For example, the time from exposure to CD4 lymphocyte count <200/mm^3 occurs more slowly in children, adolescents, and young adults than in those over 30 years of age [436, 437]; likewise, once a CD4 count falls below 200/mm^3, disease progresses more rapidly in older than in younger individuals [12, 438]. The young immune system may mount a more effective initial response or a more robust recovery.

7.2 HIV-2

HIV-2 is less pathogenic than HIV-1; it causes slower CD4 T-cell decline and a 1.5- to 3.5-fold higher than normal mortality rate compared with a 5- to 10-fold excess rate seen with HIV-1 infection in Africans [439]. Other studies have demonstrated that disease progression can be very heterogeneous with some individuals actually progressing fairly rapidly to AIDS following HIV-2 infection. At high CD4 T-cell counts, there is a clear survival advantage in HIV-2-infected individuals, but this advantage tends to wane in older age groups and when immune suppression is matched with that seen in HIV-1 patients [269, 440]. Interestingly, plasma viral load set points are about 30-fold lower in HIV-2-infected patients than in their HIV-1 counterparts [441, 442], and most asymptomatic HIV-2 patients have undetectable plasma viral load [443, 444]. Nevertheless, baseline plasma viral load appears to be a good predictor of survival [440, 443, 445], and when baseline viral loads are matched, the median

rate of CD4 T-cell decline is similar for both infections [446]. Markers of immune activation also seem to predict disease progression with HIV-2 infection [447], but levels of these markers are generally lower when compared to HIV-1 [448]. Furthermore, the manifestations of AIDS due to HIV-2 mirror those due to HIV-1 except that rates of Kaposi's sarcoma may be lower [449].

The mechanism behind the slower rates of disease progression seen with HIV-2 infection is not clear but is likely multifactorial. This virus has appeared less fit in vitro [450], and replication kinetics of the virus seem slower in HIV-2 than in HIV-1 aviremic controllers [451]. Furthermore, the immune responses made by both CD4 and CD8 T cells to specific viral antigens tend to be stronger in patients with HIV-2 infection [452–455], and neutralizing antibody responses measured in one small study were also more robust in HIV-2 infection [456–458]. Finally, innate factors involved in HIV-1 control (e.g., TRIM5α [459] and APOBEC [460, 461]) have been implicated in control of HIV-2 infection as well.

8 Patterns of Host Response

8.1 HIV-1

8.1.1 Primary Infection

An acute viral syndrome, characterized as a "flulike" syndrome with fever, rash, myalgias, mouth sores, and occasionally reversible encephalitis, has been reported among persons undergoing seroconversion [462]. Up to 2/3 of persons with primary HIV-1 infection make an acute clinical response while the rest remain asymptomatic [463–467]. It is difficult to determine the percentage of persons who exhibit the clinical syndrome of primary HIV-1 infection because many of the signs and symptoms are nonspecific. Fever (up to 40 °C) occurs in the majority (80–90 %) of symptomatic patients; malaise, anorexia or weight loss, myalgias, and arthralgias occur in about half. Fewer than half of acutely ill patients complain of headache, pharyngitis, or rash; and complaints of diarrhea or mucosal ulcers are even less common. Meningeal signs may be present, but more specific neurologic findings are rarely observed. The nonspecificity of the symptoms makes the acute retroviral syndrome difficult to distinguish from various other acute viral syndromes. They may or may not bring patients to medical attention [468]. Laboratory findings include general leukopenia with CD4 lymphopenia, atypical lymphocytosis, and thrombocytopenia in addition to abnormal liver function tests [469, 470]. Symptoms of acute HIV-1 infection generally resolve after 2 weeks; however, the number and duration of these symptoms directly correlate with worsening disease prognosis [396, 471–473]. Recently highlighted beneficial immune effects of starting early ART has provided new incentive to pursue early diagnosis of acute HIV infection [474–478].

8.1.2 Chronic Infection

While HIV-1-infected persons may remain free of serious clinical sequelae for an extended period of chronic infection, the virus is not quiescent but rather continues to replicate in the lymph nodes [361, 387]. The persistent generalized lymphadenopathy often found in a sizable percentage of HIV-infected persons relatively early in their course [479] may reflect the role of the lymph node as a harbor for HIV-1, but lymphadenopathy does not predict the rate at which opportunistic infections or HIV-associated malignancies will develop. The resolution of lymphadenopathy has been suggested as an ominous prognostic sign by some investigators [480] but not others [481].

Depletion of CD4 lymphocytes occurs throughout the course of HIV-1 infection, but the rate of CD4 lymphocyte loss varies dramatically between individuals [482]. This deficiency in CD4 cells is the primary mechanism by which HIV-1 infection leaves an individual vulnerable to life-threatening opportunistic infections and cancers. However, because the CD4 lymphocytes play such a central role in maintaining cell-mediated immunity [483], their loss may also be a more direct cause of some of the conditions seen with HIV/AIDS.

8.1.3 End-Stage Disease

Opportunistic Infections

Persons with HIV-induced immunodeficiency are more susceptible to diseases caused by a large number of commonly encountered parasites, fungi, bacteria, and viruses. In the USA, serious common opportunistic infections include *Pneumocystis jirovecii* pneumonia (formerly known as *P. carinii*), pulmonary or disseminated *Mycobacterium tuberculosis* infection, disseminated *M. avium* complex infection, meningitis caused by *Cryptococcus neoformans*, toxoplasmic encephalitis, chorioretinitis due to cytomegalovirus infection, and many others (Table 43.1). Life-threatening infections generally do not develop until an HIV-infected person has suffered considerable loss of $CD4^+$ lymphocytes (<200 total $CD4^+$ lymphocytes per microliter), but less serious conditions, including oral candidiasis (thrush), persistent herpes simplex virus infection, and herpes zoster (shingles), may present earlier in the course of HIV infection [97, 463, 467].

The incidence of specific HIV-associated opportunistic infections varies by HIV-risk group, geographic location, and time. Injection drug users are more likely to suffer *M. tuberculosis* infection than are MSM [484], while the incidence of disseminated *M. avium* complex infection does not vary by risk group [485]. Diseases such as tuberculosis

and toxoplasmosis are common in HIV-1-infected persons throughout Africa [486]. *P. jirovecii* pneumonia, originally thought to be rare in Africa, also appears to be common there [487]. The incidence of endemic mycoses (e.g., histoplasmosis, coccidiomycosis) varies geographically within the USA in association with the known geographic distribution of these infections [488]. Other mycoses are endemic in different geographic locations: *Penicillium marneffei* in Southeast Asia and *Paracoccidioides brasiliensis* in South America [489, 490]. Since cART became more widely available, the incidence of all opportunistic infections has declined significantly, but *P. jirovecii* pneumonia still remains the most common of them [491–494].

Neoplasia

Kaposi's sarcoma (KS) and non-Hodgkin's lymphoma (NHL) are strongly associated with advanced HIV-1 infection; both are AIDS-defining illnesses. In the USA, KS signaled the beginning of the AIDS epidemic among MSM and became the most common cancer occurring in HIV-1-infected patients. KS tends to appear earlier in the course of HIV-1 infection than serious opportunistic infections or NHL, and the hazard for development of KS is less strongly related to the time since infection or degree of immunosuppression [495]. The incidence rate of NHL, especially high-grade B-cell lymphoma, is markedly higher in HIV-1-infected persons [496, 497] and increases with age as it does in with non-AIDS-associated NHL [498]. Compared to KS, NHL is distributed more uniformly among all risk groups and appears later in the course of HIV-1 infection. HIV-1 infection may lead to NHL primarily because of decreased immunosurveillance, but evidence suggests that in some instances, HIV-1 may cause NHL by insertional activation of an oncogene [499]. AIDS-defining cancers have declined precipitously since cART became widely available [500]. See Chaps. 39 and 40 or more on AIDS- associated cancer.

As AIDS-defining cancers were disappearing in cART-treated HIV-1 patients, certain non-AIDS-defining cancers were increasing in incidence. The explanation for this relationship is unclear, but reactivation of infection with other viruses may be partly responsible. Hodgkin's lymphoma (due to Epstein–Barr virus), cervical or anal cancer (due to human papillomavirus), and hepatocellular carcinoma (due to hepatitis B or C) all occur at higher rates in HIV-1-infected patients [312, 501–503]. They may emerge because of HIV-1-induced immune system defects that persist despite substantial immune reconstitution with cART. In particular, when treated individuals are compared with HIV-1 seronegative controls, they exhibit a higher level of chronic immune activation, leading in turn to T-cell exhaustion and immunosenescence. Those phenomena likely contribute to the increased risk of cancer in HIV-1-infected patients just as happens with aging in persons without HIV-1 infection [504–506].

8.1.4 Healthy Long-Term Nonprogressors and Elite Controllers

A small minority (probably <1 %) of patients with HIV-1 infection can control viral replication and/or disease progression in the absence of cART [482]. Originally recognized as maintaining stable CD4 T-cell counts for ≤15 years following infection, these untreated individuals were later shown to have low levels of viremia as well [507]. A "viremic controller" group can maintain plasma viral loads at <2,000 copies/ml [367, 508], a level associated with both decreased transmission and slower rate of disease progression. An "elite controller" group can maintain plasma viral load at <50 copies/ml, the lower limit of detection in the assay used at the time this cohort was originally defined. More recently, with a detection limit at <20 copies/ml, of plasma, an even smaller subset would be categorized as having an "undetectable" viral load. Even in elite controllers, however, certain characteristics of their HIV-1 infection (e.g., immune activation) suggest progression, albeit at much slower rates [509, 510].

After intense study, although much is still unknown about why these individuals are so effective at suppressing the virus, it is clear that both viral and host characteristics contribute to this exquisite control. A fascinating, inherently defective virus with a deletion of the gene for nef generated great interest when it initially appeared that it might account for slow disease progression [511, 512]; however, most of the individuals infected with this virus eventually progressed to later stages of infection, and further research has failed to demonstrate significant viral deletions leading to replication deficits in HIV-1 controller patients [513]. In contrast, numerous studies have demonstrated the importance of different facets of the immune response in viral control. Innate factors (APOBEC3B and Trim5α), NK cells, antibodies, and T cells have all been associated with disease progression; however, the most convincing evidence for immune control in chronic HIV-1 infection has been assembled for CD8 T cells [514]. HIV-1-specific CD8 T cells from controllers tend to proliferate better, express their functional capacity more strongly, and target distinct proteins of HIV-1 [515–518]. Furthermore, CD8 T cells induce many of the mutations seen in viral proteins other than envelope, in an attempt by the virus to escape these CD8 responses. This immune pressure by CD8 cells on key viral proteins further reflects their importance in controlling viral replication [258, 423, 426]. Finally, studies show that HLA-I is strongly associated with disease progression, more so than any other human gene [253, 255]. Since a cardinal function of HLA-I is to present HIV-1 peptides to CD8 T cells for immune recognition and

control, the HLA-I associations corroborate the importance of this cell subtype in viral control.

8.2 HIV-2

As previously noted, disease in patients infected with HIV-2 progresses at a slower rate than in those infected with HIV-1 [439]; however, progression rates for both infections are similar for individuals with similar plasma viral load [446]. Furthermore, the clinical manifestations of HIV-2-associated AIDS do not appear distinctive [519–521].

Not surprisingly, a greater percentage of individuals with HIV-2 infection are able to control virus without therapy, and most asymptomatic patients have undetectable viral load [443, 444]. The reasons for this enhanced viral control seen in HIV-2-infected patients are poorly understood; however, their numbers of CD4 and CD8 T cells tend to remain higher for longer, and the function of those cells remains more intact. Moreover, HIV-2 appears to generate enhanced neutralizing antibody responses [452–458].

9 Control and Prevention

Buoyed by recent progress through simplified treatment regimens and the success of treatment as prevention, some have raised prospects of "the end of AIDS," while others emphasize the unique and formidable challenges to reaching that goal. One of the greatest challenges is the etiologic agent itself. Although other retroviral infections affect humans, no other has been so pernicious and pervasive as HIV-1. It not merely evades many of the usual immune mechanisms but actually infects and destroys the very cells vital to coordinating an effective response. In addition, eradicating HIV-1 in individuals presents an especially high hurdle compared with other chronic viral infections (e.g., hepatitis B or herpes viruses) because the former establishes latency by integrating into the host genome of these key cells so tightly as to defy complete displacement or destruction by any technique. An assault on the virus in this critical and sensitive location must be mounted cautiously. Finally, HIV-1 mutates extensively with tolerable loss in replication capacity, readily adapting to the natural immune pressure applied by successive new hosts and, in some measure, to the drugs and immunizing agents developed to date. All the more remarkable then is the steady stream of success at developing increasingly safe, effective, and affordable pharmaceuticals, and the application of these drugs on a population scale was unimaginable less than a decade ago. However, there are no illusions that the approaches to treatment currently in use will likely prove feasible or desirable as a long-range solution. Other promising breakthroughs have involved new insight into viral persistence and strategies for shrinking the reservoir virus or infected cells, as well as an ostensible proof-of-principle "cure" of HIV infection by bone marrow transplantation or extremely early antiretroviral treatment [522–524].

Those successes have impelled the US National Institutes of Health to commit substantial resources to cure research [525].

No less challenging than the biological barriers to effective control are the behavioral and social ones. Campaigns for abstention from high-risk practices and more promising risk reduction strategies have yielded mixed success at achieving and maintaining lower rates of incident HIV-1 infection: gains have been documented in certain populations (e.g., heterosexual couples), while relapses have occurred in others (e.g., young MSM). In the developed world, much of the stigma and prejudice that accompanied the original expansion of the epidemic through and beyond the MSM populations has dissipated, although it has not disappeared entirely. In less advantaged regions, the ignorance, denial, fear, ostracism, and taboos that so often shadow poverty have continued to retard prevention efforts tied to sexuality and gender. Even in the USA and some other advanced countries, it is the social, political, and legal issues that are the stubborn impediments to manifestly effective the needle exchange paradigm.

The following, in more concrete terms, is a summary of the approaches and accomplishments in dealing with these challenges at the public health level.

9.1 Public Health Approaches

The HIV/AIDS was and still is an unprecedented worldwide public health threat of HIV/AIDS has required unprecedented global public health responses. The world was unprepared to deal with the impending pandemic during its early years, but the global effort now represents an extraordinarily comprehensive, collaborative, and costly array of public health programs. Because HIV affects numerous risk groups and uses several alternative routes of transmission, it has required the multipronged attack described below.

9.1.1 Prevention of Parenteral Transmission

Screening of Blood Products

With the availability of serologic testing for HIV in 1985, widespread screening of the blood supply was added to behavioral screening of volunteer blood donors. In 1999 the USA instituted the use of nucleic acid tests (NATs) on pooled donor samples. This strategy improved detection of HIV during the so-called seronegative window, a period usually lasting weeks during which a donor may be infected but may not produce antibodies. With NATs, the risk of HIV transmission related to blood product transfusion in the USA decreased from 1 in approximately 500,000 donated units (1995) to 1 in

1.5 million donated units (2007–2008). Similar screening approaches have been adopted by most other countries with sufficient resources. For a decade, the US President's Emergency Plan for AIDS Relief (PEPFAR) has been assisting national blood transfusion services in countries with the greatest need for prevention of transfusion-transmitted HIV. From 2007 forward, varying numbers of these countries have implemented or planned safe blood collection policies, increased the numbers of whole blood units collected from voluntary uncompensated donors, decreased the proportions of HIV-reactive units [294].

Needle and Syringe Programs (NSPs)

As a measure to prevent HIV transmission among IDUs, public health programs focusing on the exchange of used for unused needles and syringes have been introduced in a few countries (e.g., the United Kingdom and Australia), often in connection with other harm reduction services. In Australia, early widespread coverage of the IDU population with NSPs has been credited with preventing a significant HIV epidemic among this risk group, despite the continuing parallel HIV epidemic due to sexual transmission in that country [526]. In 2004 a WHO panel reviewing the experience with NSPs up to that time concluded that the evidence for the efficacy, safety, and cost-effectiveness of NSPs for HIV prevention was compelling [527]. Contrary to this forceful endorsement, throughout many countries including the USA, the illegality and stigmatizing nature of injection drug use and the challenges inherent in reaching this marginalized population, along with political opposition to NSPs, have precluded the implementation or limited the impact of these programs in some of the most vulnerable populations.

Medical Injection Safety

Until very recently, many health-care facilities lacked the resources to ensure adherence to optimal medical injection practices. In recent years this gap has been addressed by programs like the WHO effort called "The Safe Injection Global Network" (SIGN) alliance, a voluntary coalition of stakeholders aiming to achieve safe and appropriate use of injections [528], and another called "Making Medical Injections Safer" (MMIS) funded in a dozen less developed countries by PEPFAR through the service and training organization John Snow, Inc [529]. These programs have been aggressively assisting governments in the training and education of health-care providers and in promoting proper use and disposal of medical injection equipment along with introduction of single dose, prefilled auto-disable syringes.

9.1.2 Prevention of Sexual Transmission

Condoms

Although research on the efficacy of condom use has varied according to subject selection, advice conveyed, methods of measurement, and other features of study design and execution, multiple studies have demonstrated that consistent and correct use of male latex condoms provide significant protection against infection with most sexually transmitted agents, including HIV. In one analysis of heterosexual HIV-1-discordant couples who reported consistent use of condoms, the HIV-negative partner was 80 % less likely to acquire HIV than persons in similar relationships not reporting condoms use [530]. Based upon this and other similarly persuasive reports, promotion of condom use and widespread distribution of condoms at sites where HIV prevention and care are delivered have been common since risk factors for HIV infection were first identified.

HIV Testing and Counseling

Once serologic screening became widely available in the mid-1980s, HIV testing was targeted to those whose behaviors increased their risk for acquiring HIV (MSM, IDUs, commercial sex workers, and others, with high-risk sexual contact) through publicly funded testing programs. Serologic testing was often linked to client-centered risk reduction counseling so that, in the event of a negative HIV test, the experience of the counseling process would promote risk-reducing behavioral change. Specialized consent procedures for HIV testing were also often required by law. Multiple diverse behavior modification approaches, combining the provision of educational information along with theory-based behavior change counseling, demonstrated some level of efficacy in clinical trials [531], but reports on their effectiveness in "real world" settings were mixed [532]. Nevertheless, public HIV testing programs focused on linking HIV testing to counseling to reduce sexual risk and increasing condom use.

As highly effective antiretroviral drugs were developed in the mid-1990s and ARV therapy became widely available in the developed world, the opportunity cost of a missed case of HIV detection became greater, because it was clear that HAART was increasingly successful in restoring health when initiated before an AIDS-defining clinical condition occurred. Over time, it also became clear in the USA that public health policies based upon targeting specific risk groups (MSM, IDU) led to many HIV-1 infections going undetected. At a practical level for various reasons, individuals often did not perceive their own HIV risk and seek testing, or staff in public HIV testing sites simply could not recognize risk status. In 2006, as noted earlier, the CDC announced a significant shift in HIV testing guidelines—to a policy of "opt out" as the standard approach to testing in clinical settings [137]. These guidelines recommended against any statute or regulation that prescribed special consent procedures for HIV testing. The guidelines did endorse counseling for those who had identifiable behaviors that put them at higher risk for HIV acquisition, but they did not link specialized risk reduction counseling to the administration of a diagnostic HIV test to an individual. Technological

advances in reliable point-of-care HIV serologic tests, which could yield results in less than 15 min, have further facilitated the public health approach of streamlined and widespread HIV testing, both in the USA and globally.

Adult Male Circumcision

Although the benefit of circumcision had been suspected previously, three well-designed and executed randomized controlled trials have demonstrated unequivocally that adult male circumcision reduces risk of heterosexual acquisition of HIV by 55–60 % [533–535]. Thus, implementation and scale up of voluntary male circumcision has become a major public health focus in parts of the world where heterosexual HIV transmission predominates and where medical circumcision of males is uncommon. Reports of long-term follow-up of cohorts who participated in circumcision trials show a very durable protective effect of circumcision [536]. However, formidable cultural and economic barriers must be overcome: variable acceptability, resource limitations, complicating infection, and potential overconfidence among susceptible men about its degree of protection.

9.1.3 Prevention with Antimicrobial Agents

Prevention of Maternal-to-Child Transmission (PMTCT)

The first clear demonstration that antiretroviral drugs could be successfully deployed to reduce HIV transmission came in 1994, when a randomized controlled trial demonstrated a 67 % reduction in HIV transmission from HIV-1-infected mothers to their infants with maternal antepartum and intrapartum use of zidovudine along with administration of zidovudine to the exposed infant during the first 6 weeks of life [32]. This landmark observation coupled with the subsequent demonstration of the safety and efficacy of combination maternal antenatal ARVs provided the impetus for routine prenatal screening for HIV-1 and the recommendation of cART for PMTCT [537]. In settings where breastfeeding is encouraged both for its nutritional advantage and for prevention of other neonatal infections, effective prophylaxis requires continuation of maternal cART along with infant ARV throughout the course of breastfeeding. When optimally implemented in the clinical trial setting, these approaches have resulted in PMTCT rates as low as 1–2 %, raising hope for its elimination under the most favorable socioeconomic conditions [538]. In fact, several international organizations have in turn set the optimistic goal of "virtual elimination" of pediatric HIV-1 infection (i.e., MTCT <5 %) in the foreseeable future [539]. The most recent available UNAIDS report on progress through the Global Plan [540] is relatively optimistic: new HIV infections have declined by an average of 38 % in 21 target countries with progress accelerating in several vulnerable ones, and new recommendations for women and children should lead to improved delivery of HIV prevention and treatment services, earlier initiation of HIV treatment, and harmonization of eligibility and regimens among various populations. Unfortunately, MTCT may persist in less developed countries at far higher rates than it should at this stage of knowledge and capacity [541] if access to cART in those places remains in adequate.

Postexposure Prophylaxis (PEP)

An early case–control study among health-care workers with occupational percutaneous injury resulting in known HIV-1 exposure showed that zidovudine given in the postexposure period was associated with a lower odds of seroconversion (OR 0.19; 95 % CI 0.06, 0.52) [323]. The safety and tolerability of combination ARV as PEP for occupational exposures has also been documented more recently. Based upon these observations, as well as animal data showing its benefits, PEP is recommended within 72 h of occupational HIV-1 exposure as well as other discrete exposure events, such as sexual assaults.

Treatment as Prevention

The strong log-linear relationship between plasma HIV-1 viral load and lower risk of heterosexual HIV transmission was noted previously [368]. Furthermore, ARV therapy in the infected partners of serodiscordant couples resulted in virologic suppression and lower transmission risk [542]. This work culminated in a landmark randomized controlled clinical trial that documented a 96 % reduction in HIV-1 transmission to the uninfected partner of a serodiscordant couple when the HIV-1-infected partner was treated early (i.e., before significant clinical outcomes or damage to the immune system was evident) [543]. This pivotal trial provided compelling justification for early identification of individuals with HIV-1 accompanied by direct linkage to early treatment. This "test-and-treat" strategy has rapidly become the cornerstone of a global public health approach to control the spread of HIV-1 and ultimately reversing the AIDS epidemic [544]. Population-level models based on this strategy predict that the widespread implementation and scale up of combinations of interventions with proven benefit—HIV testing, PMTCT, voluntary male circumcision, early treatment of HIV-infected persons—can lead to reduction of HIV incidence [545]. Widespread implementation of combination prevention packages, along with evaluation of the impact on population incidence, will be required to translate the "test-and-treat" hypothesis into significant gains at the population level. Notably, observational data on the impact of ART scale-up in communities heavily impacted by HIV-1 infection suggest that incidence decreases as ARV coverage in a population increases [546]. Reports from other populations with epidemics in such cities as Vancouver and San Francisco have described sustained declines in HIV incidence with increased testing, increased uptake of HIV treatment, and reduced

"community viral load" (an imputed surveillance measure of community infectiousness) [547, 548]. As heartening as these findings are, considerable effort and resources will need to be invested before early treatment translates into significant reductions in new HIV-1 infections worldwide [549].

9.1.4 Preexposure Prophylaxis (PrEP)

Early efforts at primary prevention in individuals prior to HIV exposure concentrated on vaginal microbicidal agents; however, hopes for success with two promising agents, nonoxynol-9 and cellulose sulfate, were deflated when clinical trials revealed not merely failure to achieve a beneficial effect but potentially increased risk of infection with their use. Lately, clinical trials have been more promising in their demonstration of benefit from various formulations of ARVs used as PrEP by high-risk HIV-uninfected persons. The use of a vaginal gel containing tenofovir applied during the time surrounding intercourse reduced the risk of HIV-1 acquisition in South African women by 39 % [550]. As noted above, daily cART given as treatment actually reduced transmission as well, although the benefit varied greatly by degree of adherence. However, other prevention trials in those at high risk of HIV-1 infection showed encouraging but mixed results with either oral PrEP [551–555] or vaginally applied tenofovir gel [555]. Taken together, these reports have generated cautious optimism that use of daily ARVs as PrEP is beneficial. However, these regimens may only work in selected populations.

Another encouraging development has been the demonstration in a randomized controlled trial among IDUs in Thailand that tenofovir prophylaxis could halve the frequency of new infections in all subjects and reduce infection in an even greater proportion of those with measurable blood levels of tenofovir. On the basis of those findings and additional data suggesting that the combination of tenofovir and emtricitabine is equally safe and optimal for prevention of sexual transmission for which IDUs are also at risk, CDC issued a formal recommendation for combination PrEP among IDUs at very high risk due to both parenteral and sexual transmission [205, 556]. Moreover, adherence to optimal regimens has been irregular, and still other barriers may limit their effectiveness [557].

Finally, other strategies for PrEP, e.g., use of long-acting injectable agents and administration of broadly neutralizing antibodies, are at early stages evaluation.

9.1.5 Government and Civil Society Commitments

As the devastating tragedy of the AIDS pandemic unfolded in the poorest regions of the world, it mobilized UNAIDS, the governments of individual nations in higher resource settings, as well as the civil society and the private sector. Innumerable local, regional, and international organizations now address the broad range of issues and needs. From repeated credits and citations throughout the chapter, the central role of WHO and its operational arm UNAIDS has been apparent. Space does not allow justice to be done to many others; discussion here focuses on two with enormous international presence and the key organization for domestic strategic policy making.

The Global Fund to Fight AIDS, Tuberculosis, and Malaria

Many of the initiatives to deliver drugs and other key resources to low- and middle-income countries have been coordinated by UNAIDS and other organizations. These initiatives have been supported by The Global Fund, which now focuses on generating the resources needed to accomplish its highly ambitious target goals, summarized for 2015 in Table 43.5. Between 2001 and 2013, paid pledges to the Global Fund totaled nearly $24 billion [44], and that does not include the numerous other public and private investments in treatment and prevention programs. On the other hand, resource deficiencies due to Global Fund donor failures to honor pledges in 2011 have highlighted the vulnerability of the global effort even after 10 years of collaboration. In the face of these shortfalls in governmental support, the Global Fund has nevertheless attracted contributions from numerous other private agencies (e.g., the Bill & Melinda Gates Foundation) as well as government entities (e.g., US President's Emergency Plan for AIDS Relief).

President's Emergency Plan for AIDS Relief (PEPFAR)

Launched in 2003, PEPFAR represents the commitment by the US government to funding the global fight against the HIV/AIDS pandemic. By 2012, through bilateral and regional partnerships in over 70 countries, PEPFAR had directly supplied 15 million people with care and support, including provision of ARVs to 4.5 million HIV-1-infected persons worldwide. In 2012, PEPFAR made antiretroviral prophylaxis for PMTCT available to more than 750,000 pregnant women who tested positive for HIV, which averted an estimated 230,000 HIV infections in children. The MMIS program for injection safety mentioned earlier is another initiative supported by PEPFAR.

National HIV/AIDS Strategy

In 2010 the US government developed and publicized the first domestically focused National HIV/AIDS Strategy (Table 43.5). Major goals included (1) reducing HIV incidence, (2) increasing access to care and optimizing health outcomes for people living with HIV, (3) reducing HIV-related health disparities and health inequities, and (4) achieving a more coordinated national response to the HIV epidemic. The plan includes clear and measurable target objectives in these areas to reach by 2015 [558].

Table 43.5 Specific targets set by UNAIDS for 2015

Public health targets	Reduce sexual transmission by 50 %
	Reduce transmission among IDUs by 50 %
	Eliminate new infections among children; substantially reduce maternal AIDS deaths
	Reduce deaths from TB among HIV + persons by 50 %
Investment targets	Reach global investment of $22–24B in low-/middle-income countries to close global AIDS resource gap
Structural and systems targets	Eliminate gender inequalities and gender-based abuse; increase capacity of women and girls to protect themselves from HIV
	Eliminate stigma and discrimination against people living with HIV by promoting laws/policies that ensure full realization of rights and fundamental freedoms
	Eliminate restrictions on entry, stay, and residence for those living with HIV
	Eliminate parallel systems for HIV-related services to strengthen the integration of the AIDS response in global health

9.2 Vaccines

With the announcement of the discovery of HIV in 1984, the Secretary of the US Department of Health and Human Services, naively, declared that an effective vaccine for HIV-1 would be produced in the next two years [559]. Far from realizing that infamous early promise, vaccinologists worldwide have not come close to accomplishing that goal in the three decades since the virus was discovered [560]. This has not been for a lack of effort; a relatively large number of investigators and organizations have accelerated work on an HIV vaccine in recent years [561, 562]. Because many highly efficacious vaccines appear to work by inducing antibody responses [563–568], the field focused on this goal early in the development phase. Antibodies that are able to neutralize virus in vitro (so-called neutralizing antibodies) are particularly desirable since such a response is theoretically able to eliminate pathogens or at least render them noninfectious. Encouraging early preclinical data demonstrated that when serum was transferred from a vaccinated chimpanzee to another chimpanzee in a passive transfer experiment, the recipient chimpanzee would be protected against HIV-1 infection following challenge [569, 570]. Antibody was clearly responsible for this protection because serum contains only proteins and no cellular components from the donor chimpanzee. This finding was initially greeted with much optimism in light of the recent success at development an effective hepatitis B vaccine using a similar approach. However, hepatitis B, a DNA virus with relatively high sequence conservation, posed a far less formidable challenge than HIV-1, an RNA virus with enormous sequence diversity [571, 572]. The realization of this critical distinction was a stark reminder that antibody responses are generally type-specific and would only be expected to protect against a few strains of virus. In fact, when the passive transfer studies were repeated using a challenge virus that was heterologous (in relation to the vaccine strain), the vaccine no longer protected the chimpanzee [573].

Nevertheless, because it was still conceivable that these preclinical studies did not accurately simulate natural HIV-1 infection, a protein-based vaccine was developed using the outer surface protein of HIV-1 termed gp120 [574]. Such a vaccine could induce broader antibody responses but generally not potent cytotoxic T-lymphocyte (CTL) responses. Bivalent HIV-1 gp120 vaccines (made from two viral strains) were tested in two separate trials. The first trial involved mostly MSM at high risk of HIV-1 infection in North America and the Netherlands; they received either vaccine or placebo in a 2:1 ratio, respectively, in a randomized double-blinded control trial [575]. Several years into the trial, the number of HIV-1 infections did not differ between vaccinees and placebo recipients. A comparable trial in a high-risk cohort of IDUs in Thailand yielded similar findings [576]. This approach presumably failed to induce antibodies that could efficiently neutralize heterologous viruses [577–580]. Furthermore, these protein-based gp120 vaccines appeared to neutralize mainly laboratory-adapted viruses rather than strains derived directly from a patient (primary viral strains). Thus, it seemed unlikely that antibody responses targeting a single or at most a few viruses could protect against the large and diverse array of viruses present in any given population.

These disappointing attempts to make a vaccine that would neutralize primary isolates partially redirected the field toward vaccines able to induce CTL responses [581]. CTLs can identify and kill virally infected cells, and they can recognize a broader range of viral strains including primary isolates. CTLs are more difficult to induce than antibodies and usually require recombinant vector technology that encodes HIV-1 proteins allowing cellular presentation of the desired antigen [582]. The best-known recombinant vector involved a recombinant adenovirus serotype 5 (Ad-5) vector developed by Merck that was genetically modified to be replication deficient but encode the gag, pol, and nef proteins of HIV-1 [583]. Preclinical studies using an SIV-1 counterpart in a nonhuman primate model did not protect against infection; however, it did appear to retard disease progression in

that the vaccinated monkeys rapidly controlled viral replication and did not develop AIDS [584]. This other similar findings renewed optimism that although a vaccine might not protect against infection, if it held viral load at lower levels and protected against AIDS, it would still be highly advantageous. This development was even more promising in light of the recent epidemiologic association of decreased transmission risk with lower viral loads [367, 368]. These encouraging developments led to an efficacy trial known as the Step Study in a high-risk MSM and female population mainly in the Americas. Surprisingly, the vaccine not only did protect against infection, it actually seemed to enhance infection risk in the vaccinated MSM who were uncircumcised and/or had preexisting immunity to adenovirus, serotype 5 [585]. Furthermore, the vaccine did not decrease viral load or protect against CD4 T-cell count loss in those who did become infected despite vaccination. As a result, the Phambil study of the same vaccine that was just beginning in South Africa at the time the Step Study findings were released was stopped prematurely [586]. While the mechanisms underlying these findings remain unknown, development of recombinant adenoviruses serotype 5 vectors for use as a commercially viable HIV-1 vaccine has essentially ceased.

More recently, another study evaluating a recombinant Ad-5 vector was undertaken using a series of three DNA primes followed by a single recombinant Ad-5 boost. Unlike the Step vaccine regimen, this vaccine also encoded Env with the hopes of inducing effective antibody responses. Although this vaccine did not protect against infection, at least there was no evidence for enhanced infection in the vaccine group [587].

Concurrent with the Step Study, another efficacy trial in Thailand was testing a vaccine regimen designed to induce T-cell and antibody responses [588, 589]. A recombinant canarypox vector encoding HIV-1 gag, protease, and gp120 (ALVAC) was given in four doses, the last two injections being coadministered with the bivalent gp120 vaccine that by itself failed to confer protection in an earlier Thai trial. About 16,000 Thai individuals at low, moderate, and high risk of HIV-1 infection received either an active vaccine or a placebo. This vaccine regimen conferred about a 31 % overall protection against HIV-1 infection [590]. As with prior vaccines, no change in viral load or CD4 counts was seen in vaccinees who became infected. The modest efficacy rates and the nonsignificant protective effect, mainly in those at low to moderate risk, raised doubts about the validity of this trial. However, subsequent immunologic studies have demonstrated a correlation between protection and vaccine-induced antibody responses to a specific region of gp120 [591]. Although many questions remain, these findings generated enough optimism to plan confirmatory efficacy studies of a similar vaccine regimen and have provided ample scientific rationale for improving upon it [562].

9.3 Antiretroviral Therapy

For virtually no other viral infection has there been either the intensity of scientific investment or the number of commercial successes in treatment or prevention as for HIV-1. Since the first antiretroviral medicine, zidovudine (originally, azidothymidine), became available for the management of HIV-1, enormous progress has been made in the areas of therapy and prophylaxis. Antiretroviral medicines are now able to target multiple parts of the HIV-1 replication cycle (Fig. 43.19).

By 2014, FDA had licensed more than two dozen different antiretroviral medications for clinical use. At the global level, therapy depends on three classes of drugs given singly or in combination: nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and protease inhibitors. However, there is rather broad agreement among the responsible US and European health organizations and WHO on the general recommendations for which HIV-positive patients should receive antiretroviral therapy. This includes recommendations about when to begin treatment in the presence of pregnancy, a history of AIDS-defining illness, tuberculosis, HBV or HCV infection, and HIV-associated nephropathy. Likewise, experts agree that therapy should be administered or strongly considered for prevention of transmission by the perinatal, heterosexual and other high-risk routes [592].

The most up-to-date recommendations and guidance for the many alternative regimens for use in adults and adolescents, children, pregnant women and several other special patient groups in the US are conveniently assembled at the website [593]. However, not all experts and advisory groups agree on an identical strategy for starting treatment with these drugs. At the time this chapter was in press, the US Department of Health and Human Services had taken a more aggressive approach by recommending initiation of therapy in all HIV-positive patients regardless of clinical status, whereas the WHO and the European AIDS Clinical Society were more cautious about treatment of asymptomatic patients who are relatively immunocompetent.

There is also on FDA-licensed agent in at least three other classes of promising drugs—an integrase inhibitor, a CCR5 receptor antagonist, and a fusion inhibitor. Because these agents are rather new and expensive, widely integrated into it is not clear whether and how they may be incorporated into regimens for use at the population level.

Treatment of HIV-1-infected adults with cART has resulted in significant reductions in AIDS-related mortality [492, 494, 594]. With optimal response to cART (a rise in CD4 T-cell counts above designated levels), patients can even safely discontinue taking medications for the primary and secondary prevention of opportunistic infections [595]. Reductions in AIDS-associated morbidity and mortality achieved by cART have been dramatic, but even the most

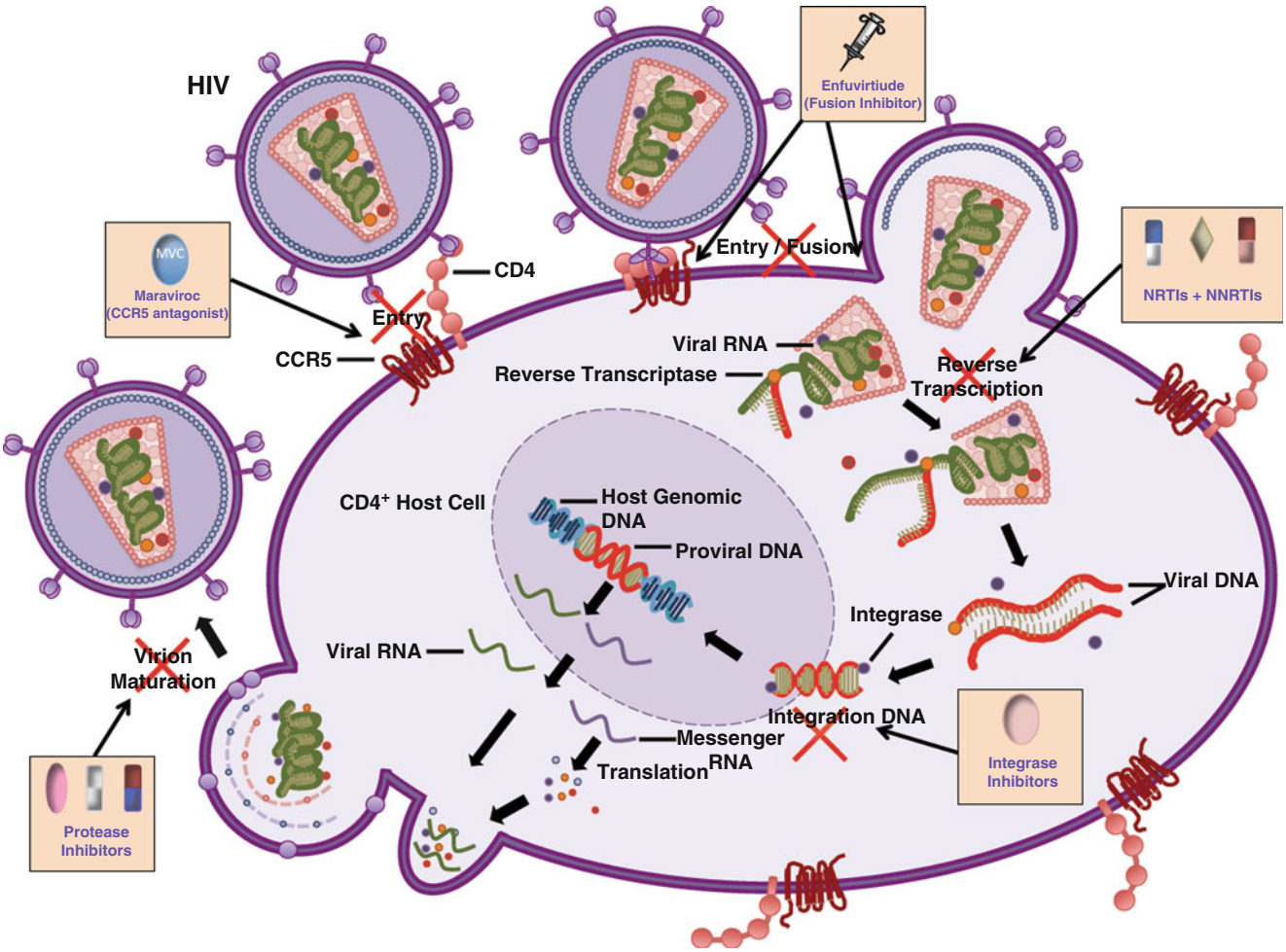


Fig. 43.19 HIV-1 replication cycle demonstrating mode of action of clinically available antiretroviral medications (Diagram is courtesy of Nilesh Amatya)

effective regimens do not completely abolish the chronic persistent immune activation that characterizes HIV-1 infection [510, 596–598], nor are these regimens entirely devoid of side effects of varying severity. Moreover, although it is premature to draw any definitive conclusions, there are indications that this chronically hyperactive immune state could be leading to side effects as described below. Furthermore, now that patients are living much longer without progression of their HIV-1 infection, coinfections such as hepatitis C virus are complicating the management of their disease (see Sect. 5) [599].

A variety of adverse responses to chemotherapeutic agents have occurred in distinctive patterns in populations. Three types of those responses illustrate the breadth of that variability. At one extreme is the case of the nucleoside analog reverse transcriptase inhibitor, abacavir, causing a severe inflammatory reaction. This complication was recognized as due to a striking, epidemiologically documented host genetic predisposition [600], which was promptly replicated in additional population studies and more recently corroborated with experimental work suggesting that drug-induced alterations of the HLA-peptide binding machinery and resulting T-cell response may be the mechanism [601]. A second response to cART is known by the name immune reconstitution inflammatory syndrome (IRIS) and by others. Its characteristic manifestation is a disproportionate immune reactivation including T-cell rebound in the presence of exposure to antigens of a variety of microbial agents and clinically significant opportunistic infections such as tuberculosis [602].

Another more insidious set of complications began to be observed soon after the introduction of the new, highly active protease inhibitors. Alone or in combination with other agents, they appeared to be associated with various patterns of fat redistribution and loss, dyslipidemia, and other metabolic derangements along with significant cardiovascular complications, that had not been previously observed in HIV-1-infected patients treated with other classes of antiretroviral agents [603, 604]. Despite intense study, the pathophysiology of these adverse consequences

of the combination of aging and treatment remains obscure. Although considerable attention has been paid to the adverse responses to agents in the cART repertoire, details about their distribution and determinants are beyond the scope of this chapter.

9.4 HIV-2

Treatment of HIV-2 infection has not been as well studied as HIV-1, but progress has been made in this area as well. While no strict guidelines have been established for HIV-2, it has been recommended that therapy starts when the CD4 cell count drops below 500/mm^3. One noteworthy distinction between the two viruses when using cART is that HIV-2 is inherently resistant to nonnucleoside reverse transcriptase inhibitors (NNRTIs) [605]. On the other hand, nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors, and integrase inhibitors all show varying degrees of activity against HIV-2 depending on the specific ART [605–610]. On the basis of both a limited number of cART trials performed on HIV-2-infected patients and in vitro suppression data, it is recommended that patients be given an initial regimen of a boosted protease inhibitor (lopinavir or darunavir boosted with ritonavir) along with two NRTIs (tenofovir plus either emtricitabine or lamivudine or zidovudine plus lamivudine). Whether or when definitive research is likely to be conducted on the efficacy of ART in preventing transmission of HIV-2 will likely depend more on the availability of resources than on the sense of urgency felt in the countries where the epidemic is most intense.

10 Unresolved Problems

After 30 years of an unprecedented worldwide mobilization of human and physical resources to understand and combat the HIV/AIDS pandemic, there are still countless conceptual uncertainties about many aspects of the virus and the disease, and there are numerous obstacles to developing and implementing control measures that hold the greatest promise. The following are a few of those that loom prominently and seem likely to be difficult to solve.

At the most fundamental level, it is not clear how the virus maintains latency and whether it can be safely dislodged from its reservoir through some combination of antiretroviral and immunobiological action short of stem cell transplantation. Despite intense investigation of both nonhuman primate and human models of viral control, it is still unknown which components of the immune system permit the host to withstand high-level viral replication, in the case of the sooty mangabey, or suppress it to undetectable levels, in elite controllers, with little or no significant clinicopathologic consequences.

The licensure of new classes of antiretroviral agents and development of increasingly effective combinations of drugs, in some cases with significantly lower costs, have raised expectations for further reversal not only of the global trends in morbidity and mortality but also of the incidence of new infections. However, the best of these regimens is not equally successful in all patients. The potential for induction of antiviral resistance in some number of treated patients will continue to challenge even countries with adequate resources, let alone those in financial and social hardship. In an additional proportion of patients without detectably resistant virus, either replication may only be partially suppressed and eventually escape from control, or control of viremia is not accompanied by a corresponding improvement in immune function. Management of these patients can be particularly difficult. For others who experience the reconstitution syndrome, even in those instances when its origins are recognized, the adjunctive therapies needed are imperfect. The dyslipidemia and the fat redistribution syndromes complicating the course of infection can also be difficult to manage. Their pathophysiology in the context of both progressive immunodeficiency and aging is still poorly understood, as is the role of ART in their appearance.

In the past few years, the evidence for the effectiveness of very important new prevention modalities has accumulated. First, clinical trials have demonstrated that male circumcision as performed in several populations can approximately halve the rate of female-to-male transmission. Second, a more recent trial has provided a remarkable proof of concept that early treatment of infected individuals can suppress viremia to the point of drastically reducing transmission to an uninfected sexual partner. These breakthrough studies have raised high expectations that, coupled with more widely instituted testing and counseling programs, the promising new approaches will be capable of preventing a large proportion of new infections. However, aggressive efforts to adopt these strategies will still have to overcome the cultural resistance to circumcision, the financial obstacles to providing treatment to ever larger numbers, and the theoretically greater potential for induction of resistance to antiretroviral agents.

The success of treatment-for-prevention programs will further depend on a solution to another major unresolved problem. Even the most advance countries, including the USA, have failed to reach as many as quarter or more of infected individuals through HIV testing programs broadly enough to ensure detection of HIV infection at an early stage. Approaches more aggressive than the current ones will likely be needed.

Persistence of replicating virus and inevitable progression to immunodeficiency in virtually every infected human who remains untreated implies that complete and lasting natural

immunity rarely occurs. The hope, as yet unrealized, is to formulate a combination of antigenic and immunobiological substances with or without adjuvants that will stimulate the cellular and humoral arms of the immune system enough to achieve long-lasting immunity. Despite years of the most intense scientific inquiry, the continuing inability to definitively establish the elusive correlates of protective immunity, the holy grail of any quest for an effective vaccine, is perhaps the most troubling of all the unresolved issues.

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Human Papillomaviruses: Cervical Cancer and Warts

44

Georgios Deftereos and Nancy B. Kiviat

1 Overview

Papillomaviruses (PV) are extremely widespread, small, non-enveloped, icosahedral, species-specific [1], double-stranded DNA epitheliotropic viruses [2, 3], which include human papillomaviruses (HPVs), known to infect humans. In humans, these viruses establish chronic epithelial infections, with each HPV type being associated with infection of specific anatomic sites and distinct natural histories [4]. The clinical manifestations of HPV infection include genital and cutaneous warts as well as genital tract lesions referred to as "intraepithelial neoplasia." Of greatest importance is that fact that a subset of HPVs (referred to as "high-risk" types of HPV) plays a central role in the development of most lower genital tract neoplasias as well as some head and neck carcinomas [5–10].

Classification of individual genotypes is based on sequence homology of the L1 region of the HPV genome [11]. More than 120 types have been identified, with additional new types being continuously cloned and characterized [11]. HPV types are classified as low-risk (LR) or high-risk (HR) types, depending on their association with cervical cancer. Currently 15 HPV types (16/18/31/33/35/39/45/51/52/56/58/59/68/73/82) are defined as HR HPV types, and 12 (6/11/40/42/43/44/54/61/70/72/81/CP6108) are defined as LR HPV types [12]. Over 40 types of HPV infect the anogenital tract, making HPVs the most common sexually transmitted viruses in both men and women.

Given its central importance in development of lower genital tract cancers, especially cervical cancers, detection of high-risk types of HPV now plays an important role in cervical cancer control, both for triage of women with equivocal cytological findings and for the follow-up of women treated for high-grade cervical lesions [13], and more recently as a primary screening test for cervical cancer [14]. Detection of HPV infection is also essential for epidemiologic studies to understand the natural history of HPV infection and the pathogenesis of cervical cancer [12].

While most genital HPV infections, including those with high-risk (HR) HPV types, are self-limited and without consequence, in some women infections persist and facilitate progression to cancer. Case–control studies among those with and without cervical cancer, as well as cohort studies examining risk of developing cervical intraepithelial neoplasia 3 (CIN 3) (the presumed immediate precursor of invasive cervical squamous cell cancer), have clearly shown that HR HPVs are central to the pathogenesis of cervical neoplasia including invasive cervical cancer (ICC) [15], with HR HPV DNA detected in 90–98 % of cervical cancers in different geographic populations. The mechanism by which HR HPV infection facilitates development of ICC is related to proteins produced by the HR HPV E6 and E7 genes. The interaction of E6 and E7 proteins with multiple cellular targets, such as the ones described later in this chapter, results in the deregulation of both the cell cycle and DNA repair [16]. Upon subsequent exposure to carcinogens and accumulation of mutations, malignant transformation occurs and ultimately leads to cervical cancer.

The direct implication of HPV in cervical carcinogenesis has led to the production of prophylactic vaccines to prevent infection with those types of HPV implicated in the pathogenesis of the majority of cervical cancers.

2 Historical Background

Descriptions of genital and cutaneous warts date from antiquity with a recognition of the viral nature of such manifestations dating from the early 1900s with the first human

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experimental model using cell-free infiltrate to cause warts dating back to 1907 [17] and preceded by animal models.

In the 1930s, Shope conducted studies on cottontail rabbits that established the contagious nature of cutaneous papillomas and led to the characterization of the Shope papillomavirus, now known as cottontail rabbit papillomavirus (CRPV). This was the first animal papillomavirus to be identified. Shope's studies, followed by those of others like Rous, represented the first studies in DNA tumor virology [18–20].

2.1 Early Studies of Human Papillomaviruses

Ancient Greek and Roman literature [21] contain descriptions of genital warts. In the 1890s transmission of human nongenital cutaneous warts from person to person was experimentally confirmed, with transmission by cell-free filtrate demonstrated in 1907 [17]. Observational studies have long recognized and generally described person-to-person infectivity of genital HPV infections, with Barrett et al. [22] in the 1950s being the first to document the development of genital warts in the wives of World War II soldiers who, before returning home, reported having had a new sexual partner and new penile warts.

Although the relationship between cervical carcinoma and sexual behavior has been suspected for over a century, it was only in the 1960s when specific variables such as "lifetime number of different sexual partners" were shown by epidemiologists to be the major sexual risk factor, in contrast to "lifetime number of sexual acts," strongly suggesting an infectious rather than traumatic etiology [23, 24].

The fact that HPV infections produce morphological changes in squamous cells (seen on both cytological and histological specimens) was observed early on, but the presence of such changes is both insensitive and non specific for HPV infection. These changes were termed *koilocytotic atypia* by Koss and Durfee (*Koilos* from the Greek κοίλος for *hollow*) [25]. Twenty years later, Meisels and Fortin [26] and Purola and Savia [27] proposed that flat cervical lesions demonstrating koilocytotic atypia were cervical equivalents of condyloma acuminatum (genital warts).

Cervical neoplasia is currently termed invasive cervical cancer (ICC) if the neoplastic (i.e., cancer) cells have extended through the basement membrane into the underlying tissue or as an intraepithelial lesion if the neoplastic cells remain limited to areas normally populated by epithelial cells and are not found below the basement membrane. ICC precursor lesions include cervical intraepithelial neoplasia (CIN) grades 1, 2, and 3, and carcinoma in situ. Our current approach to cervical cancer control is based on the concept that ICC arises from intraepithelial precursor lesions termed cervical intraepithelial lesions grade 3 (CIN 3) and/or carcinoma in situ (CIS) and that treatment of these precursor lesions prevents progression to invasive cancer. The CIN classification system, which is still in wide use, was proposed over 45 years ago, prior to any understanding of the epidemiology and natural history of HPV infections and their central role in the pathogenesis of ICC [28]. At that time it was believed that cervical intraepithelial neoplasia (CIN) grades 1, 2, and 3 represented a morphological and biological continuum of progressive, consecutive stages in the development of invasive cancer. Most women with CIN 1 were expected to progress to CIN 3 and, if untreated, to ICC [29]. We now know that most CIN 1 lesions simply represent a morphological manifestation of infection with HPV and regress within 1–2 years [30–32]. Few data are actually available on the risk progression from CIS to ICC. Data from a small series of women with CIS who inadvertently were not treated, from studies carried out before ablative treatment became the accepted standard of care, and from women repeatedly undergoing biopsies rather than definitive treatment [33, 34] suggest that, if left untreated, only a minority of cases (12 % to at most 40 %) would develop invasive disease within the span of a normal lifetime [32, 35, 36]. Furthermore, in recent studies looking at the relationship between acquisition of HPV infection and development of various grades of CIN, morphological changes that are classified as CIN 2 and CIN 3 are found early after infection with "oncogenic" types of HPV [37, 38]. Pathologists do not routinely attempt to differentiate between CIN 3 and CIS, as such morphological differentiation is known to be difficult and non-reproducible [39] and the clinical management of these lesions is similar. Unfortunately, using morphology (microscopy), we are unable to differentiate those cases of CIN 3/CIS which, if untreated, would progress from those that carry little or no risk of progression, and therefore all such lesions are treated. It is likely that in the future ICC precursor lesions will be defined by the presence of specific cancer-associated molecular changes.

2.2 Early Molecular Studies of Human Papillomaviruses

The early molecular biological studies of HPV established the plurality of HPV types, identified previously uncharacterized types, and established the association between types and lesions occurring in different tissues as well as with different degrees of neoplasia [4, 32, 40]. HPV DNA was detected in the majority of cervical neoplastic lesions, ranging from CIN 1 to invasive carcinoma and in cell lines derived from cervical carcinomas, most notably HeLa cells, one of the first established and most widely used cell lines [40].

Along with the advances in HPV DNA detection techniques, notable progress was made in the modality of noninvasive

specimen-obtaining methods. By the early 1990s more advanced and standardized DNA testing was available. This permitted more accurate detection of an increasing number of HPV types from anogenital and oral cytology specimens and permitted the characterization of new HPV types in numbers that have constantly increased from around 70 in the late 1990s [24] to more than 120 known to date [41]. Furthermore, the complete sequencing of the entire genome of many human and animal papillomaviruses has opened that way for the understanding of HPV infection pathogenesis and natural history of disease and has permitted advances in therapy and prevention. In 2006 the first FDA-approved HPV vaccine (Gardasil, Merck, Rahway, NJ) was introduced, covering types 6, 11, 16, and 18, representing the first vaccine specifically used for cancer prevention.

3 Human Papillomavirus Biology

Papillomaviruses are small (52–55 nm in diameter), nonenveloped, icosahedral viruses that have a tropism for squamous epithelial cells. They each carry an 8,000 base pair, single molecule of double-stranded DNA. Their genome guanosine–cytosine content is on average 42 %. Papillomaviruses have a capsid composed of 72 monomers (capsomers) [42]. The papillomavirus capsid is composed of two structural proteins: L1, which represents approximately 80 % of the capsid and has a molecular weight of 55kd, and L2, which has a molecular weight of 70 kd that represents the remaining percentage. Both proteins are encoded by the viral genome, and while both are normally incorporated in the capsid, only L1 is required for the capsid formation. In fact viral particles can be produced only by expressing the L1 protein and are virtually indistinguishable from those containing both proteins [43, 44].

3.1 Genome Structure and Molecular Biology

The various human and animal papillomaviruses sequenced have made possible to demonstrate that all have similar genomic structure and organization. Of the two DNA strands, only one is the coding strand and is characterized by a number of open reading frames (ORFs), classified as early (E) or late (L), based on their location on the genome.

The most upstream region of the genome, known as upstream regulatory region (UPR), is a noncoding region, representing approximately 12 % of the viral genome, and contains the origin of replication for the viral genome. The early region contains regulatory genes as well as genes necessary for the initiation of replication, while the late region is expressed (and L1 and L2 proteins are found) only in actively infected cells [45–47]. The position and organization of the various ORFs are found to be similar in the various papillomaviruses.

3.2 Viral Replication Cycle

The papillomavirus replication cycle is initiated by infecting the basal layer cells of the squamous epithelium. Viral replication goes hand in hand with the epithelial cell differentiation in that early phase proteins are the only ones detected in the basal and parabasal layers, and it is only in more differentiated squamous cells that late-stage proteins are expressed, with the production of new viral particles occurring in the highest layers [48–50].

3.2.1 Early Stages

The papillomaviruses have to infect directly the basal layers of the epithelium. In order to do so, it is believed that presence of cuts or abrasions leading to exposure of the basal and parabasal layers is necessary. Various cell membrane molecules, such as integrins and glycosaminoglycans, are possible receptors for the viral particles [51, 52]. The result of this interaction is clathrin-mediated endocytosis [51]. This mechanism of entry is mediated by L1 protein, while L2 appears to play a role in virion disassembly, genome escape into the cytoplasm, and transportation of the genome into the nucleus [51, 53–55].

3.2.2 Transcription

Papillomavirus transcription mechanisms are an expanding field, due to the presence of multiple promoter regions described to date and to the variable splice patters and mRNA end products in different cells. The majority of the mRNAs described and certainly the only ones found in warts share a common polyadenylation site (Ae) located at nucleotide 4,180, downstream of the early (E) genes, while mRNAs found in transformed cells show a polyadenylation site (Al) located at nucleotide 7,156, located downstream of the L1 and L2 ORF. The presence of this polyadenylation site can only be found in terminally differentiated keratinocytes [21].

Differences have been also described between transcription mechanisms in high-risk and low-risk HPVs. The E6 and E7 mRNAs in the high-risk HPVs share a common promoter (P97 for HPV-16 and HPV-31 and P105 for HPV-18), whereas in low-risk HPVs the E6 and E7 genes have different promoters. When E6 and E7 are transcribed with a common primer, the E6 mRNA can be intact or with splices within the E6 gene. If the E6 is transcribed intact, the E6, but not E7, is translated as there is not sufficient spacing for the E7 translation. If the E6 gene is spliced, there is enough space for the E7 ORF, so the translation of E7 can proceed normally [49, 56].

3.2.3 Transcription Regulation in Papillomaviruses

The upstream regulatory region (URR) contains a series of enhancing sequences that respond differently in various epithelial layers and have been found to contain binding sites for transcription factors as well as for cell nuclear proteins. The different layers of the squamous epithelia that are infected by papillomaviruses show different transcription patterns [57–59], and the level of keratinocyte differentiation appears to play a role in the way the viral genome is transcribed and spliced.

3.2.4 E2 Regulatory Proteins

Many of the regulatory functions in papillomavirus transcription have been found to be executed by the various E2 proteins [21, 60]. E2 has been described playing a role in transcription and DNA replication, among other regulatory functions. The first evidence of the E2 regulatory function came with the characterization of multiple response elements throughout the viral genome [61].

The E2 protein is found to be well conserved between the various viral types, being composed by two domains: one DNA-binding, sequence-specific domain, located near the C-terminal of the protein that has also dimerization function, and another one in the N-terminal that has a transactivating function. The two domains are separated by a variable hinge region. The C-terminal domain recognizes ACCN6GGT consensus sequences in promoters containing E2 binding sites. This signal is the main signal found in HPVs with some exceptions, like HPV-41 [61–63].

The E2 protein has been found to function in both activation and repression of transcription and has been well studied in anogenital HPVs. E2 can suppress the transcription of E6 and E7 leading to inhibition of growth in HPV-infected cervical cancer cell lines. This inhibition is due to the reactivation of the Rb and p53 pathways that are inhibited by E6 and E7, as evidenced by study models using bovine papillomavirus (BPV) E2 proteins [21, 64, 65]. The target elements of E2 have been widely studied, and several have been described in detail (Table 44.1) [21, 66].

Finally, E2 has an important role in maintenance of episomal viral genomes within infected replicating cells [76–78]. The TA domain is necessary for the maintenance of the viral genome in dividing cells, and specific mutations of this domain have been shown to disrupt the papillomavirus genome association to mitotic chromosomes. This interaction has been demonstrated to occur either with mitotic chromosome chromatin or even directly with the mitotic spindle [79–82].

Table 44.1 Cellular targets and functions of the papillomavirus E2 oncoproteins

Associated cellular proteins	Functional consequences	Ref.
Bromodomain protein 4	Tethers E2 and DNA to mitotic chromosomes	[67]
	Mediates transcriptional activation function	[68]
TATA binding protein (TBP)		[69]
AMF-1/Gps2	Enhances transcriptional activation	[70]
	Enhances E2 interaction with p300	[71]
YY1 transcription factor		[72]
Nucleosome assembly protein 1	Ternary complex with p300	[73]
p53	Apoptosis (high-risk HPV types)	[74]
CREB-binding protein (CBP)	Transcription activation	[65]
Papillomavirus E1 protein	Viral DNA replication	[75]

Adapted from Howley and Lowy [21], with permission

3.2.5 Late Phase Transcription

The late stages of the viral cycle occur exclusively in differentiated keratinocytes, located within the middle and higher strata of the squamous epithelium. In accordance to that the transcription of the late phase proteins (L1 and L2) is initiated only in these layers. The common L1 and L2 transcription promoter (Pl) is also shared by a series of other nonstructural genes that are similarly expressed only in more mature keratinocytes, including the E4 [83, 84]. Polyadenylation of the L1 and L2 mRNAs in HPVs has been described to start from a site (Pl) found 3′ to the L1 ORF, which is similar to the polyadenylation site described in BPV. This site has been found to be inactive in transformed cells [45]. Cis-regulatory elements for the late phase mRNA transcription have also been described in the 3′ UTR of several HPVs (HPV-16, HPV-1), and many of them have been described to be conserved among HPVs [85, 86].

3.2.6 Viral DNA Replication

HPV DNA replication is found to be dependent in great degree upon the level of differentiation of the host keratinocytes as after infection of the basal cells there is a replication of the viral genome up to a total of 50–100 copies which is maintained as a stable plasmid. In the upper layers of the stratified epithelium, the expression of viral genes required for viral genome replication is markedly accelerated, while in the terminally differentiated keratinocytes, the vegetative viral replication and packaging result in complete virion production [87, 88]. The initiation of replication necessitates the presence of the origin of replication element in cis and E1 and E2 in trans. The origin of replication is described to have a T-rich region and binding sites for E1 and E2. E1 is the central molecule in this process, and initial studies demonstrated that even in the absence of E2, the replication can be still initiated, and E2 has the function to stabilize the E1 to

the site of origin [89]. However, subsequent study models showed viral DNA replication in the absence of both the proteins [88].

The E1 protein interacts with α-primase, a DNA polymerase, and initiates the host cell initiation complex. Interactions of E1 with other molecules including histones have been described, but the significance of these interactions remains to be determined. Studies are available showing that interaction of E1 with cyclin-dependent kinases is necessary for active viral replication [90].

The E2 molecule interacts with E1 and becomes a precursor to a larger E1 multimeric complex that initiates replication and enhances E1 ability to initiate replication. E1 forms a hexameric form and demonstrates intrinsic ATPase and DNA helicase function that is essential to its function in DNA replication–initiation [91, 92].

3.3 Immortalization and Transformation of Human Papillomaviruses

Some of the papillomaviruses have been proven to be able to induce cellular transformation in vitro, and the first papillomavirus to be extensively studied was bovine papillomavirus 1 (BPV-1), using various established animal cell lines [93–95]. It was shown that in the BPV-1 DNA was maintained as a multicopy plasmid in rodent cells. The integration of the viral DNA into the cellular DNA was shown not to be a prerequisite for transformation of the cells that however kept their transformed state while expressing the viral genes [21]. In fact, previously transformed cells treated with interferon and returning to their untransformed state do not express the papillomavirus any longer [96].

HPVs have been subsequently studied in depth with regard to their role and mechanisms in cell immortalization and transformation. Studies with animal cell lines as well as human keratinocytes and fibroblasts showed the capacity of high-risk HPVs such as HPV-16 and HPV-18 to produce immortalized cells in contrast to low-risk HPVs (HPV-6 and HPV-11) that lack such capacity [97, 98]. While E7 ORF has been shown to be the most widely expressed in transformed cells, it is important to note that infection with these high-risk HPVs alone did not achieve cell transformation and that the E7 gene can act upon activated *ras* protein forms to induce transformation [99–101].

3.3.1 Human Papillomavirus E5 Gene

The E5 gene encodes for short hydrophobic peptides that are seen expressed in a variety of epithelial papillomas caused by HPVs and other papillomaviruses, and cell models have demonstrated how the E5 expression is necessary for optimal viral growth [102, 103]. Furthermore, E5 has been shown to have some transforming activity in rodent cell lines, and it has been hypothesized that it acts through modification of the EGFR as well as of other signaling pathways [104, 105]. Finally, E5 is found to be lost during viral integration, and it has been suggested that lack of E5 signifies a switch from benign lesions to establishment of malignancy and could represent a target for noninvasive (CIN2 and 3) lesions [104].

3.3.2 Human Papillomavirus E6 Gene

The E6 gene codes for a 150-amino acid protein that appears to be of similar structure in both high-risk and low-risk HPVs. The various E6 proteins seem to share similar activation elements as well as two common zinc fingerlike domains, each containing two Cys-X-X-Cys sequences. The transforming capacity present in E6 proteins of high-risk HPVs is identified in the ability to bind and complex to the p53 protein and expressed by a highly conserved C-terminus domain, able to bind to PDZ domain proteins; this capacity is not seen in the E6 of low-risk HPVs [106]. This interaction leaves p53 unable to activate its dependent promoters but also ultimately leads to the degradation of the p53 protein itself. In HPV-16 this degradation has been shown to be mediated by an E6/E6AP (an ubiquitin-protein ligase) that results in ubiquitination and degradation of p53 [107]. In fact the observed levels of the p53 detected in HPV-related neoplasms are low [108]. It has been shown that the proapoptotic function of p53 is diminished in the face of oncogene-mediated cell death signals that normally would lead to apoptosis, thus leading to immortalization of the affected cells [109–111].

The overall result is a significant decrease of the p53 levels in transformed cells. Similar results are found in vivo in HPV-positive cancer cells [108, 112]. In models that employed the use of cytotoxic agents to induce cell death, a peak of p53 expression was seen in native cells, whereas in HPV-immortalized cells expressing E6, this was not seen, as DNA damage-mediated apoptosis mechanism is deficient due to the lack of p53 [111, 112].

The E6/p53 pathway is not the only one implicated in HPV cell immortalization. Cell models using a version of E6 with a mutated p53-binding domain resulted in DNA genetic instability similar to the one seen in the loss of E6-mediated p53 loss, thus suggesting alternate pathways affected by the E6 protein [113]. One proposed mechanism activation of the catalytic domain (hTERT) of human telomerases. Lengthening of the telomeres seen in immortalized cells has been described as possible through transcriptional activation of the hTERT or through activation of the alternative lengthening of telomeres (ALT) pathway [114, 115]. This activation of hTERT can be mediated by E6AP or independent of E6AP, through Myc-binding sites within the hTERT promoter [116–119].

The p53 pathway can be inhibited by E6 in other ways as some studies proposed the interaction with p300/CREB-binding protein, a p53 coactivator, thus providing an additional

mechanism of p53 inactivation [120]. Other interactions have been described, but their clinical significance in most cases is still to be determined.

3.3.3 Human Papillomavirus E7 Protein

The E7 protein is a small, 100-amino acid protein that demonstrates two conserved regions, CR1 and CR2 and a C-terminal zinc-binding region. The conserved regions are very similar among the high-risk HPVs, as well as with other tumorigenic viruses, such as SV40. These regions are important for the transforming activity of the E7, through inhibition of retinoblastoma oncosuppressor protein (pRB), and its related pocket proteins p107 and p130 [121–123]. The E7 appears to bind preferentially to the hypophosphorylated form of pRB (inactive form) resulting in its inhibition, which results in promoting cell cycling and replication. High-risk HPV E7 proteins show different properties in inhibiting the pRB family of proteins than low-risk counterparts that appear to lack this capacity [121]. The CR1 domain has been described as the one with the capacity to bind the pRB pocket-related proteins, and CR2 contributes to their degradation [16, 100, 124].

Other targets for HPV E7 have been described including inhibition of the cyclin-dependent kinase inhibitor p27kip1. p27kip1 participates in the TGF-β-mediated growth inhibition mechanism seen in keratinocytes. With the inhibition of the p27, this mechanism is overridden resulting in growth promotion [125]. More recent studies have suggested that the inhibition of the p27 function is not a result of degradation but that of accumulation of p27 in the cytoplasm, with no effect in cell cycle arrest and contact inhibition [125]. Finally, E7 has been shown to have the capacity to inhibit the p21cip1 with loss of its inhibitory effect on DNA replication and may be essential to the replication of HPV DNA in differentiated keratinocytes, where normally the p21 pathway inhibits DNA replication [109, 124, 126–128]. Finally, E7 (along with E6) is believed to be necessary for the stable maintenance of viral episomal DNA. As shown by model forms, E6 and E7 mutant genomes may be unable to properly segregate on cell division and can be, as a result, lost from the cell [129].

4 Taxonomy and Classification

The papillomaviruses constitute the family of *Papillomaviridae*, divided in the genera of *papillomavirus*. Until recently 16 groups of papillomaviruses or individual papillomaviruses had fulfilled the criterion of genera. These genera are included in the International Committee on Taxonomy of Viruses classification and are indicated by Greek letters alpha to pi (ICTV: 2009 release). HPVs are members of five genera (Alpha-, Beta-, Gamma-, Mu-, and Nu-PVs). Each of the two genera (Eta- and Theta- PVs) contains a single bird papillomavirus, while the remaining nine genera contain one or several papillomaviruses isolated from various mammals. Research over the last decade confirmed the notion that phylogenetic congruence of virus lineages with those of the host species is an important although not the only mechanism of papillomavirus evolution. Consequently, search for papillomaviruses in previously understudied and remotely related hosts led to the identification of papillomaviruses, whose distant relationship with one another and with all previously published papillomaviruses fulfilled the criterion to establish 13 additional genera. These include the first two papillomaviruses found in reptiles (marine turtles) [41, 130]. All the animal papillomaviruses are indicated by abbreviations indicating their host organisms [130].

Since the more recently described genera exhaust the Greek alphabet, one of the proposed classifications uses the Greek alphabet a second time, employing the prefix "dyo" ("δύο," Greek for "two"), but omits the designations Dyoalpha, Dyobeta, and Dyogamma, since the Alpha-, Beta-, and Gamma-PV genera include the most common and medically important HPVs. The genera are listed in the order they were described with no necessary connection between adjacent genera. The same authors created a phylogenetic tree using the L1 nucleotide sequences of 189 papillomaviruses, which was generated using a Bayesian algorithm (Fig. 44.1) that is continuously changing with the addition of newly described species.

Within the species, HPV types are defined based on proportion of DNA sequence homology. In the 1970s and 1980s, HPV types were determined by liquid hybridization, reassociation kinetics assays [131]. Viruses with less than 50 % cross-hybridization to previously typed HPVs were called new types (and numbered sequentially in order of acceptance by the Papillomavirus Nomenclature Committee centered at the German Cancer Research Center in Heidelberg). Of note, 50 % cross-hybridization represents much more than 50 % nucleotide sequence homology. A subtype (assigned letters) was defined as a papillomavirus that cross-hybridized under stringent conditions with a given HPV prototype but had a restriction endonuclease pattern distinct from the prototype pattern. A variant was defined as differing from the prototype by an undefined but limited number of nucleotide sequences [1, 132].

The taxonomy of the HPVs, as shown in the phylogenetic tree (Fig. 44.2), agrees closely with the epidemiologic data associating specific types of HPV with different tissues and grades of disease. Specifically, the HPV types that tend to infect skin appear as a phylogenetically related group in the trees. The rare cutaneous types found mainly in patients with the immunodeficiency disease epidermodysplasia verruciformis are also shown in the tree to be closely related viruses [133].

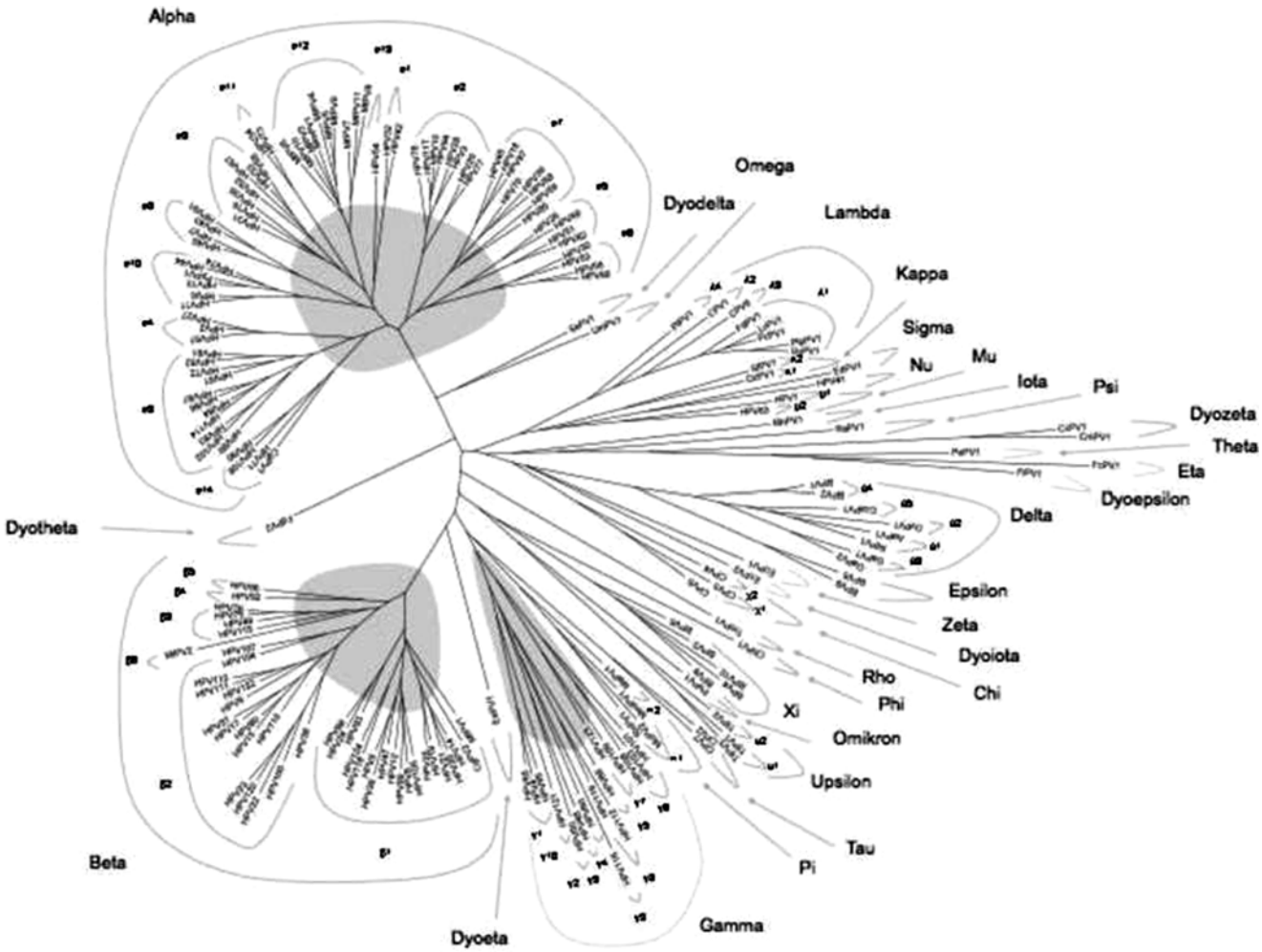


Fig. 44.1 Phylogenetic tree inferred from the L1 nucleotide sequences of 189 papillomaviruses (From Bernard et al. [130], with permission)

Among the mucosal HPV types, low-risk types found mainly in benign warty lesions cluster as a distinct group, separate from the high-risk types associated with invasive carcinomas. This concordance is evident in a comparison of the phylogenetic tree in Fig. 44.2 to epidemiologic data from large cross-sectional studies demonstrating the association between HPV type and grade of cervical neoplasia [134, 135].

In 1991, it was agreed by the Papillomavirus Nomenclature Committee that for a novel HPV isolate to be recognized as a new type, its entire genome must be cloned, and the nucleotide sequence of the three regions (URR, E6, and L1) should demonstrate less than 90 % nucleotide sequence identity with established papillomavirus types [24].

Previously described species HPV-46, HPV-55, HPV-64, and HPV-79 were proven not to meet the criteria as a unique HPV type; they were omitted and their numbers remain unassigned [41]. Among the more recently classified HPVs, HPV-101, HPV-103, and HPV-108 diverge convincingly from all other HPV types in that they lack an E6 ORF [136, 137]. In spite of this distinction, these three types are included in the genus Gamma-PV based on the present rules of sequence similarities in the L1 ORF and the resulting topology of the phylogenetic tree.

The definition and properties of PV subtypes and variants as DNA isolates with less than 10 % sequence diversity in the L1 gene have been discussed [41, 130]. While the ICTV does not implement taxonomic systems below the species level, nomenclature of variants of papillomavirus type based on complete genome sequences has been proposed in order to take into account the genomic heterogeneity, mainly within certain epidemiologically important HPV types (e.g., HPV-16, HPV-9). The proposed nomenclature systems have the intent to facilitate the comparison of variants across epidemiologic studies. Sequence diversity and phylogenies of these clinically important groups of HPVs provide the basis for further studies of discrete viral evolution, epidemiology (including geographic distribution), pathogenesis, and preventive and therapeutic interventions [138].

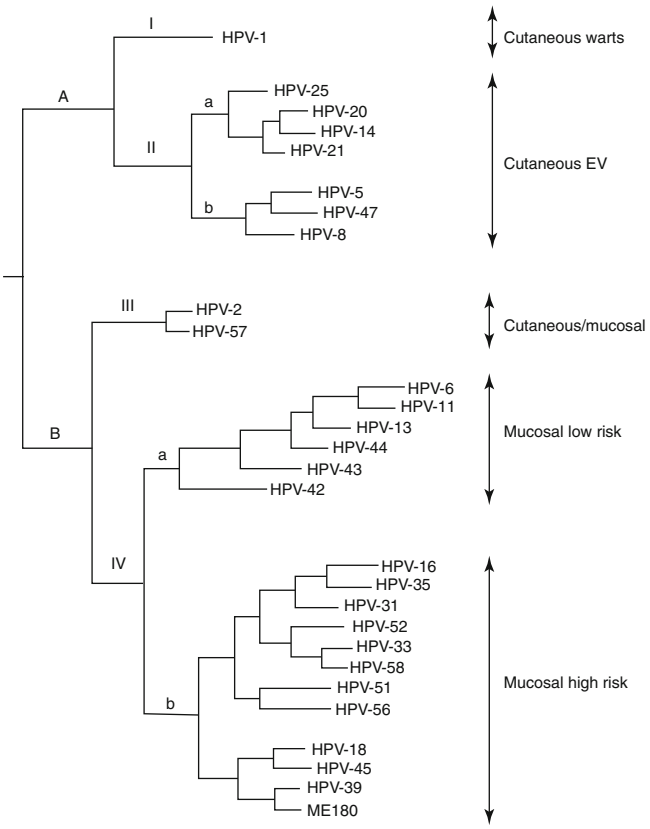


Fig. 44.2 Phylogenetic tree constructed from the alignment of 384 nucleotides in the E6 genes of 28 HPVs using maximum parsimony analysis (PAUP 3.0). To root the phylogenetic tree, bovine papillomavirus type 1 was used as an out-group (not shown). The labels on the right indicate the corresponding clinical category associated with each HPV type (From Van Ranst et al. [132], with permission)

5 Methodology Involved in Epidemiologic Analysis

Traditional virology methods, such as in vitro culture and serological assays, are not suited for detection of HPV. Replication of HPV is tightly linked to squamous epithelial cell differentiation, with capsids produced only in terminally differentiated squamous cells, making in vitro culturing of HPV difficult. Further, due to their strict species specificity, HPVs cannot be propagated in normal laboratory animals. The detection of serum antibodies to HPV is of little interest for the recognition of acute infection, due to the long time needed for the development of anti-HPV antibodies. Clinical and colposcopic examination, as well as microscopic examination of exfoliated cell samples (Pap smears) and tissue biopsies for koilocytes (cells whose nuclei demonstrate perinuclear clearing, with an increase in the density of the surrounding rim of cytoplasm), has been used for identification of HPV infections [139]. However, these morphological methods are both insensitive and nonspecific.

5.1 DNA-Based Detection of HPV Infection

HPV detection has relied on molecular assays that detect HPV DNA or RNA. Three categories of molecular assays are available for detection of genital HPV infection in tissue and exfoliated cell samples, all of which are based on the detection of HPV DNA. These include (1) non-amplified hybridization assays such as Southern transfer hybridization (STH), dot blot hybridization (DB), and in situ hybridization (ISH); (2) signal-amplified hybridization assays, such as Hybrid Capture assays; and (3) target amplification assays, such as polymerase chain reaction (PCR) and in situ PCR. Southern blot hybridization was initially used and considered the gold standard for HPV detection. However, it has serious shortcomings for routine clinical use. It requires large amounts of DNA and is extremely laborious. In addition, because interpretation of blot results is difficult and subjective, it lacks interlaboratory reproducibility. The presence of multiple or aberrant HPV types, incomplete DNA digestion by the restriction enzymes, and aberrant DNA migration and weak signals can all affect blot results. ISH has the advantage of preserving the topology of the tested tissue, thus allowing visualization of the location and physical state of HPV DNA. However, it also lacks sensitivity and specificity, as its limit of detection is approximately 5–10 HPV genomes per cell. Signal or target amplification assays are currently used in clinical and epidemiologic studies for the detection of genital HPV types. Both have a similar clinical sensitivity and specificity to detect the majority of genital HPV types.

5.1.1 Sample Collection and Preparation

HPV testing can be performed on a variety of samples, including biopsies; exfoliated cells from the female or male genital tract, urine, and blood; and paraffin-embedded materials. Exfoliated cervical epithelial cells can be collected using various devices that are available to collect cervical scrape samples for cytological examination. It is important that both the ecto- and endocervix are sampled to collect enough cells for HPV detection. Simple cotton-tipped applicators are well suited for this purpose because they do not sequester collected cells. Very little is known about the optimal devices for collecting exfoliated cells from men. In general, the collection medium of choice should be able to inactivate infectious agents and be free of PCR inhibitors. The most frequently used media include STM (Digene), 95 % ethanol, or ThinPrep PreservCyt solution. Formalin-fixed, paraffin-embedded, archived biopsy samples can also be used in PCR-based HPV tests. Several studies suggest that exfoliated cervical samples can be collected and stored as dry swabs for an extended time (up to a month) at room temperature without noticeable degradation of HPV DNA [140]. The samples are rehydrated and processed for genomic DNA isolation.

5.1.2 Signal Amplification Method

The Hybrid Capture II (HCII) assay approved by the US Food and Drug Administration (FDA) uses the sandwich enzyme-linked immunosorbent assay (ELISA) platform and a proprietary antibody against DNA–RNA hybrids. HPV DNA is released from clinical samples by denaturing in alkaline buffer and then hybridizing to a cocktail of HPV-specific RNA probes. The DNA–RNA hybrids generated are then captured by an immobilized antibody against the DNA–RNA hybrids. A second DNA–RNA hybrid antibody conjugated with peroxidase is used to recognize the DNA–RNA hybrids, and the hybrids are detected with a chemiluminescent substrate. The light emitted is measured as relative light units (RLUs) on a luminometer. The signal amplification is achieved through multivalent binding of antibody to the DNA–RNA hybrids and subsequent enzymatic reaction. Two sets of probes exist for detecting either low-risk HPV types (6/11/42/43/44) or high-risk HPV types (16/18/31/33/35/39/45/51/52/56/58/59/68), and they can be used either separately or in combination. For each assay run, the cutoff (CO) value is defined as the RLU of an appropriate calibrator sample at the concentration of 1.0 pg/ml in STM. The calibrator sample for the low-risk HPV test is cloned HPV-11 DNA, while the calibrator sample for the high-risk HPV test is cloned HPV-16 DNA. The result is reported as the ratio of RLU to CO. A sample is considered HPV positive if the RLU/CO is equal to or greater than 1 and negative if the RLU/CO value is less than 1. Samples with a RLU/CO ratio between 1.0 and 2.5 should be repeated to increase reproducibility. The assay is semiquantitative; although the RLU generated is proportional to the amount of HPV DNA present in the sample, there is no internal control for the input of cells in clinical samples.

5.1.3 Target Amplification by PCR

In PCR-based assays for HPV detection, the viral DNA is amplified in vitro by a DNA polymerase to generate an adequate amount of target, which is then directly visualized on gels, detected by hybridization using specific probes, or sequenced. The sensitivity of the PCR-based method is about 10–100 HPV viral genomes in a biopsy or smear containing 5×10^4 cells. Currently, there is no commercially available PCR-based assay for HPV testing in the United States nor are there standardized protocols for PCR-based methods. The analytical sensitivity and specificity of PCR-based assays depend on sample preparation, PCR reaction conditions (primers and polymerase used), and product detection methods. Furthermore, sensitivity varies as a function of the uneven distribution of multiple HPV-type infections in clinical samples. Type-specific PCR assays can detect only a single HPV type or a few very closely related ones. Since there are many different HPV types in any given population, this type of assay has limited utility on clinical specimens. However, being more sensitive than consensus PCR assays, the type-specific HPV PCR assays are useful in certain situations, such as in studies of persistent HPV infection. Several well-characterized consensus PCR-based assays are available for the detection of the majority of genital HPV types. The primers of these assays are designed that target the conserved regions in the HPV genome, such as the L1 region. Either non-degenerate consensus primers are used under low stringent amplification conditions, such as the GP5/6 and GP5+/6+ system, or degenerate consensus primers are used under stringent amplification conditions, such as the MY09/11 system. More recently, mixed primers have been developed to increase the sensitivity and reproducibility of degenerate primers, such as the PGMY09/11 and SPF systems [12].

The GP5/6 system has been extensively used in Europe. The primers are non-degenerate consensus primers that target the L1 region. By using a low annealing temperature during PCR, these primers are able to detect up to 27 different HPV genotypes, including types 43 and 44, which are less efficiently detected by the MY09/MY11 primer set. However, the low stringency conditions produce weak PCR signals and a relatively high background of co-amplified cellular DNA, which requires dot blot hybridization with a generic probe for unambiguous results. Using the GP–PCR consensus probe (consisting of 2 oligonucleotide probes cocktails identifying high- and low-risk HPV types), which was originally designed for GP5/6 primers, 1 fg to 10 pg of HPV DNA can be detected. An automated enzyme-linked immunosorbent format has been developed in conjunction with these primers. Biotinylated primers are used in the PCR reaction. The PCR products are captured on streptavidin-coated microtiter plates, denatured and hybridized to a mixture of digoxigenin (DIG)-labeled HPV consensus oligo probes. The DIG-labeled hybrids are then detected using an enzyme immunoassay (EIA). The sensitivity of the assay was found to be between 10 and 100 HPV-16 and HPV-18 viral genomes in a background of 100 ng of cellular DNA [141–143].

MY09/11 degenerate primers are based on conserved sequences of HPV types 6, 11, 16, 18, and 33 and target a 450-bp region of the L1 ORF of the genital HPV types [144, 145]. These primers have been widely used by researchers in North America, South America, and Asia. The amplified product contains both a conserved region and a highly differentiated region facilitating the design of both the generic and type-specific probes, respectively. HMB01 was added to increase the sensitivity for detection of HPV-51. Despite the presence of degenerate base sites, amplification across some HPV genotypes is uneven due to mismatches or low viral loads of specific HPV types. Because the random insertion of bases at degenerate positions is not a controlled process, and no analytical method is available to determine the proportion of each sequence in

each primer sample, the analytical sensitivity of the assay varies from primer batch to primer batch. For MY09/11, the generic probe was synthesized from the L1 PCR fragment of HPV-16, HPV-18, and HPV-31 using nested type-specific primers. Together, these fragments were found to detect a broad spectrum of HPV types. The presence of HPV is determined by dot blot hybridization with generic HPV probes. PGMY09/11 primers are an improved version of MY09/11 primers. They contain a pool of 18 primers (5 forward and 13 reverse), which amplify a 450-bp fragment at an annealing temperature of 55 °C. Importantly, each oligonucleotide is synthesized independently so that the pool contains a fixed proportion of each primer. Compared to the original MY09/MY11/HMB01 primers, the PGMY primers provide improved sensitivity, specificity, and reproducibility in detection of those HPV types that were not efficiently primed (especially when present at low levels) by the original MY09/11 primers [146, 147].

Another introduced system is the SPF1/SPF2 short PCR HPV consensus fragment system that originally consisted of a mixture of 6 primers (4 forward primers and 2 reverse primers used in equimolar quantities) which amplified a 65-bp fragment at an annealing temperature of 52 °C. Subsequently introduced, the SPF10 primer system improved on the original SPF1/SPF2 primers by adding four additional primers, for a total of 10 primers to increase the assay sensitivity [148]. The PCR product generated is hybridized to the generic probe in a microplate EIA assay to detect HPV-positive samples. Because the amplicon size is small, the SPF10 method is well suited for the analysis of paraffin-embedded materials or other specimens with degraded DNA.

More recently, Roche has developed a PCR-based HPV detection assay in a 96-well microplate format, the Amplicor HPV test, which was launched for clinical use in Europe. The kit uses a pool of 12 biotin-labeled primers to amplify a fragment of 165 bp in the L1 region. The HPV PCR fragment generated is then captured by immobilized probes that detect 13 high-risk HPV types. The biotin-labeled PCR product is then hybridized to horseradish peroxidase-conjugated streptavidin and detected using the chromogenic substrate. Sample adequacy is measured by including an additional primer pair for β-globin in the PCR reaction and detected with a separate microplate coated with the β-globin probe. The reaction mixture is read at 450 nm. An absorbance of greater than 0.2 is considered positive for the presence of HPV and β-globin.

Finally, PapType™ HPV test (Genera Biosystems, Australia) is a polymerase chain reaction (PCR)-based qualitative in vitro diagnostic test for the simultaneous detection and genotyping of 14 high-risk and 2 low-risk HPV genotypes with analytical sensitivity of 500 copies per reaction (PapType™ package insert).

5.1.4 Quantitative PCR

Although the HCII assay is quantitative, it does not normalize input cellular DNA. Current methods of HPV DNA quantitation rely on type-specific real-time quantitative PCR reactions using TaqMan probes (Applied Biosystems, Fall City, CA). Cellular DNA input is normalized by quantitating a housekeeping gene, such as β-actin. Since these assays are type specific, HPV genotyping of the sample is required beforehand. Multiplexing allows the detection up to 10 HPV types simultaneously. Similarly, HPV viral gene (E6, E7) expression can be quantitated using quantitative reverse transcription PCR on RNA samples. In general, the level of viral gene expressions is highly correlated with the viral DNA load. Quantitation of HPV DNA and RNA is of potential interest for several reasons. Since only 5–10 % of HPV-infected patients eventually develop cervical cancer, it is hypothesized that the level of HPV virus present (perhaps over time) might be associated with persistent infection and subsequent development of cervical neoplasia. Several studies showed that the viral load of HPV-16, HPV-18, and other high-risk HPVs increases with the severity of the lesion. Further, the viral load is associated with progression to cervical cancer and patient survival. More recently, it has been shown that HPV-18 E6/E7 transcript is associated with progression of cervical lesion [149–151]. However, due to the low prevalence of these other HPV types, larger studies are required to confirm this association for each individual HR HPV type.

5.2 Methods for HPV Genotyping

Currently, the well-established methods for HPV genotyping are various reverse line blot assays. These assays are based on solid phase hybridization of amplified HPV sequences to a slot blot membrane. The Liquid Bead Microarray (LBMA) assays are liquid-based hybridization assays and represent an alternative method, suited for high throughput and automation, being comparable to reverse line blot assays for HPV genotyping in clinical samples.

5.2.1 Roche Linear Array

The Roche Linear Array is a commercially available reverse line blot assay based on the PGMY09/11 PCR system and detects 37 HPV types (6/11/16/18/26/31/33/35/39/40/42/45/51/52/53/54/55/56/58/59/61/62/64/66/67/68/69/70/71/72/73/81/82/83/83/IS39/CP6108) (Roche Diagnostics, Pleasanton, CA). Specifically, HPV PCR products are generated using biotin-labeled primers, hybridized to a strip that contains type-specific HPV probes, and detected using the streptavidin-conjugated horseradish peroxidase and a chromogenic substrate. The sufficiency of the sample is monitored by the detection of β-globin gene.

5.2.2 Inno-LiPA

The Inno-LiPA HPV Genotyping (v2) assay is based on the SPF PCR system (Microgen Bioproducts, UK). The sequence variation in the inter-primer region has permitted the development of genotype-specific probes for 25 HPV genotypes (6/11/16/18/31/33/35/39/40/42/43/44/45/51/52/53/54/56/58/59/66/68/70/73/74). A recent study showed that the SPF10-INNO LiPA assay and Roche Linear Array assay are highly comparable and reproducible [152].

5.2.3 Liquid Bead Microarray

The LBMA assay is based on the Luminex-based technology (Luminex, Austin, TX), where HPV DNA is amplified using biotin-labeled consensus primers, then hybridized to a set of types of beads [153]. Each type of bead is attached with a probe specific to one HPV type. The beads with HPV DNA hybridized to them are then labeled with phycoerythrin-conjugated streptavidin and analyzed in the Luminex 100 instrument. Because of the unique fluorescent dye carried by each type of beads, HPV genotyping can be unambiguously determined. A recently developed and validated LBMA assay is available for genotyping 37 HPV types (6/11/16/18/26/31/33/35/39/40/42/45/51/52/53/54/55/56/58/59/61/62/64/66/67/68/69/70/71/72/73/81/82/83/83/IS39/CP6108) that can be useful for future clinical and epidemiologic research [153].

5.2.4 Other Novel Genotyping Methods

Several novel detection methods have been developed for HPV genotyping using DNA microarray, pyrosequencing, or mass spectrometry technologies. Based on DNA microarray technology, various HPV genotyping biochip assays have been developed, using different PCR consensus primers and detection method [154–157]. For example, Biomedlab in South Korea has developed HPV chips for genotyping of 15 high-risk (16/18/31/33/35/39/45/51/52/56/58/59/66/68/69) and 7 low-risk (6/11/34/40/42/43/44) HPV types [157]. In this assay, HPV is amplified using consensus primers in the presence of fluorescent-labeled nucleotides (Cy5-dUTP) and then hybridized to a DNA chip. The hybridized HPV DNA is visualized using a DNA chip scanner. Xu et al. developed an HPV biochip using spin valve sensor array and magnetic nanoparticle labels for genotyping four HPV types (16/18/33/45) [156]. The PapilloCheck test is a commercial DNA chip developed by Greiner Bio-One (Frickenhausen, Germany), which uses consensus primers targeting the E1 HPV gene and detects 15 high-risk HPVs (16/18/31/33/35/39/45/51/52/56/58/59/68/73/82), 2 probable high-risk HPVs (53/66), and 7 low-risk HPVs (6/11/40/42/43/44/70) [154]. More recently, a polymer-based HPV biochip has been developed using new consensus primers targeting the HPV L1-region and detecting 12 HPV types (6/11/16/18/31/33/35/43/52/56/58/73) [155]. The accuracy of these HPV biochip assays is determined by the sensitivities of the consensus primers to amplify all HPV types and the specificities of the probes to discriminate each HPV type. Early application of DNA pyrosequencing technology on HPV genotyping suggests that pyrosequencing is not only suitable for large-scale clinical application, but also able to identify novel HPV types and HPV variants. More recently, type-specific multiple sequencing primers are implemented to improve the ability of pyrosequencing assays to detect multiple HPV infections in clinical samples [158, 159]. Matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry (MS)-based assays have been also developed for HPV genotyping. Hong et al. described a method for HPV genotyping based on the restriction fragment mass polymorphism (RFMP) assay [160], while Soderlund-Strand et al. developed an HPV genotyping assay based on Sequenom MassARRAY platform [161]. These new technologies hold great promise in the future of clinical diagnosis. However, the successful implementation of these HPV genotyping assays requires the performance of studies to cross-compare different assays on a large number of clinical samples and the standardization of HPV genotyping assay protocols [162].

5.3 Recommendations and Limitations of Available Methods

In the United States, HCII is the only FDA-approved assay for detection of HPV. Furthermore, since there is no target amplification involved, the chance of sample contamination is low. This simple assay format is well suited for clinical settings because it does not require sophisticated equipment. Although the analytical sensitivity of the assay is lower than PCR-based assays, the HCII assay demonstrates a similar clinical sensitivity to target amplification methods. Therefore, HCII is the recommended assay for HPV testing in clinical settings. On the other hand, PCR-based HPV detection methods are recommended for epidemiologic studies where high analytical sensitivity and HPV genotype results are required. Currently, there is no commercially available PCR-based assay for HPV detection in the United States, although the Roche Amplicor HPV test has been launched in Europe. For clinical use, available PCR-based methods have shortcomings. Since there is no standardized protocol for sample preparation, PCR reaction conditions, and product detection, the analytical sensitivities and specificities of PCR-based assays vary from laboratory to laboratory. In addition, agreement between the HCII assay and various PCR-based assays for HPV detection in clinical samples is high.

It is important to recognize the limitations of both assays. For the HCII assay, cross-hybridization of the high-risk probe mix to low-risk HPV types, such as HPV-53 and HPV-66, can cause false-positive results. The assay also lacks

reproducibility for low viral load samples. In these circumstances, PCR-based methods might be used to corroborate the HCII result. Finally, the HCII assay cannot distinguish between single and multiple HPV infections since it does not provide viral genotype information; therefore, the assay is not suitable for detection of persistent HPV infection. Though PCR-based methods in general tend to have high analytical sensitivity, in comparison with the HCII assay, they have similar clinical sensitivity and lower specificity to detect cervical high-grade lesions and invasive cancer.

Several studies suggest that viral load, detection of persistent HPV infection, or detection of HPV integration into the host genome might be better markers for the risk of progression to cervical cancer. However, these assessments are not possible using currently available methods. Although the HCII assay is semiquantitative, it does not control for cell input. Even if cell input is controlled, it is still not possible to predict lesion size and percentage of abnormal cells in a clinical sample. It is impossible to determine whether the detection of a previously detected HPV type reflects persistent infection or a new infection because very little is known about the HPV life cycle and how fast the host immune system clears an HPV infection. Finally, because HPV integrates randomly into the genome, there is no molecular method that can be employed to assess the physical status of HPV (episomal or integrated) on large numbers of clinical samples [12].

5.4 Cytopathologic Diagnosis of Human Papillomavirus Infections

HPV infection can be diagnosed based on the histological or cytological recognition of characteristic cytopathic effects induced by the virus. In light microscopy, HPV can cause epithelial hyperplasia and a cytopathic effect in the skin or squamous epithelia that involves the superficial epithelial layers. This effect is related to the assembly of viral particles, followed by their release that occurs only in the superficial strata of the epithelium as described above. The cytopathologic changes are referred to as koilocytotic atypia [25]. Koilocytotic atypia, the hallmark of productive HPV infection, is characterized by nuclear hyperchromasia and wrinkling (described at times as "resinoid") associated with thick-walled perinuclear cavities that give the impression of "hallow" cytoplasm, hence the name as described previously. Virions in crystalline array are also visible by electron microscopy in the nuclei of degenerated cells. Hyperkeratosis (excess acellular keratin) or parakeratosis (nucleated keratinized cells) may be seen at the epithelial surface. The spiny or prickle layers may be particularly thickened (finding referred to acanthosis), particularly in exophytic, wart type lesions induced by certain types of HPV.

Recognition of exfoliated koilocytes, as part of cervical cancer screening, is considered characteristic for HPV infection. Analogous screening of other sites for HPV infection is also possible. For example, anal cytological screening can be performed in populations at high risk for anal carcinoma, such as homosexual men.

However, the low sensitivity and specificity levels of histological or cytological preparations for HPV-specific changes make the diagnosis of anogenital HPV infection impossible, relying solely on morphological diagnosis of HPV, because the findings are neither sufficiently accurate nor reliable. For example, based on conventional cervical cytological diagnostic practices, only about 10–20 % of HPV infections detectable by consensus primer PCR have concurrently abnormal cytological diagnoses of koilocytotic atypia. Cytological diagnosis of HPV is dependent on subjective interpretation of subtle morphological criteria, and the interpretation of the findings is hampered by wide interobserver variability [163, 164].

5.5 Clinical Diagnosis of Human Papillomavirus Infections

At the clinical level, some anogenital lesions can be diagnosed as possible HPV infections macroscopically. Skin warts have a characteristic raised appearance. Even when the lesions are flat and uncolored, on mucosal or skin surfaces, HPV-induced lesions may be diagnosed by application of 5 % acetic acid. HPV-induced lesions are usually "acetowhite," in that their color changes to white after local application of acetic acid. The mechanism behind this change in color is not known for sure, although several theories exist [165]. Acetowhitening is frequently used by clinicians examining the cervix, vagina, and vulva under magnification (colposcopy) following an abnormal cytological result, in order to identify and biopsy lesions. Cervicography is a clinical method of cervical cancer screening that also depends on acetowhitening to diagnose HPV in magnified photographic images of the cervix [166–168]. Because it represents a low-cost version of colposcopy, cervicography is adaptable to epidemiologic studies. However, acetowhitening is not specific for HPV infection as other types of epithelial conditions show similar tinctorial properties. Like colposcopy, anoscopy and peniscopy are possible in populations at risk of anal or penile carcinoma. Studies investigating the value of molecular testing of HPV from peniscopic and anoscopic samples are available [169–172].

The morphological diagnosis of HPV-related changes in anal lesions is the standard for defining the severity of anogenital HPV-related disease. However, the indications and modalities of histological and cytological specimen retrieval and diagnostic parameters related to these sites still remain to be fully defined [173].

5.6 Serological Assays

HPV types 16 and 18, the two most frequent cancer-associated types, as well as HPV-6 and HPV-11, the most common HPV types of genital warts, have been the HPV types most extensively studied, due to their higher epidemiologic impact. Associations have been described between HPV seropositivity and HPV-associated precancerous lesions like CIN [174] or carcinoma containing HPV-16 DNA [175–178].

While there is a relationship described between viral load and antibody response as well as persistent versus transient HPV infection and the capacity to produce HPV antibodies, epidemiologic studies have indicated that the proportion of women who develop these antibodies in the setting of a persistent infection varies and may be less than 60 % within 18 months of infection with HPV-16 and HPV-18 [179]. Although other studies have shown higher percentages [180–182], the response is not only patient dependent but also HPV-type dependent [179]. While HPV types differ in their immunogenicity, genetic and immunologic factors in individual patients regulate their capacity to produce antibodies against HPV, even in the setting of multiple HPV-type coinfections [179]. The chronologic relation between incident infection and antibody development varies; an interval of about 12 months following initial HPV DNA detection has been reported for HPV-16, HPV-18, and HPV-6 [179]. Furthermore, the antibody response tends to decline over time, despite the continued presence of detectable HPV DNA [179].

A variety of immunologic tests have been employed for the detection of antibodies produced, including enzyme-linked immunosorbent assays (ELISA), Western blot, and radioimmunoprecipitation assays (RIPA). Mainly, IgG and IgA antibodies have been assayed in serum, with a few studies of IgA in cervical secretions [174].

Among the HPV ORFs, E2, E6, E7, and L1 [183] are the ones most extensively studied, and for each ORF, several different epitopes have been tested. Researchers have attempted to define "HPV type-specific" epitopes as well as "type-shared" or "group-specific" epitopes reflecting exposure to any HPV [184]. The existence of apparently type-specific epitopes has been demonstrated by producing the same proteins (e.g., L1) from a series of HPV types and showing that antibody responses are restricted to a single HPV type or a few closely related types [179, 185].

Most antigens for seroepidemiologic studies of HPV have historically been produced as bacterial fusion proteins [185], chemically synthesized peptides [186], and in vitro-translated proteins [176, 178]. In the attempt to study the antigenicity of viral proteins in their native conformational state, viruslike particles (VLPs) have more recently been used for these immunoepidemiologic studies [179, 187, 188] and used instead of linear epitopes. Investigators have proposed that the neutralizing antibody response to HPV is directed predominantly against conformational antigens, created by the folding of the capsid (L1 and L2) proteins during virion assembly. As these antigens usually are not completely represented by linear epitopes, the use of VLPs has permitted a more accurate assessment of the immunologic response to the natural HPV antigenic epitopes. A number of investigators have successfully synthesized HPV VLPs for viral types 1, 6, 11, 16, 18, 31, 33, and 45 [48, 189, 190]. In the last years, specific antigenic peptides and their possible use as serological markers for HPV infection and HPV-associated malignancy have been studied [188, 191, 192], but the advancements in this field are far behind compared to DNA testing for HPV, mainly due to the extensive variability and the partial understanding of the immune response against these viruses and even more so its clinical significance. The major advancements in DNA testing for HPV detection and typing make HPV DNA testing the mainstay for epidemiologic and clinical methodology.

6 Descriptive Epidemiology

6.1 Prevalence and Incidence Data

HPV infections are extremely common in the general population. However, in the estimation of the exact burden of HPV, several factors should be taken in account: the type of tissue under study, the methods employed in the sample collection and analysis, and the geographic and other population characteristics. All the factors that are implicated in the natural history of disease caused by HPV infection complicate its epidemiologic study. Due to the clinical impact of cervical cancer, extensive studies and more reliable HPV prevalence estimates have been generated for the genital region, particularly the cervicovaginal epithelium, with increasing data available for other tissues.

6.1.1 Prevalence of Cervical Human Papillomavirus Infections

Studies looking into HPV cervical infection incidence and prevalence have shown highly disparate results, depending on the modality of collection of specimens, the analytical methods, and the age and sexual practices of the population. Individuals of younger and with higher levels of sexual activity appear to be at greatest risk; among the various populations studied, these groups appear to have the highest prevalences of HPV infection. This emphasizes the importance of accounting for these factors in reporting observed prevalence, rather than summarizing figures for whole populations [193–195].

Using the cytological diagnosis of koilocytotic atypia as the measure of HPV infection, the point prevalence varies

considerably among independent studies, and different populations with similar characteristics have shown different prevalences [196–198]. Conversely, studies employing more than one analytical technique have yielded different results when studying the same population [197]. Furthermore, the diagnostic criteria employed in the various studies are not uniform with regard to accepting the sole detectable HPV DNA in the population versus using clinical and cytological criteria for the definition of HPV infection. Early studies demonstrated significant percentages of detectable HPV in women with normal pap smears. Cervical HPV infection as defined by HPV DNA detection in cytologically normal women was higher in younger women with negative cytologies, while older women with negative cytologies showed lower percentages of HPV positivity. These findings were attributed to transient HPV infection with no clinical significance. Conversely, among women with cytological atypia, the percentages of HPV positivity did not change with age [199].

Table 44.2 presents data from large-scale HPV testing of cytologically normal women screened at Kaiser Permanente health clinics in Portland, Oregon [24]. Two different testing methods were used to study this population. The first group of women was tested for HPV using a consensus primer PCR, followed by typing of the amplification product by dot–blot hybridization. The second group was tested for 16 specific types of HPV by Hybrid Capture. Using either test method, HPV-16 was observed to be the most common individual type among cytologically normal women. Most HPV types individually accounted for only a small proportion (<10 %) of the total infections of this population. Unknown types at that time, cumulatively considered as a group, still accounted for a sizable fraction of infections among cytologically normal women tested by PCR.

The estimated prevalence values of HPV infection among women around the world range between 2 and 44 % [33]. The wide variation in estimates is largely explained by differences in the age range of the populations studied and the sensitivity of the DNA assay used for detection of HPV infection. Various methods used in these studies and the improvement of techniques over the course of the years also account for these different results. These DNA-based studies, when combined with measurements of type-specific antibodies against HPV capsid antigens, have shown that more than 50 % of sexually active women have been infected by one or more genital HPV types at some point in their lives. In a Planned Parenthood clinic population in the United States with mean age of 25 years, the prevalence of high-risk HPV infection was 27.4 % [200]. Similar prevalence estimates have been found among female university students in the United States and Canada [201, 202]. A recent study in Scotland showed the prevalence of PCR-detected HPV DNA in women with a mean age of 36.6 years attending routine cervical cancer screening to be approximately 20.5 % for all HPVs and 15.7 % for HR-HPVs [203], while a more recent study in Turkey showed a 17.9 % PCR-detected HPV DNA prevalence among women with normal cervical smears [204]. HPV-16, which is one of the more common types among cytologically normal women, is also the most common type among cervical cancer cases [36, 199, 201, 202, 205–208]. The prevalence of type-specific HPV infections among HPV-infected population-based controls from the International Agency for Research on Cancer (IARC) cervical cancer study and from a US Planned Parenthood population is shown in Table 44.3.

Table 44.2 Prevalence of individual HPV types among cytologically normal women, assayed by L1 consensus primer PCR or Hybrid Capture in cervical specimens from two random samples of Portland Kaiser gynecology patients (individual prevalence estimates expressed as percent of total positives)

	PCR ($n=453$)	Hybrid capture ($n=12{,}366$)
Total HPV positivity	17.6 %	4.6%[a]
HPV types (% of positives)		
6/11	9.7 %	9.0 %
16	14.6 %	16.8 %
18	1.2 %	7.9 %
31	4.8 %	9.0 %
33	1.2 %	5.4 %
35	1.2 %	5.1 %
39	2.4 %	12.1 %
40	2.4 %	N/A
42	N/A[b]	15.8 %
43	N/A	6.7 %
44	N/A	7.0 %
45	4.8 %	7.9 %
51	3.6 %	12.3 %
52	6.1 %	11.9 %
53	7.3 %	N/A
54	7.3 %	N/A
55	3.6 %	N/A
56	7.3 %	11.4 %
58	4.8 %	5.8 %
Unknown or uncharacterized[c]	23.0 %	N/A
Multiple types	26.1 %	26.1 %

Adapted from Schiffman and Burk [24]: Data from Schiffman M, Lorincz A, Manos M, unpublished at time of publication (1997). With permission

[a]Hybrid Capture was performed for 16 specific types of HPV; thus, the total percentage of positivity cannot be compared to PCR without adjusting for which types were included in the respective tests

[b]*N/A* not assayed

[c]Types not characterized at the type of original PCR testing. Several of these types have since been assigned numbers, e.g., HPV-66 and HPV-68, or have been found to be subtypes or variants of known types

6.1.2 Incidence of Cervical Human Papillomavirus Infections

It is important in natural history studies to distinguish incident (newly acquired) cervical HPV infections from prevalent infections, because prevalence reflects the duration of

Table 44.3 Type-specific HPV DNA prevalence among HPV-infected women

HPV types	Percentage of all infected women (median age of 46 years) (IARC)[a]	Percentage of all infected women (median age of 25 years) (USA)[b]
16	24.3	23.7
18	7.3	7.2
31	4.2	6.0
45	3.5	4.7
35	2.7	3.7
58	2.3	5.1
6	2.3	6.3

Adapted from Baseman and Koutsky [209], with permission
[a]Population-based control group of women from IARC cervical cancer studies in nine countries [208]
[b]Planned parenthood population from Western Washington, unpublished data [209]

infection as well as the incidence rate. However, this distinction has proven difficult to make for HPV. To distinguish HPV incidence from recurrence requires the knowledge at the outset that a woman has not been previously infected. With no marker of lifetime HPV exposure, the possibility of viral reactivation from a "latent" state below the level of current molecular detection must always be considered as an alternative interpretation of apparent incidence [24]. New acquisition of HPV is very common, and sexually active young females are the population group with the highest risk. The incidence of infection with high-risk HPV types appears to be higher than with low-risk types [201, 202, 205, 210]. In a prospective study in the United Kingdom, the cumulative incidence of HPV infection among women aged 15–19 was found to be 44 % over a 3-year period and increased to 60 % at the end of 5 years [36]. Similar studies in the United States have yielded similar results. Among two populations of college women in the United States, the 3-year cumulative incidence of HPV infection was 43 % in one study [201] and 42.8 % (95 % CI: 38.0, 47.9) in another [211]. In a cohort study of Brazilian women, the cumulative incidence of HPV infection was 23.6 % over 18 months [205], an estimate similar to the 18-month cumulative incidence figures in previous studies.

The differences in age-specific incidence rates for cervical HPV infection seem to parallel the differences in age-specific prevalence rates presented above. In particular, the apparent incidence of cervical HPV infection, as measured by new DNA detection or first cytological diagnosis under observation, is highest in sexually active young women, going hand in hand with the observations made on the prevalence rates of HPV in the various population groups [212, 213].

6.1.3 Prevalence and Incidence of Genital Warts

The clinical manifestation of a number of HPVs belonging in the low-risk category is an exophytic lesion known as *condyloma acuminatum* or simply genital warts. These are histologically characterized by arborescent, spiky papillae surrounding thick fibrovascular cores and showing normal maturation of the keratinocytes. These lesions show sharply demarcated base, with no evidence of invasion, and may present koilocytic changes. HPV types 6, 11, and related types [213] are the most commonly associated types with these lesions. However, the data available regarding the prevalence and incidence of genital warts are essentially based on clinical observation, as in common clinical practice these patients do not undergo DNA testing for HPV. Thus, the prevalence and incidence of clinically diagnosed genital warts, certainly a measure of infection with these HPV types, most likely represents an underestimation of the true infection rates.

A study by the California Family Planning Access Care and Treatment (Family PACT) program, looking at a little over 2 million people between 2007 and 2010, showed a prevalence of 0.7 % for females and 3.3 % for males in the general population [214] with the highest rates in the 20–25 years age range. Among a series of women in another clinical setting, genital warts are most commonly found in the following sites (in rough order of decreasing prevalence): the posterior part of introitus, labia minora and clitoris, labia majora, perineum, anus, vagina, urethra, and cervix. In men, the order of frequency is reported to be the frenum, corona and glans, prepuce (foreskin), meatus, shaft, anus, and scrotum [24, 215]. Finally, it has been noted that the clinical manifestation of genital warts usually involves more than one site in the genitourinary area, and very commonly these lesions are associated with concomitant HPV infection of the vagina and cervix, as documented by clinical and DNA testing [216–218].

6.1.4 Prevalence and Incidence of Nongenital Cutaneous Warts

Skin warts are so common and demonstrate so benign behavior that they raise very little epidemiologic interest. As a result of that, there are very few studies giving exact epidemiologic estimates available. Furthermore the lack of PCR assays used in these clinical settings makes the data less reliable.

One of the largest population surveys of the prevalence of skin diseases in the general population was conducted as part of the US Health and Nutrition Examination Survey (HANES) of 1971–1974 [219], studying over 20,000 subjects, and showed an estimated population prevalence of 850 per 100,000. More recently a study employing a PCR-based assay examining HPV types involved in cutaneous warts demonstrated that HPV types 1, 2, 27, and 57 were most commonly associated with cutaneous warts, being present in 89 % of cases [220].

6.1.5 Prevalence and Incidence of Other Human Papillomavirus Infections

The prevalence of vaginal HPV infection cannot be easily separated from cervical HPV infection, because vaginal cytological

specimen collection is characterized by admixture of exfoliated cervical cells. While the few small studies of HPV DNA prevalence in women with hysterectomies have found vaginal HPV infection to be common [221], the range of HPV types detected by cervicovaginal lavage is virtually identical to the types found in cervical swabs and scrapes [222] supporting the theory of concurrent infection of the urogenital tract by the various HPV types. HPV-related koilocytic cytopathic effect is well described, but, as cytological surveillance of vaginal lesions is not standardized as it is for cervical HPV infections, the assumption is that albeit the frequencies of infection by HPV for the vagina and cervix are similar, the malignant potential of the various HPV types is tissue specific.

In estimating the prevalence of HPV infection, the vulva must be considered as two separate subsites, the introital mucosa of the labia minora versus the cornified skin of the labia majora. The HPV types that cause exophytic genital warts (6, 11, 42, 44, and related types) commonly infect both subsites of the vulva, while the viruses associated with cervical carcinoma (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 54, 55, 56, 58, 59, 64, 68, and related types) infect the labia minora with higher prevalence, comparable to that of cervical infection [223], although high-grade intraepithelial and invasive vulvar neoplasias are relatively rare compared to cervical neoplasia. Other sites potentially infected include the urethra [224].

Similarly in the male, infection of the penis with cancer-associated types of virus appears to be very common. As with the vulvar skin, noninvasive sampling of the penile epithelium is difficult. Prevalence estimates of HPV infection (as measured by PCR-based assays of HPV DNA) in young men attending sexually transmitted disease clinics have given estimates as high as 84 % [225]. HPV-containing lesions of the penis have reportedly been found in a high percentage of men whose sexual partners have CIN [226, 227].

In a recent study conducted in the United States, Mexico, and Brazil with a combined study group of 1159 men, the incidence of a new genital HPV infection was 38.4 per 1,000 person-months, defined as newly diagnosed HPV infection by HPV DNA PCR detection. The presence of high-risk HPV infection was found to be significantly associated with a higher number of lifetime female sexual partners but also with a high number of male anal sexual partners. This study also looked into the median duration of infection and concluded that the clearance of high-risk HPV infection decreased in men with a high number of lifetime female partners. Age was seen to play a role in prognosis as clearance of high-risk HPV was more rapid in increasingly older patients [228].

HPV infection of the anus has been studied particularly in the context of human immunodeficiency virus (HIV)–HPV interactions in homosexual men [15, 173, 229]. HPV infection of the anal epithelium is very common in homosexual men, as diagnosed by HPV DNA detection or by morphological characteristics in cytological or histological specimens, independent of HIV status. Studies in HIV-negative homosexual men have shown that the prevalence of anal HPV infection is around 75 %, while that of high-risk HPV types is more than 50 % [230]. Positivity for HPV in squamous carcinomas of the anus has been reported as high as 93 % [173]. In accordance to the findings of urogenital lesions, HPV-16 is the most prevalent, while the most common types are the ones observed in the genital areas. The prevalence of HPV infection of the anus in the general male population is unknown. A few studies of anal carcinoma in women have suggested correlations with cervical carcinoma in the same populations, implying a common etiology most likely to be anogenital HPV infection [231].

HPV types showing tropism for the anogenital mucosa as well as nongenital cutaneous types can infect the oral cavity and other regions of the upper aerodigestive tract [232]. HPV can be found in biopsies from a variety of lesions of the oral cavity and appears to be the etiologic agent for one type of benign lesion called focal epithelial hyperplasia [233]. Despite studies available where HPV infection is tested in patients with oral squamous cell carcinomas, a cause-and-effect role for HPV in these carcinomas has not been established [234]. Studies examining the prevalence of HPV DNA in the oral cavity in the general population show rather discrepant results [235].

Described presence of detectable HPV in various other human tissues, including the esophagus [236], lung [237], prostate [238], and bladder [239], remains to be defined with regard to a possible clinical significance.

6.2 Epidemic Behavior

HPV infections are most commonly transmitted by person-to-person contact. It has been suggested that minor epithelial tearing is a prerequisite to transmission, permitting the virus to reach the target basal cells that are the virus initial targets. Transmission through the sharing of fomites HPV could be possible, and few studies have shown presence of HPV DNA on underclothes and gynecologic equipment [240] suggesting a mechanism similar to the transmission of plantar warts among school children. Similarly, airborne transmission of viral particles appears plausible in very special settings, such as laser ablation of genital warts in a gynecologist's office, with resultant virus-containing laser plumes [241]. The viability of HPV outside of the human body under varying conditions and the deriving risk of transmission are still unknown. Vertical transmission has been recently evaluated and the possibility of transplacental HPV infection has been reported as high as 70 % in immunocompromised mothers [242]. Other studies examining vertical HPV transmission have detected HPV DNA in amniotic fluid, cord blood, and fetuses. HPV transmission have detected HPV DNA in amniotic fluid, cord blood, and fetuses. These studies demonstrated that the virus can infect children during the perinatal period [243–246], while other

studies showed the possibility of horizontal, nonsexual transmission between family members and particularly between parents and neonates [247].

The age-specific prevalences of different types of HPV infection support the view that most infections are passed from infected to uninfected individuals. Common cutaneous warts (HPV-2 and HPV-4 and other types) are most frequently seen in school-aged children and adolescents [248]. Plantar warts have a later modal age peak in adolescence and early adulthood [248]. Male and female genital HPV infections (as measured by DNA, cytological diagnosis, or presentation of overt genital warts) peak in early adulthood concurrent with the age of usual onset of sexual intercourse [193, 199]. These peaks in age-specific point prevalences appear to represent the combined effects of exposure and immunity [24].

6.3 Geographic Distribution

6.3.1 Cervical Cancer Geographic Distribution

The geographic distribution of HPV DNA detection has been studied mainly in correlation with cervical cancer incidence rates, and efforts have been made to determine whether variation in prevalences of HPV measured by DNA would be reflected in cancer rates. In earlier studies, geographic differences in HPV did not correlate consistently with geographic differences in cervical cancer incidence [249].

In 2008 cervical cancer was the third most common cancer in women in the world, and the seventh overall, with an estimated 530,000 new cases in 2008. More than 85 % of the global burden occurs in developing countries, where it accounts for 13 % of all female cancers. High-risk regions are Eastern and Western Africa with an age-standardized rate (ASR) greater than 30 per 100,000, Southern Africa (26.8 per 100,000), South-Central Asia (24.6 per 100,000), South America, and Middle Africa (ASRs 23.9 and 23.0 per 100,000, respectively). Rates are lowest in Western Asia, Northern America, and Australia/New Zealand (ASRs less than 6 per 100,000). Cervical cancer remains the most common cancer in women only in Eastern Africa, South-Central Asia, and Melanesia. Overall, the mortality–incidence ratio is 52 %, and cervical cancer is responsible for 275,000 deaths in 2008, about 88 % of which occur in developing countries: 53,000 in Africa, 31,700 in Latin America and the Caribbean, and 159,800 in Asia (World Health Organization – International Agency for Research on Cancer, GLOBOCAN 2008 data).

6.3.2 Cervical HPV Infection Geographic Distribution

Studies on the prevalence of HPV DNA in cervical specimens from women with negative cytological results have findings that vary considerably, due to the selection of study subjects and to the different tests used to detect HPV DNA. A recent multicenter, centrally coordinated international study conducted by the International Agency for Research on Cancer (IARC) has provided data from 15 areas in 4 continents regarding women aged from 15 to 74 years. The age-standardized prevalence ranged from less than 5 % in some Mediterranean and Southeast Asian countries to more than 15 % in several countries in Latin America and among a few African populations [250]. In a comprehensive review of studies that used standardized inclusion criteria and controlled for variables that may have challenged the comparability of the studies, prevalence estimates of HPV infection among women with negative cytological results ranged from 10 to 15 %.

Age-specific prevalence estimates showed HPV DNA to be more prevalent among very young women, with a decline in young adult women and a variable pattern afterwards. In some countries, notably in the Americas, the prevalence increased again in postmenopausal age groups. In Europe, a plateau in the middle-aged groups was maintained, whereas in other high-prevalence countries in Asia and Africa, the prevalence remained fairly constant across all age groups [251].

6.4 Temporal Distribution

6.4.1 Cervical Human Papillomavirus Infections

In order to examine trends of HPV infection over time, sequential datasets submitted by different cancer registries are being used. The vast majority of data available involves cervical cancer, and while some conclusions can be made for high-risk HPVs, there is relatively little data for low-risk HPV infections. Although there is substantial variation in terms of the age-standardized incidence rates observed among different countries, generally, in developed countries, the incidence is declining at a broadly similar rate in the more recent time period. While the absolute incidence rates will, to a certain degree, reflect the prevalence of high-risk HPV infection in the respective populations, the decline is thought to be predominantly a result of the introduction of effective screening procedures combined with a range of sociocultural and economic factors [252]. Unfortunately, most of the data available comes countries with higher socioeconomic status, and while they may suggest that there is an overall worldwide decline in incidence, this is not universally the case. In some populations, mainly from parts of East and Southeast Asia and Eastern Europe, where data is available and in the absence of effective screening, incidence has remained stable or increased in successive generations of women as a result of changing sexual behavior and increasing risk of persistent HPV infection [252].

The GLOBOCAN 2008 database gives the possibility to make projections of the expected burden of cervical cancer up to 2030. These projections are based entirely on predicted variations in the demographics of the various populations,

including expected growth variations and changes in age distribution. Based on these estimates from global burden for cervical cancer of 530,000 new cases in 2008, it is projected that the annual incidence rates will increase by approximately 2 % annually to an estimated 770,000 new yearly cases by 2030 (World Health Organization – International Agency for Research on Cancer, GLOBOCAN 2008 data). However, these estimates are an average of the various regional trends and that again are very different between developed and developing countries [252].

Even within the same population however, different subgroups present their own particular trends. In a study taken place in Finland, the Finnish Maternity cohort, comparing between the time periods 1983–1985 and 1992–1994, the incidence of HPV-16 among women age ≤22 was seen to drop from 25/1,000 person-years to 13/1,000 person-years before increasing again to 31/1,000 person-years in the period 1995–1997. In particular, for women between the ages of 23 and 31, the HPV-16 incidence increased steadily from 5/1,000 person-years to 13/1,000 between 1983–1985 and 1995–1997 [253]. For noncancerous HPV-related disease, far less global data is available. In the United States, the age- and gender-adjusted incidence of genital warts increased from 13/100,000 to 106/100,000 between 1950–1954 and 1975–1978 [254] and appears to be on the rise even more recently [252]. A renewed interest for the trends of incidence of genital warts has been developed as a result of the introduction of HPV-6 and HPV-11 vaccination.

6.4.2 Other Human Papillomavirus Infections

The incidence of genital warts appears to have increased substantially over the past few decades, according to data available from the United States and the United Kingdom [255–257]. The incidence of condylomata in the United States increased about five times between 1966 and 1981, with most of the increase occurring prior to 1976. A similar tendency was observed in the United Kingdom between 1972 and 1982. Data on other types of HPV infections are less reliable because they rely mainly on clinical observation, and specific HPV DNA testing is normally not performed for these conditions [24].

6.5 Age

6.5.1 Cervical Human Papillomavirus Infections

At the simplest level, it appears that cervical HPV infection rates measured by DNA detection or cytology decline sharply with age, from a peak at 16–25 years of age [199, 258, 259].

The prevalence of HPV infection is highest among young women and appears to decrease with increasing age. In a meta-analysis using data from multiple international studies, the median high-risk HPV prevalence among all women was found to be 15.1 %, while the median high-risk HPV prevalence among women age 30 and older was 9.2 % [260, 261]. Since the occurrence of HPV infections in most cases is closely connected to the initiation of sexual activity and most are transient, the over-age-30 group of women who are HPV positive includes those who are persistent carriers as well as those with new infections. While most studies indicate a decrease in HPV prevalence with age, some data from studies conducted in several different international regions attest to the pattern of a peak prevalence of HPV infection in women below age 25, a decrease among women aged 35–54 and a second peak after age 55 [262]. This difference is highlighted in Fig. 44.3. This observed increase in HPV prevalence in older age groups (age group 45–54 years old) could be attributed to a cohort effect or to reactivation of latent virus [209].

The prevalence of HPV infection in women under 16 years of age is difficult to assess, because this is generally the minimum age of obtainable informed consent for most epidemiologic studies. To further support the connection between initiation of sexual activity and onset of HPV infections, albeit through limited DNA test data collected before sexual debut, it appears that cervical HPV infection is extremely uncommon in both girls and adult women who are sexually naïve [194, 263].

In many study populations, the prevalence of HPV infection as measured by DNA or cytology decreases strongly with age, from a peak at 15–25 years [199]. The profound drop in cervical HPV prevalence in women over age 30, seen in many different studies, is thought to be due to immunologic clearance or suppression of existing infections. This implies a transience of most HPV infections, in combination with less exposure to new HPV types from fewer new sexual partners after that age. The "sexual acquisition/immunologic clearance" explanation of the HPV age trend is supported by prospective data suggesting rapid, sexual acquisition of HPV [212, 264] and by other prospective data indicating that most HPV infections are only transiently detectable [213, 264].

The majority of women infected with a specific HPV type will not show evidence of that same type 6–12 months after initial detection [201, 205, 212, 264, 265]. In a prospective study of female college students, approximately 70 % of women no longer showed detectable HPV DNA within 12 months of follow-up after the incident HPV infection, while after 18 months over 80 % of the subjects appeared to have cleared their infections [201]. These findings have been supported by several other cohort studies having a median duration of HPV detectability of approximately 1 year [36, 205, 212, 266]. Some studies compared high-risk and low-risk HPV duration of infection and showed similar average duration [202], while others showed longer durations of infection for high-risk than for low-risk HPV types [201, 205, 212]. It appears that HPV-16 has a particularly long time to clearance relative to other HPV types [202, 207].

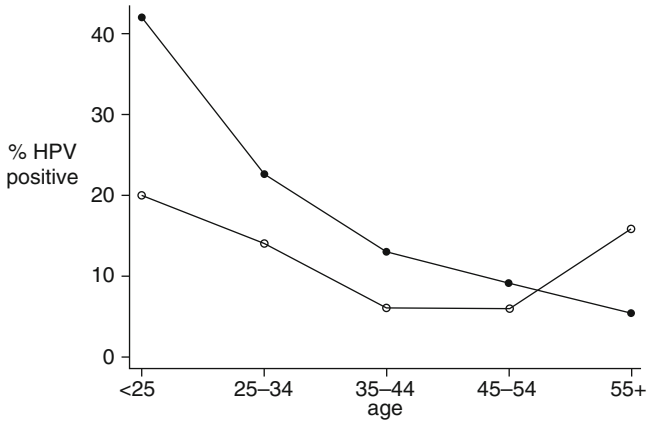


Fig. 44.3 Age-specific prevalence of HPV in a routine screening population in the UK, data source: Cuschieri et al. [203] (*solid circles*) and in rural Costa Rica, data source: Herrero et al. [262] (*open circles*) (Modified from Baseman and Koutsky [209], with permission)

When interpreting data on duration of HPV infection, it is important to keep in mind that there is no consensus regarding what constitutes a "persistent" HPV infection. The interval between follow-up visits varies among studies, and many aspects of the natural history of HPV remain unknown. Therefore, what constitutes an appropriate assessment interval, the significance of being positive at two points, and the distinction between "persistence" and "transience" of infection all still remain to be defined. Moreover, it is unclear whether undetectable HPV infections have been acquired and entirely cleared by the host or whether there is a period of viral latency in which HPV levels are maintained below the detectable threshold of current HPV DNA assays, a threshold which varies among these assays. If viral latency occurs, a woman who appears to have cleared her infection between follow-up visits would still be at risk for potential development of HPV-associated disease. In the same way, it is unclear whether HPV infections that are detectable over a certain interval of time result from persistent HPV infection or HPV clearance and reinfection. Reinfection with the same HPV type appears to be uncommon: three cohort studies of sexually active young women showed that the same HPV-16 variant was always detected in the same woman during follow-up examinations [267].

6.5.2 Other Human Papillomavirus Infections

Age trends in other genital HPV infections (e.g., condyloma acuminatum) tend to parallel the trends in cervical infections, with the peak rates occurring between ages 15 and 25 and connected to the usual onset of sexual intercourse. In accordance to that, genital warts in both men and women tend to peak at these ages [255, 257, 268], as does the prevalence of penile HPV DNA detection and the occurrence of flat HPV-containing lesions of the penis [225].

Age-specific prevalence rates of nongenital cutaneous HPV infections have been less well documented, and it is widely accepted that these lesions are extremely common. Historical reports include the HANES study of 1971–1974, and the prevalence of nongenital cutaneous warts was observed to peak in the 12–17-year age group and to decline thereafter [219]. The age peak in males (18–24 years) was later than in females (12–17 years). Plantar warts were not distinguished from common cutaneous warts; thus, these prevalence estimates are not site- or HPV-type specific, and also in these studies the diagnosis is clinical and corresponds to a cutaneous wart, without the confirmation of HPV infection. A more recent population-based British study of nongenital cutaneous warts was consistent with the HANES study, finding a prevalence of 3.9 % at age 11 and 4.9 % at age 16 [269]. Again, the sites and kinds of warts seen were not distinguished. From other school-based series, it appears that the peak age of plantar warts occurs a few years later than the peak age of common warts [270]. In the last few years, studies have been made available in multiple countries, and a recent Australian study in pediatric patients showed that the incidence of cutaneous warts among that population is that of 22 %, with common warts being 16 % alone, and that the prevalence changes with age as in the 2–4 years old group the prevalence was that of 12 %, only to double to 24 % in the 16–18 years old group [271].

6.6 Sex

6.6.1 Genital Human Papillomavirus Infections

Reliable prevalence estimates for genital HPV infections in males are more difficult to obtain than for females. It appears from the available data, which are sometimes conflicting, that genital HPV infections are about equally common in both sexes [255, 257, 272].

HPV-induced penile lesions can be found in the urethral meatus, on the glans and shaft of the penis, and on the scrotum [273], but, unlike with the localized, easily exfoliated mucosal surface of the cervix, the cornified epithelium of the penis and scrotum does not permit a reliable, standardized collection of relevant cells.

Since the advent of PCR-based HPV test methods, analysis of HPV infection in men is more feasible. A recent study tested male partners of women with genital HPV lesions and found that warts and high-grade intraepithelial neoplasia were diagnosed in 83 (78 %) and 23 (22 %) of cases, respectively. The prevalence of clinical HPV lesions in men ranged from 34 % in case of HG CIN to 80 % when the female partner suffered from genital warts. The authors concluded that peniscopy in males with female partners having an HPV-related lesion could be a feasible solution [274].

6.6.2 Nongenital Cutaneous Human Papillomavirus Infections

Data from the early HANES study (1971–1974) suggest that males have slightly higher prevalence of nongenital cutaneous warts (10.3 per 1,000) when compared to females (7.2 per 1,000) [219]. A strong male predilection was noticed only in the 18–24 age (21.5 per 1,000 for males compared to 4.0 per 1,000 for females). However, other surveys have not generally confirmed this disparity. In fact, a female excess for plantar warts among teenagers has been reported [275].

6.7 Race and Ethnicity

The correlation of HPV prevalence with race, controlling for known risk factors for infection, is not clear. To study the correlation of HPV with race demands random, strictly comparable sampling of different racial populations is necessary. Clinic-based studies are prone to confounding by correlates of race, such as differences in socioeconomic status and patterns of self-referral.

Epidemiologic studies in the United States have been able to compare different ethnic groups living in the same geographic areas, and the data produced show that African-American women have higher rates of cervical cancer and mortality rates compared to other races (US Cancer Statistics Working Group: United States Cancer Statistics: 1999–2009). Age-adjusted death rates show 4.3 per 100,000 for African-American women in comparison to 3.0 for Hispanic women and 2.2 for Caucasian women (US Census Bureau: 2000 Census). Paradoxically, data on Pap test use in 2008 show that African-American women have the highest screening rates nationally of all racial and ethnic groups at 80.1 % in contrast to 74.9 % for non-Hispanic white women and 75.4 % for Hispanic women (National Center for Health Statistics) [276]. This discrepancy between mortality and screening rate suggests a number of explanations including the continued existence of confounders that could distort certain comparisons between these groups. Potential confounders include socioeconomic, educational, and health system barriers impacting access and utilization of quality health care, as well as the failure of current interventions to account for the cultural diversity within this population and how it impacts their cervical cancer knowledge, attitudes, and practices [276].

The resulting increased rates of CIN and cervical carcinoma among African-Americans, Hispanics, and Native Americans (compared to non-Hispanic whites) are intertwined with socioeconomic differences in risk [277]. Another discrepant result was noticed from these studies: in contrast to cervical cancer rates, the frequency of genital warts [255, 257] and nongenital cutaneous warts [269, 278] has been reported to be higher in whites than in blacks.

6.8 Other Factors Influencing the Prevalence of Genital Human Papillomavirus Infections

6.8.1 Occupation

The only professions linked to HPV infection are those associated with an increased risk of exposure to the virus, and accordingly sex workers are at increased risk of clinical outcomes of genital HPV infection [23]. However, the prevalence of HPV DNA was not elevated in one survey of immunocompetent (HIV-uninfected) sex workers [279]; this finding may reflect their development of immunity. Meat and fish handlers are reported to be at increased risk of infection with HPV type 7 [280, 281], but the mode of transmission in this case remains obscure.

6.8.2 Socioeconomic Status

Cervical HPV prevalence appears to be increased in women of lower socioeconomic status [282], in accordance with their higher risk of cervical carcinoma [277]. One study of nongenital cutaneous warts also observed increasing prevalence in children of lower socioeconomic status [269].

6.8.3 Pregnancy

Current pregnancy, especially advanced pregnancy, has been found to increase the detection of cervical HPV DNA, although different studies show significant variability [283–285]. If confirmed, increasing susceptibility to HPV infection during pregnancy could suggest the possibility of viral reactivation.

6.8.4 Smoking

HPV infection has been associated with current smoker status [286] and past history of smoking [287]. However, only one study found that formerly smoking women had a significantly lower prevalence of HPV infection than women who had never smoked [282]. A more recent study investigated the relationship between smoking and oncogenic HPV infection and found no association between number of cigarettes smoked per day and presence of HPV DNA [288], and most other studies investigating the relationship between smoking and HPV infection have failed to detect an association [209, 289–292].

7 Mechanisms and Routes of Transmission

7.1 Evidence for Sexual Transmission of Genital Types of Human Papillomavirus

Sexual partners of women or men with HPV-related lesions are more likely themselves to have clinical, cellular, or DNA evidence of HPV infection [226, 273], and despite the fact

that clinically diagnosed lesions are often absent, studies have suggested that the majority of regular sexual partners of infected individuals show themselves detectable HPV infection [193, 194, 283].

The most consistent risk factor for HPV infection is believed to be an increased number of sex partners, and studies of women have demonstrated strong associations between lifetime number of sex partners and detectable genital HPV in both sexes [286, 289, 292–296]. Although HPV type-specific analyses have been limited by small sample sizes, the prevalences of both cancer-associated and noncancer-associated HPV types are associated with sexual history.

According to a few epidemiologic studies available, the epidemiologic variables "*lifetime* numbers of *different* sexual partners" or "*recent* numbers of *different* sexual partners" are superior predictors of risk of prevalent cervical HPV infection compared to "*lifetime* numbers of *regular* sexual partners," suggesting that long-term association with a partner is less likely to increase risk [283].

7.2 Nonsexual Transmission of Genital Human Papillomavirus

Although the sexual transmissibility of HPV has been extensively studied, the nonsexual person-to-person transmission and the possibility of indirect transmission are only partially understood.

In the study of nongenital HPV infections, documented transmission of HPV in laryngeal infections (often due to types 6 and 11) supports the notion that vertical transmission of genital HPV infections is possible [297]. The occurrence of laryngeal papillomas in children has been associated with genital warts in the mother, and cesarean section has been associated with some protection against laryngeal papillomas. Moreover, the types of HPV in laryngeal papillomas (types 6 and 11) are the same as in genital warts. However, the reported increase in the incidence of genital warts has not been followed by an appreciable increase in laryngeal papillomatosis.

Among 329 recently studied pregnant women, HPV genotypes present in the cervix of the mothers, their newborns, cord blood, and placenta samples were determined by molecular and immunologic techniques [298]. HPV DNA was detected in 17.9 % of oral samples from newborns and in 16.4 % of the cervical samples of the mothers. At delivery, mother–newborn pairs had similar HPV-genotype profiles, but this concordance disappeared in 2 months, while oral HPV carriage in newborns was most significantly associated with the detection of HPV in the placenta. The association between HPV in cord blood and oral HPV was also significant at delivery but disappeared within 1 month. Finally, the HPV antibodies detected in the infants were found to be of maternal origin. The study concluded that HPV is prevalent in oral samples from newborns and that the genotypic profile of newborns is more restricted than that of the maternal cervical samples. The close maternal–newborn concordance could indicate that an infected mother transmits HPV to her newborn via the placenta or cord blood.

7.3 Transmission of Nongenital Cutaneous Types of Human Papillomavirus

Direct skin-to-skin transmission is known to be the predominant mechanism of transmission of skin warts [248]. Activities associated with minor skin trauma promote person-to-person transmission and subsequent autoinoculation [275], which permit access for the HPV to the basal layer of the epidermis that the virus has a tropism for.

Although direct contact is the established route of transmission of most nongenital cutaneous HPV infections, the mode of transmission of the rare HPV types found in epidermodysplasia verruciformis is still a subject of study. Only a few hundred cases of the condition have been reported [133], and individual studies involve a very limited number of cases. The disease is believed to be an autosomal recessive genodermatosis with an increased susceptibility to specific HPV genotypes [299]. It has been associated with more than 20 known EV–HPV types, including 3, 5, 8, 9, 10, 12, 14, 15, 17, 19–25, 28, 29, 36, 46, 47, 49, and 50 [300].

8 Pathogenesis and Immunity

8.1 Pathogenesis

8.1.1 Definitions Related to Studies of Human Papillomavirus Pathogenesis

As knowledge surrounding HPV infections and diagnostic modalities evolved, the need to redefine the terms in which the HPV genesis of disease is described arose. These terms changed to better reflect the viral biology and the natural history of viral infection and eventual subsequent disease manifestations. This newer way to classify HPV pathogenesis, almost 20 years old now, had also the role to avoid the use imprecise terminology, in the face of lack of understanding of the various facets of HPV biology. The use of terms such as "latent" or "subclinical" infection is being abandoned, and, to facilitate the discussion of HPV pathogenesis, a classification system was proposed based on the diagnostic method that establishes the HPV infection [301].

According to the proposed system, the *molecular* evidence of HPV is established by the demonstration of viral particles, nucleic acids, or proteins, using methods such as electron microscopy, PCR, or other nucleic acid hybridization

techniques, but also Western blot and immunocytochemistry. *Cellular* (or microscopic) evidence of disease requires morphological evaluation for the presence of viral cytopathic effect in cytological and/or histological specimens. *Clinical* evidence of disease is based on the presence of symptoms or signs observed on physical examination with or without aids such as magnification and acetowhite staining. Finally, *immunologic* evidence can be added to this schema [24].

While these levels of detection may reflect the various phases of HPV infection, multiple diagnostic modalities are employed to characterize and confirm the presence of the virus in the host and to confirm its causality of the morphological and clinical findings associated with disease.

8.1.2 Pathogenesis of Genital Human Papillomavirus Infection at a Molecular Level

The mechanism of entry of the HPV into cells is still a subject of study. As mentioned in Section 3, the observation of early phase proteins along with the presence of episomal viral genome copies in the absence of late phase proteins and complete viral particles in the basal layers has suggested that the virus needs to infect proliferating cells found in the basal epithelial layers. In order to do so, the presence of microtraumatic tears of the overlying layers, with exposure of the basal layer for viral inoculation, is believed to be necessary. The evidence of HPV-related lesions in previously traumatized skin [302] and especially the microtrauma produced in the genital mucosa during sexual intercourse, as studied for other sexually transmitted agents such as human immunodeficiency virus (HIV), is thought to be the way of entry for the HPV viral particles. The completion of the full viral cycle is thus connected to the differentiation process [303].

Cervical HPVs are the most widely studied, and evidence suggests that in lesions that progress to high-grade dysplasia, a limited number of ORFs are preferentially expressed, and particular emphasis has been given to E6 and E7. E6 and E7 share a common primer site, and their differential expression is depended on alternate splicing patterns [304]. Progression to high-grade disease has been linked to higher levels of E7, and it has been suggested that a specific splicing pattern (named E6*I) is the one that promotes E7 production and is responsible for E7 production in high-risk HPVs. Ultimately, E6 and E7 regulate the expression of a series of genes and play a key role in the progression to high-grade dysplasia and cancer [305]. Integration of the HPV DNA into the cellular genome appears to be necessary in order to stabilize the HPV infection, thus permitting the progression of disease and finally the evolution to high-grade dysplastic manifestations. Studies trying to investigate HPV genome integration in different grade premalignant cervical lesions and cervical cancer showed that only a small percentage of grade 2 cervical intraepithelial neoplasias (CIN 2) have integrated HPV genetic material [306], with increasing proportions in CIN3 [18, 306].

Although the potential integration sites within the human genome are multiple, it has been observed that for any given cervical cancer, only a few contain integrated HPV genetic material. Frequently, these sites correspond to genomic fragile sites [307, 308]. Regulatory elements and the E6 and E7 ORFs are always preserved, with frequent disruption during integration of the E1 and E2 genes that normally inhibit E6 and E7 [309]. Thus, continuous production of E6 and E7 proteins appears to have a role in HPV carcinogenicity, and, in fact, the E6 and E7 regions of the HPV genome are transcriptionally active in HPV-associated cervical carcinomas and derived cell lines [4, 309]. It is also possible that the virus uses the genetic expression regulatory mechanisms, such as promoters, to increase viral gene expression. The necessity for viral genome integration in tumorigenic capacity appears to vary between HPV types. Viral integration occurs in almost all tumors and cell lines containing HPV-18. While the same holds true for most tumors and cell lines, a significant number exhibit episomal HPV-16 genomes [310].

The protein products of the E6 and E7 ORFs of HPV appear to be principally responsible for HPV neoplastic effects. The E6 and E7 proteins have been shown to be cooperative transforming proteins in vitro and appear to be necessary for the HPV-positive cell line viability and proliferation [97, 311, 312]. The E6 protein binds to and promotes the degradation of the p53 tumor suppressor protein by forming a complex requiring the cellular protein E6-AP [107, 313]. The E7 protein binds to and inactivates the retinoblastoma tumor suppressor protein [314]. The transforming proteins of cancer-associated HPV types such as types 16 and 18 have greater binding affinities for tumor suppressor proteins and greater in vitro transforming abilities than E6 and E7 proteins from low-risk HPV types like HPV-6 and HPV-11 [97]. Another biochemical demonstration of dysplasia is the expression of p16, which appears to be elevated in dysplastic cells, as a response to the pRB inactivation mediated by E7 [315–317]. p16 is thus used as an indirect marker of HPV-induced dysplasia by immunohistochemistry [38].

E6 and E7 are believed to be involved in the production of centrosome abnormalities and resulting aneuploidy which contributes to the dysplastic process. Abnormalities of the centrosomes can produce deregulatory effects in the mitotic sequence of events which may lead to aneuploidy. Studies have suggested aneuploidy, seen in many cervical lesions, has a high degree of association to the presence of integrated HVP genome in the cells [318].

As mentioned previously, telomerase activity is another factor that appears to be involved in cervical cancer pathogenesis [319, 320], and increase of this activity is mediated primarily by E6, with a p53-independent mechanism, but

also by E7 [321, 322]. Increased allelic imbalance in the locus 6q14-6q22 is found in CIN3 and invasive cervical carcinoma, suggesting the presence, on this chromosome 6 locus, of a gene that may antagonize HPV-induced telomerase activation [323].

Finally an association between increased E5 levels and elevated EGFR and its related protein ErbB4 has also been described, in both episomal and integrated HPV-16 DNA lesions, and it is possible that HPV-16 E5 plays a role in stimulating these factors, thus promoting cell transformation [101, 324].

It is not yet known how the interactions of HPV-transforming proteins with tumor suppressor genes and proteins translate in the spectrum of HPV growth-altering effects, starting from the production of benign warts and early intraepithelial neoplasia to the development of carcinoma. Many examples of human cancers result from the accumulation of genetic mutations and epigenetic alterations. In the molecular pathogenesis of cervical carcinoma, the persistent infection with an oncogenic HPV that expresses E6 and E7 can be viewed as the "first hit" event. Additional somatic genetic changes may then occur, including loss of heterozygosity. Loss of heterozygosity studies have suggested that tumor suppressors on chromosome regions 11q [325] and 3p [326, 327] may be involved in cervical cancer development.

8.1.3 Pathogenesis of Genital Human Papillomavirus Infection at a Chromosomal Level

Besides interaction of E6 and E7 proteins with cellular proto-oncogenes and oncosuppressor genes, other regulatory mechanisms are in play. Epigenetic deregulation of various genes, such as methylation [328], has been identified in cervical high-grade dysplasia and cancer, and its role is being defined [329–331] along with that of cytogenetic alterations. The genes affected by these cytogenetic alterations have not been identified in most cases. The c-Myc gene, frequently overexpressed in other tumors, can be upregulated in cervical cancer. About 10 % of cervical cancers present HPV DNA integration close to the c-Myc site with associated upregulation of this proto-oncogene [332].

A variable percentage of cervical cancers, between 25 and 50 %, present recurrent cytogenetic alterations, the most common of which is loss of heterozygosity (LOH) in the following regions: 3p14, 4p16, 6p21-22, 11p15, 11q23, 17p13.3, and 18q12-22. One of these loci, 3p14.2, has been described also in dysplastic areas, adjacent to the cervical cancer lesions examined [333]. This might imply that this particular LOH is an early event in the progression of HPV-related disease, and in fact the levels of the affected gene (FHIT), an oncosuppressor, have been described to be lower in cervical cancer [334, 335] and also to be inversely proportional to the degree of dysplasia demonstrated morphologically by the tissue [120, 336]. Gain of 3q24-28, per contrast (whose gene is part of the PI3 kinase/AKT signaling pathway), has been reported in cervical cancer, in very high percentages, whereas in high-grade dysplasia is rarely seen [337], suggesting it might represent a late event in carcinogenesis.

Loss of HLA class I antigens has finally been reported as well and has been tied to more advanced disease and poorer prognosis. In particular, HLA-B7/40 allelic loss is thought to be connected to metastatic disease, suggesting that this kind of alteration might be a way for the tumor cells to evade immune response [338–341]. Among the mechanisms possibly involved in this common loss of HLA antigens as described above [342] is the loss of region 6p21.3, where class I genes are located.

8.1.4 Pathogenesis of Genital Human Papillomavirus Infection at a Cellular Level

In cattle, experimental inoculation of BPV by scarification leads to microscopically evident epidermal papillomatosis in about 2–3 months [2, 3], and the pathogenesis of human skin warts may have a similar incubation period. As shown in Fig. 44.4, among cytologically normal women who are HPV DNA positive at enrollment using the Hybrid Capture assay, the absolute risk of incident abnormal smears rises to a very high level (approximately 25 % of smears taken) at 1–2 years following enrollment and declines thereafter, returning to baseline (1–2 % of smears taken) at about 4 years. This peak at 1–2 years may approximate the incubation period of cervical HPV infection [343].

The earliest and mildest cellular changes associated with cervical HPV infection are extremely subtle and nonspecific. It is moreover possible that many HPV DNA-positive women have lesions too small to be diagnosed. In this interpretation, the incidence of CIN following HPV infection shown in Fig. 44.4 could represent the growth of a focus of HPV-infected cells to cytologically detectable size. Alternatively, HPV DNA might be detectable before and after the briefer appearance of cytopathic changes. In other words, it is not clear whether molecularly diagnosed HPV infections, especially high levels of viral DNA, imply the existence of cellular abnormalities somewhere in the infected tissue. In cases that are confirmed positive by cytology however, HPV DNA is found in 90 % or more of cytologically diagnosed HPV infections, identified using the Bethesda system criteria [163].

A possible explanation to the pathomorphological changes that characterize dysplasia is that the expansion of the spiny layer of the epithelium in HPV lesions, particularly exophytic ones, results from a minor degree of cell differentiation and reduced squamous cell sloughing, rather than

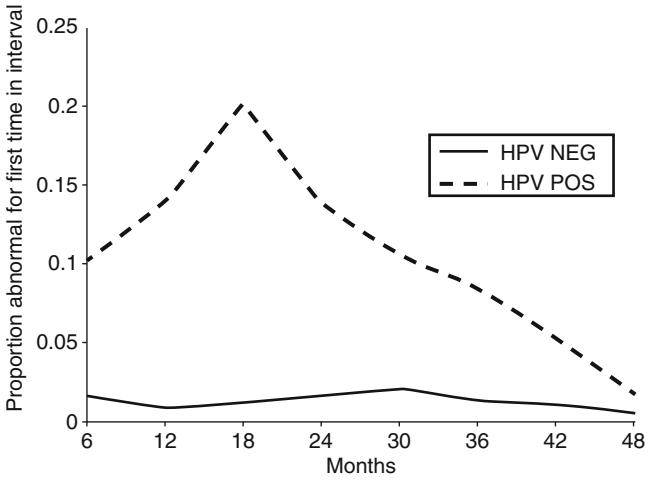


Fig. 44.4 Risk of new diagnosis of SIL by HPV DNA test result at enrollment. A cohort of 17,654 women with normal cytological diagnoses and no known past history of SIL were tested for 16 types of cervical HPV DNA using primarily Hybrid Capture. For each subsequent half-year time interval, the numbers of incident SIL diagnoses were calculated as a proportion of all women in the cohort obtaining smears during the interval. The relative and absolute risks of SIL were greatly increased in the 1,279 women testing positive at enrollment for HPV compared with the larger group testing HPV negative. The greatest risk of SIL following HPV DNA detection appeared in the second year of follow-up (From Schiffman et al. [24], with permission)

increased rate of cell turnover [344]. The result of this would be that the proliferative compartment (the number of cells dividing) is increased in HPV-infected epithelia, but the rate of cell division may not be [24].

Analysis of data from a large prospective study showed that the cumulative risk for a woman with negative cytology at enrollment of developing high-grade dysplasia or cervical cancer within 10 years of detection of HPV-16 or HPV-18 on HCII was 17.3 and 11.8 %, respectively. This was much higher than the 3 % cumulative risk for women with non-HPV-16 or HPV-18 high-risk types on HCII or the 0.8 % risk for women with negative HCII [343] (Fig. 44.5).

8.1.5 Pathogenesis of Genital Human Papillomavirus Infection at a Clinical Level

Most HPV-induced cellular abnormalities are transient and barely detectable at the clinical level. For example, flat HPV lesions of the penis are usually not recognizable, although they can be seen by peniscopy as acetowhite areas following the application of acetic acid [225]. Similarly, cervical intraepithelial neoplasia is asymptomatic and requires colposcopy to be seen.

The most commonly diagnosed clinical outcomes of HPV infection are nongenital cutaneous and genital warts. Warts tend to grow from an initially inapparent size to an appreciable but delimited lesion that then persists or regresses over the course of a few months to a few years. Clinically evident wart disease has been noted to worsen during pregnancy [345] and in response to immunosuppression, for example, secondary to renal transplantation [346, 347].

Compared to warts, the occurrence of HPV-related cervical carcinoma is a much rarer event, while infection with HPV explains a smaller fraction of the other HPV-related malignancies, whose etiologies may be more multifactorial than the etiology of cervical carcinoma.

8.2 Immunity

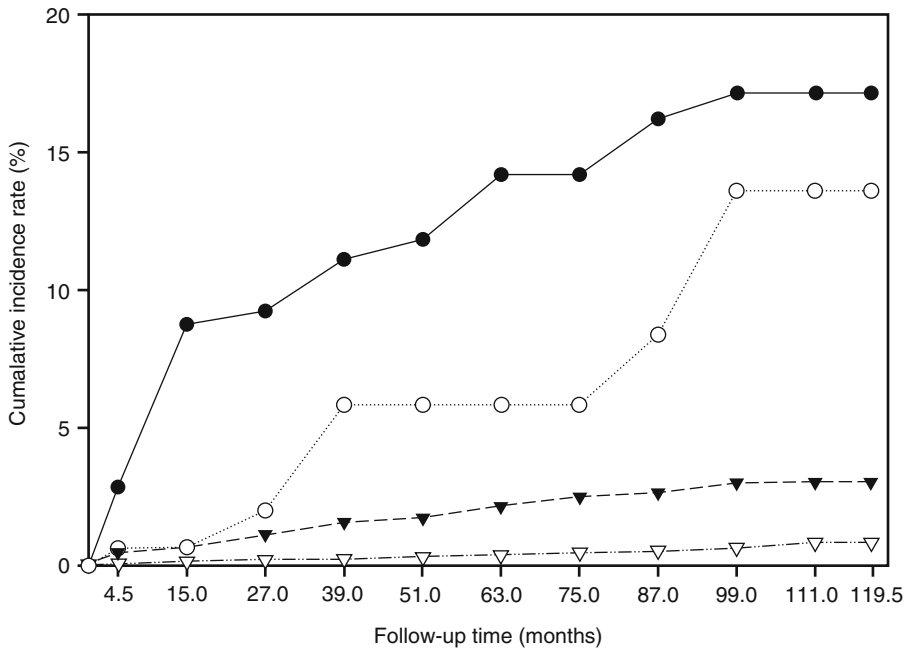
HPV infections, except as seen in carcinomas, are limited to mucosal and cutaneous epithelium above the basement membrane. Even after numerous studies conducted over many years, it is still unknown whether and how systemic or local immune factors affect HPV infections, nor are the mechanisms involved in the immune response to HPV or their efficacy yet fully understood. The fact that there is no known viremia and the impossibility of directly detecting in vitro antigens have meant that experimentation on HPV immunity relies greatly on constructs such as viruslike particles (VLPs), and to date there is no standardized test for the measurement of HPV antibodies [348]. Also unclear is whether infection with one type of HPV protects against infection with other types.

8.2.1 Immunity in Animal Papillomavirus Infections

Studies in animals, including experimental HPV infection models, have shed some light on the immunologic mechanisms of HPV infections. In the rabbit, development of a systemic immune reaction to papillomavirus infection is supported by the following observations: (1) All papillomas regress concurrently once regression begins; (2) suppression of the immune system inhibits regression, while vaccination with tumor preparations increases regression frequency; (3) regressing papillomas have a mononuclear infiltrate; and (4) rabbits with regressing papillomas are resistant to reinfection with CRPV DNA, while rabbits with persistent papillomas are not resistant to additional infection. In cattle, concurrent bovine fibropapillomas also regress simultaneously, accompanied by infiltrating mononuclear cells and by resistance to reinfection [2, 3].

8.2.2 Human Papillomavirus Infections in Immunodeficient Patients

Human immunodeficiency virus (HIV)-positive patients, posttransplant, and other immunodeficient patients are more prone to infections and tend to have more severe manifestations of disease. This has been observed in HPV infections of immunodeficient patients. For example, an excess in cutaneous and genital warts among subjects with iatrogenic



No. of women seem during follw-up interval

HPV16+	455	247	190	144	125	112	94	84	89	35	3
HPV18+	154	85	74	51	43	41	36	37	35	16	1
HC2+	2211	1208	1016	862	755	701	600	528	547	256	17
HC2–	17391	9759	8672	7813	7136	6479	5960	5551	5278	2621	156

Fig. 44.5 Cumulative incidence of cervical intraepithelial neoplasia grade 3 and cancer (≥ CIN 3) over a 10-year period in 20 514 women according to oncogenic HPV status at enrollment. HPV status is defined hierarchically as positive for HPV-16 (*closed circles*), else positive for HPV-18 (*open circles*), else positive for the non-HPV-16/HPV-18 oncogenic types in Hybrid Capture 2 (*closed triangles*), and else oncogenic HPV negative (open triangles) (From Khan et al. [343], with permission)

immunosuppression following renal transplant has been demonstrated [346, 347, 349].

As mentioned above, patients with the rare syndrome epidermodysplasia verruciformis are subject to extensive HPV skin lesions containing characteristic types of HPV. Not only these types are typical to this clinical manifestation and rare in others, but the lesions are more prone to develop into squamous cell carcinoma. Although a specific immune dysfunction has been sought, the prevalence in HIV-positive patients, especially those that have acquired HIV through vertical transmission, is much higher [350].

HIV infection provides perhaps the most important example of the effect of immunosuppression on HPV infection. Individuals infected with HIV through sexual contacts are likely to be exposed to genital types of HPV as well, because both viruses are sexually transmissible and prevalent in sexually active populations. Furthermore, patients with one sexually transmitted disease are more prone to develop another sexually transmitted disease as well. Thus, an increase in HPV-related clinical outcomes would be expected in certain HIV-infected cohorts even if there were no causal connection. However, the diagnosis of HPV in HIV-infected individuals is definitely increased and associated with increasing immunosuppression, as measured in terms of progressively decreasing CD4 counts [9, 231, 351, 352] (Fig. 44.6). Concurrently, CIN is commonly diagnosed. Parallel situations are seen in studies of anal infection among homosexual men [231]. It is not known whether HIV immunosuppression permits reactivation of previously suppressed HPV infection, as opposed to allowing rapid infection or reinfection from environmental sources.

Although the association between HIV infection, HPV infection, and anogenital intraepithelial neoplasia is established, a causal role for immunosuppression in the risk of progression of intraepithelial lesions to invasive carcinoma is less clear. Anal carcinoma rates are increasing in the homosexual male population, probably related to HIV infection [171, 229], though progression from dysplasia to cancer occurs in much lower rates than in the cervix [353].

8.2.3 Serological Studies of Genital Human Papillomavirus Infections

Epidemiologists and immunologists are attempting to differentiate the serological responses conferring natural immunity to HPV infections from responses simply representing biomarkers of infection or disease progression. The available data from animal studies do not indicate a major role for antibody responses in wart regression [2, 3], while immunization of animals with papillomavirus capsid proteins blocks reinfection but does not appreciably affect existing warts.

No convincing evidence of a protective serological response in humans has been reported. Rather, several groups

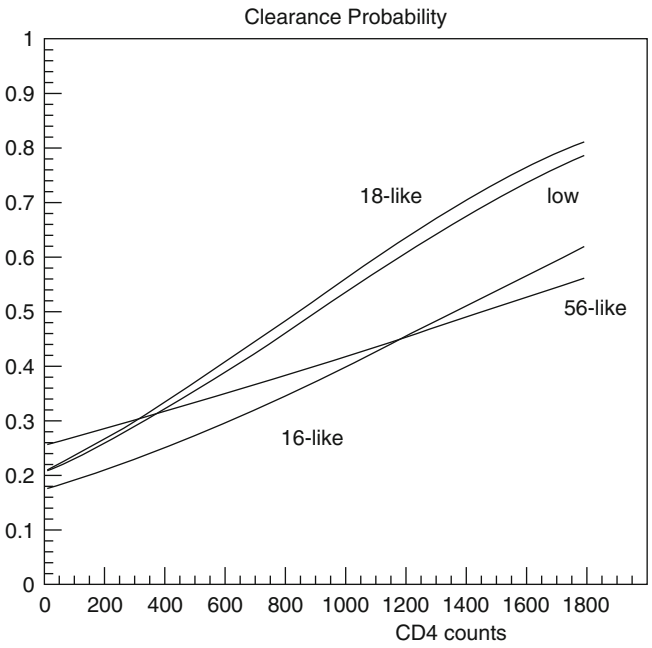


Fig. 44.6 The 3-month HPV type-specific probability of clearance depending on CD4 T lymphocytes in HIV-1-positive adolescent girls from the REACH cohort (From Kravchenko et al. [9], with permission)

have reported elevated HPV antibody levels in subjects with clinically evident HPV-associated disease compared with controls [183]. For example, elevated titers to HPV-16 E7 are observed in about half of HPV-16-associated cervical carcinoma patients [176, 178, 185, 354]. Measurement of HPV-16 and HPV-18 antibodies in women with positive DNA found prevalence rates of 63 and 57.5 %, respectively [355]. In the same study, women with positive cytology and/or high viral loads had higher antibody titers compared to women with negative cytology and/or low viral loads, but neither observation suggests that this might represent a systemic immune response to HPV.

As mentioned above, the majority of women with CIN or cancer, but also genital warts or nongenital cutaneous warts, have antibodies to the same HPV types found in their lesions. But in contrast to HPV DNA-positive women, with regard to HPV-16, women with very low probability of exposure, assessed by DNA testing and interview, have a very low HPV-16 seroprevalence, whereas individuals infected with other genital types of HPV have an intermediate seroprevalence [356] with the exception of those with in situ penile cancer [179]. The persistence of cervical HPV DNA positivity, even in the absence of cytological abnormalities, is highly predictive of seropositivity. Investigations so far have confirmed the associations of HPV infection with anal cancer [357] and other anal lesions [358].

8.2.4 Cellular Immune Responses to Human Papillomavirus Infections

Based on the animal experiments and the immunosuppression data, it is assumed that the key immune response involved in the clearance of HPV infections is cell mediated. Mouse models showed the evocation of a cellular immune response to HPV-6, HPV-11, HPV-16, and HPV-18 after DNA immunization [359, 360]. As in animals [2, 3], once multiple human warts of a single kind begin to regress, others regress concurrently [361], although warts of different kinds (e.g., plantar vs. common skin) may not regress concurrently. Histologically, regressing flat skin warts show infiltration with mononuclear leukocytes and other features suggestive of a cell-mediated immune response [268]. This is in accordance with the observation that while genetic defects affecting humoral immunity have little effect on the natural history of HPV infections, deficiencies of cell-mediated immunity profoundly elevate the prevalence of HPV infection assessed clinically, microscopically, or molecularly [361, 362].

The two classes of cells thought to be involved in the cellular immune response to HPV are antigen-presenting cells and lymphocytes. The usual antigen-presenting cells of the epithelium are the Langerhans cells. Langerhans cells are less numerous near HPV-induced epithelial lesions compared to normal epithelium; a possible mechanism is that HPV manages to downregulate proinflammatory cytokines, and in particular type I interferons, resulting in an attenuated cellular immune response, which includes these cells [302].

Finally the possible associations of specific HLA locus genotypes with the risk of invasive cervical carcinoma and the loss of HLA class I antigens as a possible HPV mechanism of cellular immune evasion point towards the primary role that cellular immunity has in response to HPV infections.

9 Patterns of Host Response

9.1 Warts

9.1.1 Nongenital Cutaneous Warts

Skin warts are a well-known and common response to infection with HPV cutaneous types, including 1, 2, 4, and related types. The most frequent skin wart is the common variety (verruca vulgaris), which occurs mainly on the back of the hands and fingers, but can occur anywhere on the skin [268]. Common warts are firm papules ranging in size from less than a millimeter to over a centimeter in diameter. They may occur singly or in groups. About two-thirds of common warts spontaneously regress within 2 years [363]. Malignant conversion is extremely rare [268].

Deep plantar warts (verruca plantaris) are most commonly associated with HPV type 1, which is phylogenetically and molecularly quite distinct from the viral types more commonly found in common skin warts [132]. Plantar warts are usually single but may be multiple. These lesions are sharply demarcated from the adjacent normal skin and possess a smooth hyperkeratotic collar. Regression is common but as variable as for common warts [268].

A less common kind of skin wart is the flat wart (verruca plana), which is smooth and flat or mildly elevated. Flat warts occur multiply, mainly on the face, back of the hands, and the shins. They may disappear suddenly after weeks or months or persist for years [268].

Multicentric nongenital cutaneous wart disease is very common, sometimes with mixed kinds of warts. Multicentric wart disease of the same kind is apparently related to autoinoculation by scratching, hand washing, and nail-biting [275].

9.1.2 Genital Warts

Almost all exophytic genital warts, also called condylomata acuminata, contain HPV types 6, 11, or related types [364]. Like warts of nongenital skin, they tend to regress spontaneously and only rarely are associated with malignant progression. However, genital warts can be multifocal, can become very large, at times gigantic (giant condyloma acuminatum of Buschke and Löwenstein). They may recur after treatment, creating significant discomfort and psychosexual morbidity in some patients.

9.1.3 Respiratory Papillomas

Respiratory (laryngeal) papillomatosis is a rare disease with a bimodal age occurrence [365]. Thirty to 50 % of cases become evident before the age of 5 years (juvenile onset), linked most probably to vertical transmission in children born to HPV-positive mothers, most often to HPV-6 or HPV-11 [297]. Another group of cases associated with the same HPV types occurs in adulthood (adult onset), with an unproven mode of infection. Adult-onset respiratory papillomas are more often solitary, recur less frequently, do not tend to spread, and exhibit a male predominance [365].

Respiratory papillomas most commonly affect the larynx, particularly the true vocal cords, and become clinically evident with hoarseness being the major symptom. The true vocal cords contain a junction (or transformation) zone, present between the stratified squamous epithelium and the columnar epithelium, very similar to that found on the cervix. The papillomas uncommonly undergo malignant transformation, although one report noted cases of malignancy following irradiation therapy [366]. Although considered benign, the papillomas may sometimes become life-threatening due to airway obstruction, particularly in young children. Laryngeal papillomas tend to recur in many cases despite repeated excision. This rare presentation of warts is among the most severe and debilitating [24].

9.2 Cervical Intraepithelial Neoplasia

The cervix, though a well-studied epithelial model, is a special histological case (as are the true vocal cords and anus), containing a transformation zone between the squamous epithelium and ectocervix that transitions in to the columnar epithelium of the endocervix. This transformation zone is particularly susceptible to neoplastic transformation following HPV infection. In vivid contrast, the neighboring mature vaginal epithelium of the ectocervix, though prone to HPV infection, very rarely develops carcinoma [367]. The reasons for the special susceptibility of the transformation zone are unknown.

9.2.1 Defining Cervical Intraepithelial Neoplasia

The morphological response of the cervical epithelium to HPV infection is usually not a raised wart but rather a flat lesion with warty colposcopic and cytopathic features. As a corollary, the flat lesions of the cervix contain mostly types of HPV not found in exophytic venereal warts (e.g., 16, 18, 26, 31, 33, 35, 39, 45, 51–56, 58, 59, 64, 66, and 68), although flat lesions may also contain the types associated with exophytic genital condylomata (e.g., 6, 11, 42).

The HPV-induced, flat intraepithelial lesions of the cervix form a gradient of risk of malignancy, first formalized by Richart and colleagues when they introduced the term cervical intraepithelial neoplasia (CIN) [368]. Usually, CIN is classified as CIN 1 (mild dysplasia), CIN 2 (moderate dysplasia), and CIN 3/CIS (encompassing severe dysplasia and carcinoma in situ). As illustrated on Fig. 44.7, the spectrum of CIN has no perfect "cut points" and is, therefore, a subjectively defined gradient that is difficult to categorize reproducibly, although relative recommendations are available.

In normal epithelium, proliferation is restricted to the basal layer. Epithelial cells differentiate on a fixed and orderly program that leads to their progressive differentiation moving towards the more superficial strata of the epithelium, their programmed death and sloughing. In low-grade lesions, squamous differentiation in the more superficial layers of the epithelium becomes abnormal, but the cells continue to differentiate, such that there is only a minimal effect on the expansion of the proliferative, basal layer. Histologically, in low-grade lesions, the proliferative layer remains less than one-third of the full thickness of the epithelium. Fundamentally, low-grade lesions can be thought of as viral infections that are common, minor, and usually transient.

For the most part, lesions designated as CIN 1 are virtually indistinguishable from koilocytotic atypia. The relatively recent combination of CIN 1 and koilocytotic atypia as LSIL, in the Bethesda classification of cytopathology, is supported by epidemiologic data demonstrating that the two subsumed diagnoses share the same broad HPV type spectrum, have similar demographic characteristics such as early age peaks, and share a transient and benign natural history [199].

A one-third to two-thirds replacement of the epithelium with proliferating cells is termed CIN 2. Virtual full-thickness replacement of the epithelium with proliferating cells, with loss of epithelial differentiation, is termed CIN 3, which includes carcinoma in situ. In CIN 2 and CIN 3, in addition to expansion of the proliferative compartment, there is also an increased appearance of progressively more serious

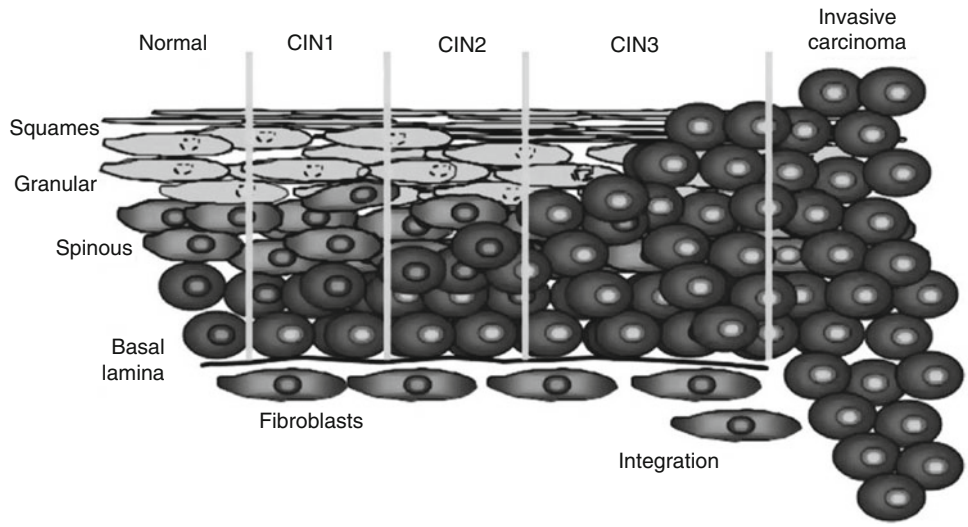


Fig. 44.7 Progression from a benign cervical lesion to invasive cervical cancer. In the diagram, HPV-positive cells are depicted by yellow nuclei. Infection by oncogenic HPV types, especially HPV-16, can cause formation of a benign wart, low- or high-grade dysplasia. CIN 1 and CIN 2 designations are reversible forms of precancerous lesions, and CIN 3 is irreversible. Carcinoma in situ occurs many years after an infection. This results from the effects of HPV genes, particularly those encoding E6 and E7, which are the two viral oncoproteins that are preferentially retained and expressed in cervical cancers by integration of the viral DNA into the host genome (From Angelletti et al. [369], with permission)

nuclear abnormalities. CIN 2 and CIN 3 are combined for cytology in the Bethesda system because (1) the categories are not reliably distinguished in practice and (2) both diagnoses lead to the same practical outcome, i.e., immediate colposcopic referral and biopsy, with ablative treatment if confirmed, through cold-knife conization or, more recently, loop electrosurgical excision procedure (LEEP) or other procedures.

9.2.2 Progression of Low-Grade to High-Grade CIN

A common conception in the beginning was that women who developed cervical cancer always progressed through distinct consecutive stages from low- to moderate- to high-grade intraepithelial lesions. Natural history studies however have raised doubt about the notion of a progressive continuum of cervical precancerous stages and led to the conclusion that low-grade and high-grade cervical lesions are distinct HPV infection processes. The cytological finding of low-grade squamous intraepithelial lesion (LSIL) appears to represent a transient manifestation in the course of viral infection, where the HPV-infected epithelium undergoes differentiation and maturation, exhibiting only minor cellular abnormalities. High-grade intraepithelial lesion (HSIL), the true cervical cancer precursor, instead occurs when HPV infection of replicating immature cells prevents squamous epithelial maturation and differentiation leading to continued replication of immature cells with consequent accumulation of genetic abnormalities that could ultimately lead to a clonal proliferation of cancer cells [209].

Although low-grade lesions caused by HPV are usually transient, statistically, women with low-grade cervical lesions are nonetheless at significantly increased risk of developing higher-grade CIN compared to the general population [31]. High-grade CIN usually emerges in women with low-grade CIN, but may sometimes arise in women with preceding, repeatedly normal cytology or only equivocal lesions [139, 370].

A diagnosis of low-grade CIN predicts an increased risk of a subsequent diagnosis of CIN 2–3, but it is not totally clear just how frequently women with low-grade lesions progress. While it is still not clear if, and in what measure, low-grade dysplasias will progress to high-grade dysplasias and cervical cancer, the type of HPV found in the cervix appears to predict the risk of the woman progressing to a diagnosis of CIN 2–3. This association has been verified in both cross-sectional [134, 135, 371] and prospective [372, 373] studies. While many HPV types are found in low-grade lesions, fewer types are found in CIN 2 and even fewer in CIN 3. Generally the types of HPV associated with CIN 3 are the same types found in invasive carcinomas. In lower-grade lesions, the cancer-associated HPV types increase steadily in relative prevalence with the severity of the diagnosis.

A recent prospective cohort analysis looked into recorded covariates as potential risk predictors of incident CIN 1, CIN 2, and CIN 3 using multinomial logistic regression models. In univariate analysis, the covariate profiles for incident CIN 1, CIN 2, and CIN 3 were unique. According to their finding, the only covariate significantly associated with all three CIN

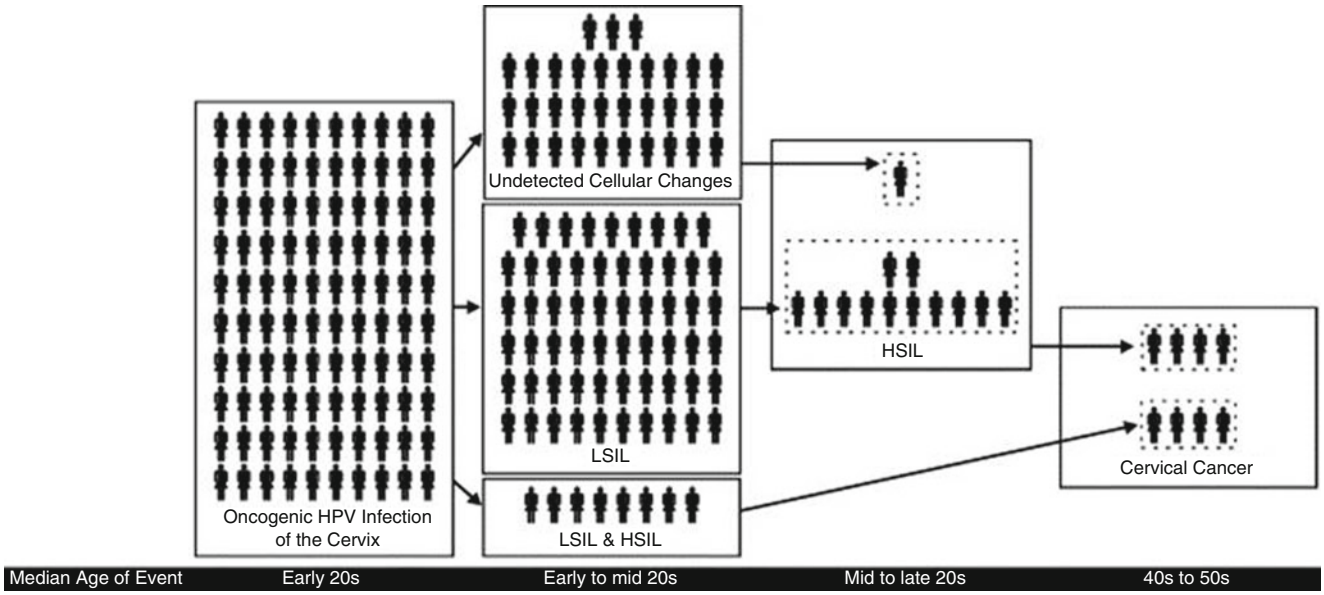


Fig. 44.8 Natural history of cervical HPV-associated disease (From Baseman and Koutsky [209], with permission)

end points was the baseline HR HPV status, which limits the use of HR HPV status as a predictor of CIN progression [374]. Moreover, in accordance to previous studies, the covariates associated with baseline CIN 1, CIN 2 and CIN 3, considered separately, are different [286]. As the data available suggests, cofactors associated with progression to CIN1 are different from those associated with incident CIN 2 or incident CIN 3, while there is no HPV cofactor significantly associated with all three outcomes [209].

Use of oral contraceptives has been suggested to be the only cofactor associated with two of these outcomes (incident CIN 2 and CIN 3). Factors such as current oral contraceptive use and Pap smear history were found to be individually confined to incident CIN1, whereas incident CIN2 was linked to the status of having ever been a smoker. Incident CIN3 was seen to be associated with different age groups and a baseline ASCUS (atypical squamous cells of undetermined significance) cytological diagnosis [209]. This diversity of HPV cofactors associated with progression to CIN1, CIN2, or CIN3 would seem to suggest that CIN1, CIN2, and CIN3 are three separate nosographic entities.

The cases that progressed to CIN2 and CIN3 were compared with those that progressed to CIN1 only, and incident CIN3 was compared with incident CIN2 in multinomial regression models. Also these results substantiate our concept that the three grades of CIN are, indeed, distinct entities with unique risk profiles, though specific clinical risk factors of progression seem to be identified. A schematic representation of the natural history of disease is provided in Fig. 44.8.

9.3 Invasive Cervical Carcinoma

Much attention has been given to the natural history of disease for cervical cancer. The mean age of women with invasive cervical cancer is around 50 years, while the mean age of women with HSIL is only approximately 28 years (Fig. 44.8), suggesting a long precancerous state. Epidemiologic studies have not been able to consistently identify risk factors for invasion, even though, as mentioned above, cofactors to HR HPV infection, such as smoking, long-term oral contraceptive use, infection with other sexually transmitted infections, and multiparity or integration of HPV into the host chromosome, have been hypothesized to play some role in this transition [8, 290, 375–379].

Historical literature suggested that between one- and two-thirds of women with HSIL will develop invasive cancer if left untreated [380, 381]. It is now known from histological evidence and a few early follow-up studies that invasive cervical carcinoma develops frequently from CIN 3. However, the absolute risk of untreated CIN 3 developing into invasive disease is still argued, with estimates averaging about 30 % but ranging from 10 to 90 %.

HPV-16 is consistently the most common cancer-associated type, and the epidemiologic definition of cancer-associated types correlates well with the relative transforming properties of the various viral types as defined in vitro and with "phylogenetic" studies grouping HPV types by genetic relatedness, suggesting HPV type itself to be a possible risk factor for progression [1, 132]. HPV-negative cervical carcinoma, on the other hand, appears to exist as a separate entity, with a worse prognosis [382] and possibly distinct molecular features [383].

9.4 Other Carcinomas Associated with Human Papillomavirus Infections

9.4.1 Other Genital Carcinomas

Genital HPV infection is associated with carcinomas and premalignant lesions of nearly all other parts of the female and male lower genital tracts, including vaginal, vulvar, penile, and anal neoplasia. Analogous to the concept of CIN for the cervix, pathologists recognize a spectrum of HPV-related intraepithelial pathologic changes called vulvar intraepithelial neoplasia (VIN), vaginal intraepithelial neoplasia (VaIN), penile intraepithelial neoplasia (PIN), and anal intraepithelial neoplasia (AIN). Multifocal intraepithelial genital lesions, associated with different HPV infections of cancer-associated types, are often diagnosed. As a result, some authors have coined the term of "lower genital neoplasia syndrome" for these pathological entities [216].

However, when investigating these premalignant lesions in these other genital carcinomas, the proportion attributable to HPV infection may be lower than for cervical carcinoma. It has been thus hypothesized that the other genital carcinomas may be more multifactorial than cervical carcinoma. In vulvar carcinoma, for instance, different histological subtypes of squamous cell vulvar carcinoma, with distinct epidemiologic profiles, are recognized [384, 385]. While typical squamous cell vulvar carcinoma in older women is not HPV associated, the basaloid and warty, two of the more rare subtypes, are found more often in younger women and are found to be HPV associated [385]. The role HPV in vulvar neoplasia is further supported by the fact that HPV-associated vulvar carcinomas are often found adjacent to intraepithelial lesions that may represent vulvar HPV infections.

Both vulvar and penile premalignant lesions are however less studied, and the amount of elevation in risk of subsequent carcinoma posed by these lesions on the vulva or penis is unclear. In the case of the penis, the term *bowenoid papulosis* is used to indicate multiple lesions that occur with a histological appearance resembling intraepithelial neoplasia [386]. Bowenoid papulosis occurs mainly in patients under 40 years of age and is thought to be benign, contrary to the fact that HPV-16 is the predominant HPV type [386].

Bowen's *disease*, in contrast, occurs at generally later ages and is synonymous with PIN 3 or VIN 3. Bowen's disease occurs most often as a solitary plaque shows a slowly progressive course [386]. Epidemiologic studies of intraepithelial HPV-associated lesions of the penis and vulva are limited so that prevalences and risks of progression are still not well defined. The role of HPV infection in the etiology of penile cancer is especially unclear [387]. In some regions, chronic inflammation appears to be a more important risk factor [388].

9.4.2 Nongenital Carcinomas Associated with Human Papillomavirus Infections

Benign HPV lesions of the oral cavity are quite common and are often asymptomatic. Genital HPVs, most commonly HPV types 6, 11, and 16, have been retrieved from the oral mucosa, in about 50 % of papillomatous oral lesions, with types 6 and 11 being responsible for most benign lesions. HPV types 13 and 32 are the most commonly found in focal epithelial hyperplasia (FEH). In any case, the remaining 50 % of lesions appear to be negative for HPV. Maternal transmission of genital HPV types to children with respiratory papillomatosis is better documented [389]. Furthermore, a variety of benign lesions of the oral cavity have been linked to HPV infection, including cases of focal epithelial hyperplasia, papillary hyperplasia, fibrous hyperplasia, lichen planus, and leukoplakia [390].

In head and neck pathology, HPV appears to play a role in at least some cancers [5–7, 10, 391] trailing well behind smoking and alcohol in order of importance. Out of the HPV-positive head and neck cancers, HPV-16 accounts for about 90 %. While most of these cases are squamous cell carcinomas, as is true for head and neck cancers, the HPV-related types have been described as showing a more basaloid pattern.

10 Control and Prevention

10.1 Primary Prevention of Human Papillomavirus Infections

The licensure and widespread adoption of HPV vaccines was an important scientific achievement and a monumental public health success. Work on the development of an HPV vaccine started in the 1980s, and ultimately, the vaccine was developed, in parallel, by multiple centers. In 2006, the US Food and Drug Administration (FDA) approved the first preventive HPV vaccine, Gardasil (Merck and Co., Whitehouse Station, NJ). In 2007, a second HPV vaccine, Cervarix (GlaxoSmithKline, London, UK), was approved in Australia and the European Union, while its approval in the United States came in 2009.

Both these HPV vaccines are based on hollow viruslike particles (VLPs) assembled from recombinant HPV coat proteins. Gardasil is a tetravalent recombinant vaccine (referred to also as HPV-4) that targets the two high-risk HPVs, types 16 and 18, which combined account for around 70 % of cervical cancers, along with HPV types 6 and 11, most commonly seen in genital warts. It contains inactive L1 proteins from these four different HPV types. Cervarix is a bivalent vaccine that targets HPV types 16 and 18 (referred to also as HPV-2).

Gardasil and Cervarix are designed to elicit virus-neutralizing antibody responses that prevent initial infection with the HPV types represented in the vaccine. The vaccines have been proven to be safe and to offer essentially 100 % protection against the development of cervical precancers and genital warts caused by the HPV types in the vaccine. The protective effects of the vaccine are expected to last a minimum of 4.5 years after the initial vaccination [392]. Between the years 2007 and 2009, HPV vaccines entered the recommended, and in some cases mandatory, vaccination schedules of most developed countries. The two vaccines are recommended for women who are 9–25 years old who have not been exposed to HPV. However, since it is unlikely that a woman will have already contracted all four viruses, and because HPV is primarily sexually transmitted, the US Centers for Disease Control and Prevention has recommended vaccination for women up to 26 years of age. HPV vaccines are approved for males in several countries, including Australia, South Korea, and the United Kingdom. The US Food and Drug Administration (FDA) has approved Gardasil for use in males age 9–26 for prevention of genital warts and anal cancer with advisory panel recommendations for male vaccination being available prior to that [393]. According to the current US Centers for Disease Control and Prevention vaccination schedule, a 3-dose series of HPV vaccine on a schedule of 0, 1–2, and 6 months is to be administered to all adolescents aged 11–12 years. Either HPV-4 or HPV-2 may be used for females, and only HPV-4 may be used for males. The vaccine series can be started beginning at age 9 years. The second dose is to be administered 1–2 months after the first dose and the third dose 6 months after the first dose (at least 24 weeks after the first dose). For cases of catch-up vaccination, the vaccine series is to be administered to females (either HPV-2 or HPV-4) and males (HPV-4) at age 13 through 18 years if not previously vaccinated with the same recommended routine dosing intervals [394].

10.2 Treatment of Human Papillomavirus Infections

Standard treatment for HPV-associated, precancerous cervical lesions is either expectant management or treatment, aimed at the destruction of the lesion and the remaining cervical transformation zone by means of loop electrosurgical excision procedure (LEEP), cold-knife excision, cryosurgery, or laser [395]. Research for the use of therapeutic vaccines and targeted gene therapy is still in an early stage.

For genital wart disease, destruction of the lesions remains the major clinical modality, although the indications for therapy and the efficacy of current techniques can be questioned. Eradication of the virus is not an achievable goal through destructive means. Destructive methods include chemical applications, such as podophyllin or acids, and surgical means, including laser, cryosurgery, and electrosurgical loop excision. Trials of adjunctive interferon or antimetabolite administration have been conducted for severe recurrent genital warts, with variable success.

Nongenital cutaneous warts, when treated, can be destroyed by a wide variety of destructive methods including keratolytic agents, cytotoxic agents, chemotherapy, immunotherapy, cryosurgery, and various other surgical removal techniques [396].

10.3 Use of Human Papillomavirus DNA Testing in Cervical Cancer Screening

It has been demonstrated that HPV testing can be used in several ways to clarify and triage inconclusive or “atypical” Pap smear diagnoses [37, 164, 397, 398]. Even in the Bethesda system, which has attempted to sharpen the detection process and restrict the number of inconclusive diagnoses [399, 400], the triage and treatment of women with borderline smears consumes a disturbingly disproportionate amount of clinical resources. HPV testing is used to clarify inconclusive cytological diagnoses, providing quality control of the diagnosis of ASCUS in cytopathology laboratories and, most importantly, clarification and triage of the individual inconclusive cytological result. The current guidelines for the prevention and early detection of cervical cancer as per the American Cancer Society, the American Society for Colposcopy and Cervical Pathology, and the American Society for Clinical Pathology [401] are summarized in Table 44.4.

Table 44.4 American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer

Population	Recommended screening method[a]	Management of screen results	Comments
Aged <21 years	No screening		HPV testing should not be used for screening or management of ASC-US in this age group
Aged 21–29 years	Cytology alone every 3 years	HPV-positive ASC-US[b] or cytology of LSIL or more severe: refer to ASCCP guidelines [402] Cytology-negative or HPV-negative ASC-US[b]: rescreen with cytology in 3 years	HPV testing should not be used for screening in this age group
Aged 30–65 years	HPV and cytology "cotesting" every 5 years (preferred)	HPV-positive ASC-US or cytology of LSIL or more severe: refer to ASCCP guidelines [402] HPV positive, cytology negative: Option 1: 12-months follow-up with cotesting Option 2: test for HPV-16 or HPV-16/HPV-18 genotypes If HPV-16 or HPV-16/HPV-18 positive: refer to colposcopy If HPV-16 or HPV-16/HPV-18 negative: 12-months follow-up with cotesting Cotest-negative or HPV-negative ASC-US: rescreen with cotesting in 5 years	Screening by HPV testing alone is not recommended for most clinical settings
	Cytology alone every 3 years (acceptable)	HPV-positive ASC-US[b] or cytology of LSIL or more severe: refer to ASCCP guidelines [402] Cytology-negative or HPV-negative ASC-US[b]: rescreen with cytology in 3 years	
Aged >65 years	No screening following an adequate negative prior screening		Women with a history of CIN2 or a more severe diagnosis should continue routine screening for at least 20 years
After hysterectomy	No screening		Applies to women without a cervix and without a history of CIN2 or a more severe diagnosis in the past 20 years or cervical cancer ever
HPV vaccinated	Follow age-specific recommendations (same as unvaccinated women)		

Modified from Saslow et al. [401]. (Table 44.1), with permission
[a]Women should not be screened annually at any age by any method
[b]ASC-US cytology with secondary HPV testing for management decisions

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Human T-Cell Leukemia Viruses Types 1 and 2

45

Edward L. Murphy and Roberta L. Bruhn

1 Introduction

Human T-lymphotropic virus (HTLV) is a member of the RNA virus Retroviridae family, genus *Deltaretrovirus*, which causes chronic infection of humans, and is divided into four currently recognized genotypes and serotypes (HTLV-1 through HTLV-4). Discovered several years prior to human immunodeficiency virus, HTLV is actually an ancient virus of humans, having been transmitted from simian species thousands to tens of thousands of years in the past. It causes a chronic, latent infection with integration of proviral DNA into the host lymphocyte DNA and expansion of the infection predominantly by means of lymphocytic proliferation rather than cell-to-cell transmission of virus. Pathogenesis has been well-defined for HTLV-1 and HTLV-2. Most HTLV-1-infected humans are asymptomatic, but serious disease occurs in approximately 5 % of those infected. Unique among human viruses, HTLV-1 causes T-lymphocytic malignancy manifested as a peripheral T-cell lymphoma with or without leukemia in adults. A chronic inflammatory myelopathy characterized by lymphocytic infiltration and degeneration of the thoracic spinal cord, paraplegia, and bladder dysfunction occurs in both HTLV-1 and HTLV-2 infection. Finally, a variety of inflammatory syndromes including uveitis, pneumonitis, and dermatomyositis have been linked to HTLV infection.

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However, knowledge of and research into the epidemiology and pathogenesis of HTLV has been overshadowed by that directed at HIV, another human retrovirus which has caused a global pandemic since its discovery using techniques originally used to isolate HTLV. HTLV is most prevalent in low- and middle-income countries and, because of its relatively modest pathogenesis in comparison to the more famous lentivirus, receives little resources for prevention and diagnosis. In the USA and Europe, HTLV may practically be considered an orphan infection due to its very low prevalence, unfamiliarity to most practicing physicians, and small portfolio of research funding and involved laboratories.

However, HTLV is important in several respects. First, research on HTLV-1 and adult T-cell leukemia/lymphoma (ATL) provides insights into the mechanisms of hematologic malignancy. Despite three decades of research, the mechanism of malignant transformation remains unclear. Alternative mechanisms include: the action of viral proteins including trans-acting viral regulatory protein Tax or the HTLV-1 basic leucine zipper protein HBZ; an imbalance between lymphocyte proliferation and host immunologic control; the accumulation of somatic mutations in lymphocytes proliferating due to HTLV; or, less likely, insertional mutagenesis due to insertion of HTLV-1 genes in sensitive regions of the host genome. Second, HTLV myelopathy provides an interesting model for other inflammatory neurologic conditions, including multiple sclerosis. In this case, a high HTLV proviral load has been clearly associated with increased risk. Specific human leukocyte antigen (HLA) genotypes in the host have also been linked to increased or reduced risk for myelopathy. Finally, HTLV provides both biological and epidemiologic parallels to HIV infection that may be useful in the control of the latter pandemic. For example, current research into the eradication of minimal, latent HIV infection may benefit from research into the predominantly latent infection of HTLV. In addition, the practice of interrupting HTLV mother-to-child transmission, by substituting bottle-feeding for breast-feeding by infected mothers, may be applicable to the HIV setting provided that safe infant formula is available.

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This chapter will give historical background on this human retroviral infection and then continue with a synopsis of current knowledge relevant to HTLV biology, epidemiology, and public health control. Wherever possible, we have attempted to include original references for key scientific contributions and to identify new work of relevance to the field. We shall cover in somewhat less detail the clinical diagnosis and management of HTLV-related diseases, as these have been reviewed recently in other textbooks [1, 2].

2 Historical Background

In 1970, Temin and Baltimore reported the discovery of a novel reverse transcriptase enzyme associated with retroviruses, for which they were jointly awarded the Nobel Prize in 1975 [3, 4]. This enzyme defined a class of viruses in which virally encoded reverse transcriptase reverses the normal flow of nucleotide transcription, allowing a DNA sequence to be copied from the viral RNA template and inserted into the host genome. Gallo et al. subsequently developed sensitive assays for the detection of reverse transcriptase, facilitating the search for retroviruses in human and animal specimens [5]. Cancer-causing retroviruses, first discovered in animals at the beginning of the twentieth century, had proven elusive to researchers seeking a viral etiology for human cancer and leukemia and most had abandoned this quest and focused instead on viral transforming genes that occur as oncogenes in human tumors. However, the new molecular assays for reverse transcriptase combined with astute translational investigation of lymphoma cases in the USA and Japan soon led to the successful end of this search [6].

The human T-cell lymphotropic virus type 1 (HTLV-1), the first recognized human retrovirus, was described in 1980 by Poiesz, Gallo, and coworkers [7, 8], who isolated retroviral particles with type C morphology budding from fresh cultured lymphocytes of a 28-year-old American man with a diagnosis of cutaneous T-cell lymphoma similar to cases seen in the Caribbean. Soon thereafter, Yoshida et al. similarly isolated a new retrovirus from cases of adult T-cell leukemia, which had been recognized as common in southwestern Japan, and the two isolates were subsequently found to be the same virus [9, 10]. The techniques used to isolate and characterize HTLV-1 provided the intellectual and technical basis for the discovery of the human immunodeficiency virus (HIV) in 1983 [11, 12], which was shown to be the cause of acquired immunodeficiency syndrome (AIDS) in 1984 [13]. In fact, the Gallo HIV isolate was originally named "HTLV-III" and renamed HIV when it was recognized to be a lentivirus, not an oncovirus. This, in part, led to the persistent misimpression that HTLV is associated with AIDS.

Consistent with the geographic distribution of ATL, it was soon demonstrated that HTLV-1 is endemic to the Caribbean and southwestern Japan. Cases of ATL were shown to represent a high proportion of T- lymphocytic lymphomas in both of these areas [14]. Then, in 1985, Gessain recognized that HTLV-1 antibodies were frequently demonstrated in patients with a myelopathy in Martinique then known as tropical spastic paraparesis or TSP [15]. Japanese investigators soon realized that a similar neurologic syndrome, which they called HTLV-1-associated myelopathy or HAM, was occurring among HTLV-1-infected patients in Japan [16]. It is now accepted that these two syndromes are the same disease, which is generally referred to as HAM or HAM/TSP.

The second HTLV type, now known as HTLV-2, was isolated 2 years after HTLV-1 from a patient with an unusual T-lymphocytic case of hairy cell leukemia [17, 18]. However, several years passed before it was recognized that HTLV-2 had become epidemic among injection drug users in the USA and Europe [19, 20], and it was several more years before HTLV-2 was known to be endemic among Amerindians and African Pygmies [21–23].

The most recent chapter in HTLV research has included the discovery of two new HTLV types: HTLV-3 (not to be confused with the former HTLV-III renamed HIV) and HTLV-4 in Central African Pygmies and bushmeat hunters [24, 25]. As with past HTLV discoveries, two separate teams of investigators published articles on these new HTLV types within a short window of time. As of this writing, these new viruses have been recognized in only a handful of humans and it is unclear whether they represent recent simian-to-human transmissions or the new establishment of retroviruses transmitted among humans [26–28].

3 Methods for Epidemiologic Analysis

3.1 Sources of Mortality Data

While HTLV-related mortality data are still sparse, there have been significant contributions to the literature within the last three decades. In 1984 the Miyazaki Cohort Study (MCS) was established to determine the natural history of HTLV-1 infection in the endemic population of Miyazaki Prefecture, Japan [29, 30]. This cohort probably has the largest number of HTLV-related deaths, and cohort investigators have published estimates of mortality related to adult T-cell leukemia as well as on the interaction between HTLV-1 and hepatitis C-related liver mortality [31–33]. The Retrovirus Epidemiology Donor Study (REDS) HTLV Cohort was established in 1990 and continued as the HTLV Outcomes Study (HOST), a prospective study of health outcomes including mortality in HTLV-1- and HTLV-2-infected former US blood donors as well as their sexual partners, with

follow-up from 1992 to 2008 [34]. This study found a non-significant increase in mortality among HTLV-1-infected persons, but a significant increase in both overall and cancer mortality among HTLV-2-infected persons [35]. The Nagasaki Cohort Study prospectively monitored subjects with HTLV-1 infection for cancer incidence and survival from 1993 to 2000 [36]. They found increased all-cause mortality but not increased cancer mortality (excluding ATL) in HTLV-1-infected patients. Finally, a case-control study suggested higher mortality in the mothers of HTLV-infected persons in Miyasaki, Japan [37].

3.2 Sources of Morbidity Data

In addition to the studies listed above, several new studies are providing information on morbidity related to HTLV-1 and HTLV-2. The Interdisciplinary HTLV-1/2 Research Group (GIPH) Cohort Study (1997–ongoing) is an open prevalence prospective cohort study conducted by the Hemominas Blood Center in Brazil to assess behavioral and environmental risk factors for seropositivity [38–40]. The UK HTLV National Register, established in 2003, is the first prospective study in Europe designed to capture progression and onset of disease, consisting of subjects recruited from UK blood service donors and recipients as well as patients from specialty HTLV clinics [41]. Another contribution to morbidity statistics came from a nationwide survey of neurologists regarding HAM/TSP incidence in Japan [42].

Because of the rarity of some HTLV outcomes, consortia of several studies are promising new approaches to quantifying HTLV-1 morbidity. The Study of Adult T-Cell Leukemia/Lymphoma Among Carriers of HTLV-1 is a collaborative study of the immunologic and genetic risk factors for ATL with participants drawn from eight studies of HTLV-1 carriers including the Jamaica Mother-Infant Cohort Study, Jamaica Family Study, Jamaica Food Handlers Study, Miyazaki Cohort Study in Japan, Nagasaki Cohort Study in Japan, Japan Public Health Center-based Prospective Study on Cancer and Cardiovascular Disease, the HOST cohort in the USA, and GIPH Cohort Study in Brazil [43]. A large consortium for the prospective study of ATL has also been formed in Japan [44]. The Comprehensive Oncology Measures for Peripheral T-cell Lymphoma Treatment Registry (COMPLETE) is a prospective, longitudinal, multinational, observational study that will collect data on how patients with peripheral T-cell lymphoma (PTCL) are treated in academic and community practices (www.clinicaltrials.gov). While not exclusive to HTLV-1 carriers, it is the first comprehensive assessment of PTCL treatment and outcomes.

Case-control studies have also proved an efficient approach for studying the etiology of ATL and HAM/TSP, albeit with the drawbacks of the retrospective design, including inability to access pre-diagnostic biological specimens. Examples include case-control studies of HTLV-1 proviral load and antibody titers as risk factors for ATL [45–47] and of mode of infection, HTLV-1 proviral load and HLA genotype as risk factors for HAM/TSP [48–50].

3.3 Prevalence Data

Seroprevalence surveys for HTLV have often utilized convenience samples in concert with other surveillance studies, most frequently HIV surveillance in high-risk populations, intravenous drug users (IDUs), and pregnant women. Analysis of blood samples from Japanese case series of ATL, HAM/TSP, or neurologic abnormalities of unknown etiology provided the first evidence linking HTLV seropositivity with adverse outcomes [51, 52]. Studies that took advantage of special sampling frames such as infant screening for metabolic diseases or food handling safety have contributed prevalence data from a broader population base [53, 54]. Blood donation screening allowed initial estimation of population prevalence, assembly of research cohorts, and initiation of larger population-based surveys in many countries [55–57]. However, truly population-based surveys remain rare [58].

3.4 Laboratory Diagnosis

Detection of antibody to HTLV-1/2 in serum or plasma is the primary method of establishing infection as nearly all anti-HTLV-positive individuals harbor virus, attributable to HTLV persistence via host genome integration. Several standard serologic techniques have been employed as anti-HTLV screening and diagnostic assays, including the enzyme immunoassay (EIA), the chemiluminescent immunoassay (ChLIA), the immunofluorescence assay (IFA), the radioimmunoprecipitation assay (RIPA), the particle agglutination assay (PAA), and the Western blot (WB). While high sensitivity and specificity have been demonstrated in clinical evaluation of most screening assays, false-positive results are common when these assays are used in low-prevalence populations. The most common algorithm for diagnosing seropositivity is to repeat the initially reactive screening assay in duplicate, and then test any repeatedly reactive (minimum two of three replicates) sample with a supplemental or confirmatory test. An alternative, potentially more economical, strategy is to insert a screening assay from a different manufacturer between repeatedly reactive and confirmatory testing steps [59, 60]. HTLV-1 and HTLV-2 antibodies often cross-react on screening serologic assays in common use. For discrimination, testing with an HTLV-1 and HTLV-2 antigen-specific EIA, ChLIA, IFA, Western blot, or RIPA or subsequent testing by polymerase chain reaction (PCR) is required.

The most common qualitative screening assays used in the USA are the EIA, which utilizes viral lysate and recombinant antigens in a colorimetric assay, and the ChLIA, which incorporates the same antigens in a light-emitting assay. Both assays perform well and comparably in terms of sensitivity and specificity when used for initial screening [61]. In Japan, a PAA, consisting of purified HTLV antigens coated on artificial gelatin particles, was developed and performs quite well, especially in their HTLV-endemic population [62]. IFA or RIPA may be used for confirmatory testing, but are not suited for high-volume screening as they are more labor and cost intensive than EIA or ChLIA. PCR may be useful as confirmatory testing in endemic areas with a high frequency of WB indeterminate results following a repeatedly reactive EIA. In Africa, the abundance of indeterminate WB results may be influenced by the high rate of coincidental *Plasmodium falciparum* infection [63].

Precursor proteins in provirus production have characteristic molecular weights and at least one *env* gene product and one *gag* gene product should be used as the immunologic targets in WB analysis. Modified WB assays use both group- and type-specific proteins to allow simultaneous confirmation and differentiation of HTLV-1 and HTLV-2 with high accuracy [64–68]. Antibody quantification may be done with serial serum dilutions testing with EIA, PAA, or IFA.

HTLV-1 and HTLV-2 proviral detection and quantification may be done with PCR, where proviral DNA is amplified in repeat enzymatic reactions and HTLV-specific nucleic acid sequences are detected with targeted probes. PCR is an excellent research tool and very sensitive in detection but is not recommended for clinical screening due to false-positive results in low-risk populations. Culturing of HTLV-1, HTLV-2, and other retroviruses may be done from PBMCs obtained from human and simian blood samples together with uninfected lymphocytes with or without cell growth factors [69]. However, the technique is cumbersome and is therefore used mainly for research. For the establishment of relatedness and presumed viral evolution between HTLV viruses, proviral DNA sequence variation may be determined by DNA sequence analysis and/or restriction fragment length polymorphism [70, 71]. However, these analyses are limited by the relatively low degree of sequence variation within the major HTLV-1 genotypes.

4 Biological Characteristics

4.1 Genomic Structure and Replication

The HTLV are diploid retroviruses, consisting of two molecules of single-stranded positive RNA (ssRNA) per virus particle. HTLV-1 and HTLV-2 are approximately 100 nm in diameter with a nearly spherical ribonucleoprotein inner core surrounded by a lipid envelope with glycoprotein projections. Structurally, the complete provirus genome of HTLV-1 contains 9,032 nucleotides and HTLV-2 comprises 8,932 nucleotides (Fig. 45.1). Their genomes are 65 % homologous, but their encoded amino acids are less so, especially between viral capsid and envelope proteins. In general, retroviral genes code for large overlapping polyproteins that are processed by viral protease into functional peptides.

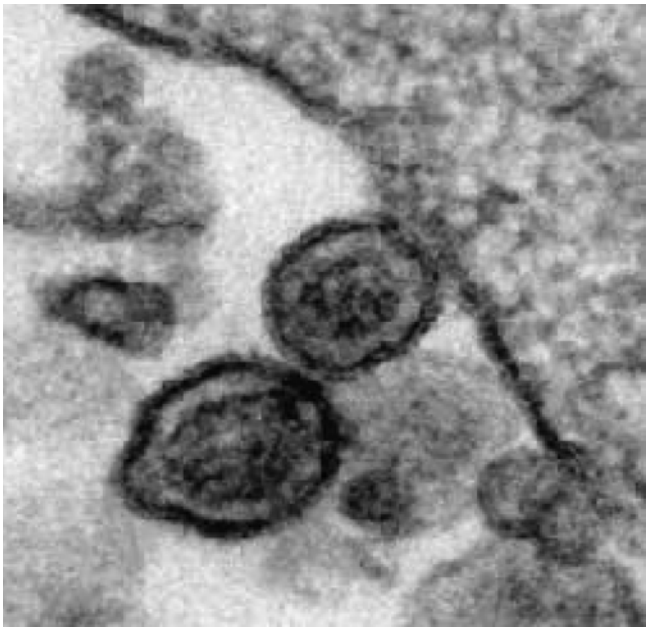

Fig. 45.1 HTLV-1, just after release from an infected T cell; electron micrograph from the National Institutes of Health

The replication strategy of HTLV is typical of members of the Retroviridae family, wherein the RNA genome undergoes reverse transcription into a DNA provirus that integrates into the host genome. Once in the cell, the virally encoded RNA-dependent DNA polymerase (reverse transcriptase) complexed to the genomic RNA of the virus transcribes viral RNA into double-stranded DNA. This double-stranded viral cDNA is transported to the nucleus where, through a complex process mediated by the viral integrase, it is inserted into the host genome. Subsequent virion production is via this integrated DNA under control of the viral regulatory genes. When the DNA provirus is expressed (transcribed by a cellular RNA polymerase), viral genomic mRNA and, subsequently, viral proteins are made by the cell. New genomic RNA is assembled at the cell membrane and packaged for release. During the budding process, the envelope incorporates some of the cell's lipid bilayer, producing an infectious virion of about 100 nm. The host-cell genomic integration of HTLV establishes a lifelong infection and is crucial to both the virus replication cycle and amplification of provirus.

The HTLV genome includes the coding regions of *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope)

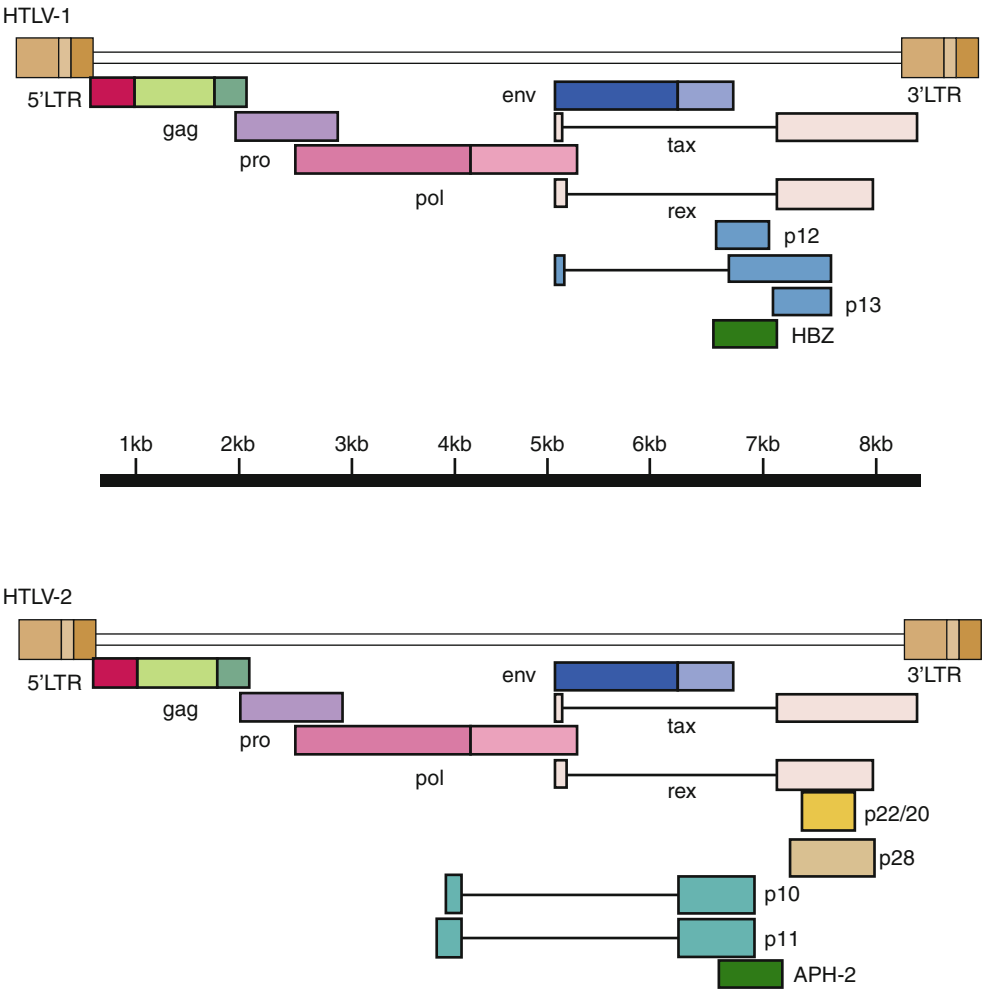

Fig. 45.2 Genomic organization of HTLV-1 and HTLV-2

common to other retroviruses, plus an additional coding region, *pX*, consisting of four open reading frames (ORFs): pX-I, pX-II, pX-III, and pX-IV. The long terminal repeat (LTR) with three distinct regions (U5, R, and U3) flanks the genome at the 5′ cap and 3′ poly-A tail, and these sequences have been shown to be identical between the HTLV-1 and HTLV-2 genomes (Fig. 45.2).

4.2 Molecular Biology

The *gag* gene encodes structural proteins of the nucleocapsid, capsid, and matrix. The *pol* gene encodes a protease that cleaves Gag and Gag/Pol polypeptides into proteins of the mature virion; a reverse transcriptase that generates double-stranded DNA from the RNA genome; and an integrase that incorporates viral DNA into the host-cell chromosomes. The *env* gene encodes the major components of the viral coat including a surface glycoprotein (gp46) and a transmembrane glycoprotein (gp21).

The *pX* ORFs I and II code for accessory and regulatory proteins with putative functions of cell-cycle regulation. The *pX* ORF I codes for the 12-kDa protein, $p12^{I}$, and *pX* ORF II results in the production of two nuclear proteins, $p13^{II}$ and $p30^{II}$. The protein of *pX* ORF I enhances T-cell activation and is essential for establishing persistent infection while proteins of both *pX* ORF I and II impact cell proliferation and modulate host immune responses to HTLV-1 infection.

The pX ORFs III and IV encode the trans-acting proteins Tax and Rex, which are the major regulators of HTLV replication. HTLV-1 Tax is a 40-kilodalton (kDa) protein (p40), and HTLV-2 Tax is a 37-kDa protein (p37). These proteins localize primarily to the nucleus of infected cells, although small amounts of Tax have been found in the cytoplasm. The Tax proteins are responsible for enhanced transcription of viral and cellular gene products. The Tax viral regulatory protein for HTLV-1 plays an important role in promoting viral replication and disease pathogenesis via trans-activation of cellular genes, such as those encoding interleukin-2 (IL-2) and the IL-2 receptor (IL-2R), which

promote cell proliferation; the proto-oncogenes c-fos and c-erg; the gene encoding granulocyte-macrophage colony-stimulating factor; the human lymphotoxin gene; the parathyroid hormone-related protein gene; and an array of early response genes [72]. The process is also modulated by simultaneous trans-repression of other genes, including the β-polymerase gene. The Tax protein interacts with both nuclear regulatory elements (the NF-κB pathway) and cytoplasmic elements through induced nuclear translocation of transcriptional activation factors [73, 74]. The *rex* gene of HTLV-1 and HTLV-2 encodes two proteins in each virus. In HTLV-1, a 27-kDa protein (p27) and a 21-kDa protein (p21) are formed, while in HTLV-2, a 26-kDa protein and a 24-kDa protein are formed. Previous studies have suggested that while the Tax proteins activate transcription, Rex proteins act only posttranscriptionally to regulate viral gene expression via cytoplasmic export of unspliced or incompletely spliced viral mRNAs encoding *gag/pol* and *env* and that nuclear export and expression of the doubly spliced tax/rex RNA is Rex independent. However, Tax expression dependent on the Rex-response element (RxRE) and the RNA binding property of Rex has recently been demonstrated as well [73]. The overall balance of tax and rex expression may influence the rate of viral replication and possibly disease progression [74].

Elements in the viral LTR are essential to integration and replication; they form the sites for covalent attachment of the provirus to cellular DNA and provide important regulatory components for transcription. The LTR U5 and R regions form the leader sequence encoded at the 5′ end of mRNA. An overlapping region of R and U3 constitutes the binding site for Rex (RxRE) and contains sequences that control transcription of provirus as well as termination and polyadenylation of mRNA. Specifically, the 5′ end of the U3 region encodes the carboxyl terminus of the Tax protein. Transcription initiated from the 3′ LTR (antisense transcription) of HTLV-1 produces the HBZ (HTLV-1 basic leucine zipper (bZIP) factor) protein, while antisense transcription in HTLV-2 and in the recently discovered HTLV-3 and HTLV-4 governs production of a protein with some similarity in structure and function to HBZ, the antisense protein of HTLVs (APH), denoted APH-2, APH-3, and APH-4 for each virus, respectively [75–78]. The HBZ protein is capable of inhibiting Tax-mediated activation of the HTLV-1 LTR, activating cellular transcription, and promoting T-lymphocyte proliferation [79]. The APH-2 and APH-4 proteins localize in the nucleus of transfected cells, as does HBZ, while APH-3 localizes in both the nucleus and cytoplasm. All APH proteins discovered thus far demonstrate the ability to repress Tax-mediated viral transcription [77, 78]. Recent research has also shown that while APH-2 is associated with higher proviral load, it neither promotes cell proliferation nor causes lymphocytosis [75, 76].

4.3 Cellular Biology

While recent work has identified three proteins expressed on human cells that are important for HTLV-1 entry (glucose transporter 1, heparin sulfate proteoglycans, and neuropilin-1), the precise interaction with the virion and/or each other remains uncertain [80–82]. Natural infection occurs in Old World primates, and numerous animal species can be infected experimentally (mice, rats, rabbits, New World and Old World primates as well). Although HTLV-1 can infect a number of different cell types in vitro, its growth and propagation in vivo are supported mainly by CD4+ cells and to a lesser extent CD8+ cells. In contrast, HTLV-2 preferentially infects CD8+ cells over CD4+ cells.

Based on epidemiologic data demonstrating that HTLV-1 transmission is strongly cell-associated and supported by in vitro cocultivation studies, transmission of HTLV is via live cells and not cell-free body fluids. For this reason, HTLV-1 is not easily transmitted and universal biohazard precautions are adequate for handling potentially infectious blood or bodily secretions. Because HTLV is highly cell associated, the mechanism of initial viral attachment is not well characterized but cell-surface adhesion proteins and cell-to-cell contact are important for facilitating transmission, and, similar to other retroviruses, fusion of the virion with the host-cell membrane results in uncoating of the diploid RNA genome of the virus [83].

Wattel, Wain-Hobson, and colleagues first demonstrated that expansion of HTLV-1 proviral load occurs mainly through expansion of infected lymphocytes rather than through viral replication and infection of new lymphocytes [84]. HTLV-1 carriers with high proviral load as well as patients with HAM/TSP often have oligoclonal expansion of a limited number of large clones [85]. Clonal expansion may also play a role in the pathogenesis of ATL via the accumulation of somatic mutations in host DNA flanking the HTLV-1 clonal integration site [86]. The relationship between the number of clones and the size of each clone may be determined by mode of transmission, with vertical transmission leading to large oligoclonal expansion and sexual transmission leading to polyclonal expansion [87, 88].

4.4 Immunology

Upon initial infection with HTLV, the first detectable antibodies are directed against the transmembrane Env glycoprotein and multiple distinct regions of the Gag polyprotein. Over a longer time-course (weeks to months), subsequent development of antibody to the surface Env glycoprotein occurs and around half of infected individuals will exhibit detectable levels of antibodies against the Tax protein as well. Antibody titers vary from patient to patient in both HTLV-1 and HTLV-2

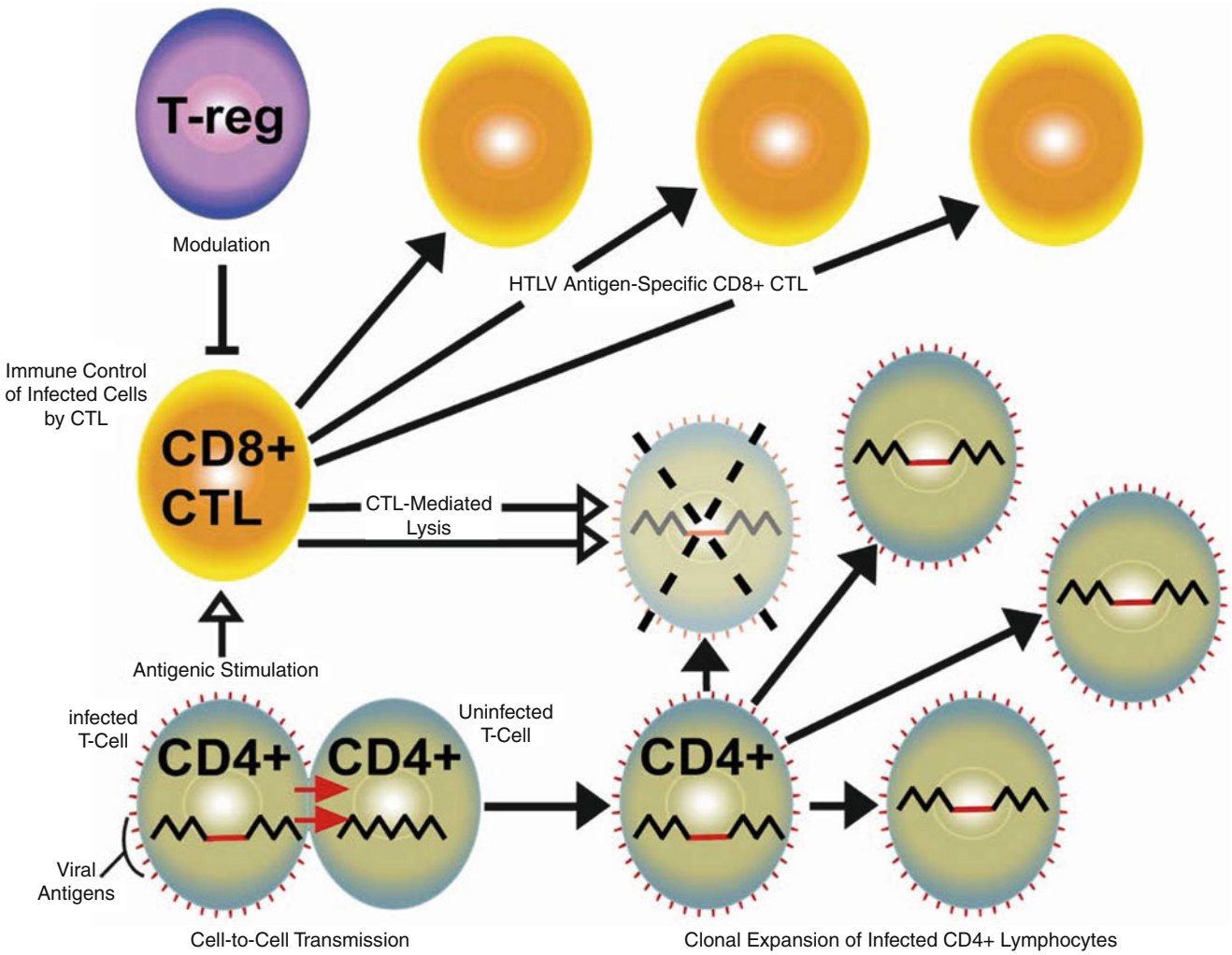

Fig. 45.3 Cell-to-cell transmission, clonal expansion of infected lymphocytes, and cell-mediated immune response to HTLV-1 infection

infection but in general correlate with proviral load, although a protective effect of antibody on HTLV proliferation or disease progression has not been seen in humans [89–91]. Instead, cytotoxic T lymphocytes (CTL) drive the cell-mediated immune response in primary HTLV infection (Fig. 45.3). CTLs in primed patients can recognize numerous epitopes of HTLV, including the Tax antigen. As viral replication results in expression of Tax and $CD4^+$ cell proliferation, the subsequent destruction of infected $CD4^+$ cells via CTLs releases HTLV proviral particles that are then free to infect additional $CD4^+$ cells. As Tax-mediated $CD4^+$ cell expansion escalates, so too does the CTL response, with corresponding $CD8^+$ cell expansion. In an attempt to keep the HTLV infection in check, this process repeats cyclically in infected individuals [92].

Thus, cell-mediated immune response to HTLV infection is inadequate to abrogate persistent viral infection. The effect of $CD4^+$ T-helper 1 (Th1) cell response in HTLV infection has not been extensively characterized, but recent work demonstrated that the HBZ protein, which is constitutively expressed in infected cells, directly suppressed production of Th1 cytokines in a murine model, diminishing the effect of cell-mediated immunity and resulting in higher rates of opportunistic infection when challenged [93]. Cell-mediated immune response to HTLV may also be responsible for the pathogenesis of HAM/TSP (see below).

5 Descriptive Epidemiology

5.1 Molecular Epidemiology and Simian Origins

HTLV and the related simian T-lymphotropic viruses (STLV) together are designated as the primate T-cell lymphotropic viruses (PTLV). It is generally accepted that HTLV resulted from cross-species STLV infections either through a nonhuman

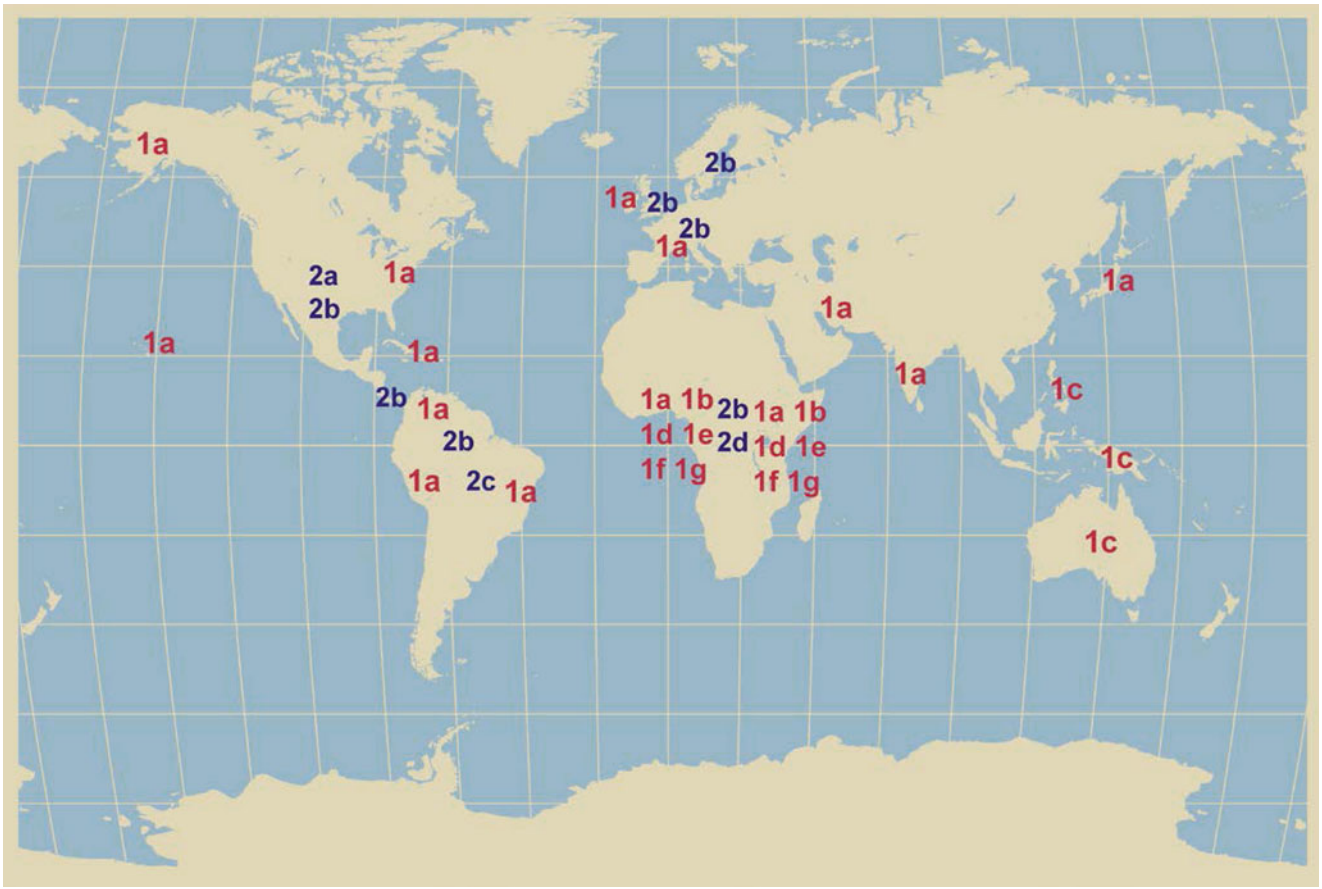

Fig. 45.4 Geographic distribution of HTLV-1 (indicated by "1") and HTLV-2 ("2"). Subtypes indicated by letters

primate bite or through the process of hunting and butchering a primate carcass. Africa is the only continent where all known PTLV have been identified, and this evidence, along with phylogenetic analyses, strongly suggests a common ancestor of Central African origin [26, 70, 94–97]. Currently, four types of HTLV are recognized: HTLV-1, HTLV-2, HTLV-3, and HTLV-4, but only three related STLV counterparts have been identified (STLV-1, STLV-2, and STLV-3). The first HTLV was isolated in 1979 and subsequently identified as HTLV-1 in 1980. Of the remaining types, HTLV-2 was identified in 1982, while HTLV-3 and HTLV-4 were both discovered in 2005 [6, 26]. All HTLV types exhibit a low level of genetic drift, reflected in high genetic consistency among isolates maintained in cell culture from the same geographic area or within a related strain. The broad geographic dispersion of HTLV-1 is thought to be linked primarily with the slave trade while HTLV-2 clusters are more closely aligned with geographically segregated indigenous peoples and IDUs (Fig. 45.4) [70, 95, 96]. Seven molecular subtypes of HTLV-1 have been identified, primarily distinguished by nucleotide sequence within the LTR region: the cosmopolitan subtype, 1a, has worldwide distribution and is further separated into subgroups (A–E); five African subtypes (1b and 1d-1 g); and a Melanesian/Australian subtype (1c) (Fig. 45.5) [98]. Some HTLV subtypes cluster by racial and ethnic group within a limited geographic region, but closely related isolates have also been found in widely disparate locations, including the Caribbean, Japan, Chile, Iran, and Kuwait [99]. Four subtypes of HTLV-2 (a–d) have been identified; although there is still controversy as to whether 2-c is a separate subtype, a *tax* gene of very different length supports a distinction (Fig. 45.6). Two subtypes of HTLV-3 (b and d) have been isolated [26, 94], but, as yet, no subtype or STLV counterpart has been identified for HTLV-4, and extensive genetic analysis has clearly demonstrated its monophyletic nature. HTLV-4 is also considered the oldest known strain of the PTLV lineage [26]. Fairly extensive data are available on the seroprevalence of HTLV-1 and HTLV-2, but there are only limited data on the extent of HTLV-3 or HTLV-4 infection, mostly from studies of Pygmies and those exposed to nonhuman primates in Central Africa [100, 101].

5.2 HTLV-1 Prevalence by Geography and Risk Group

Prevalence data are primarily collected via cross-sectional serologic surveys and convenience samples from targeted groups that may not be representative of the general

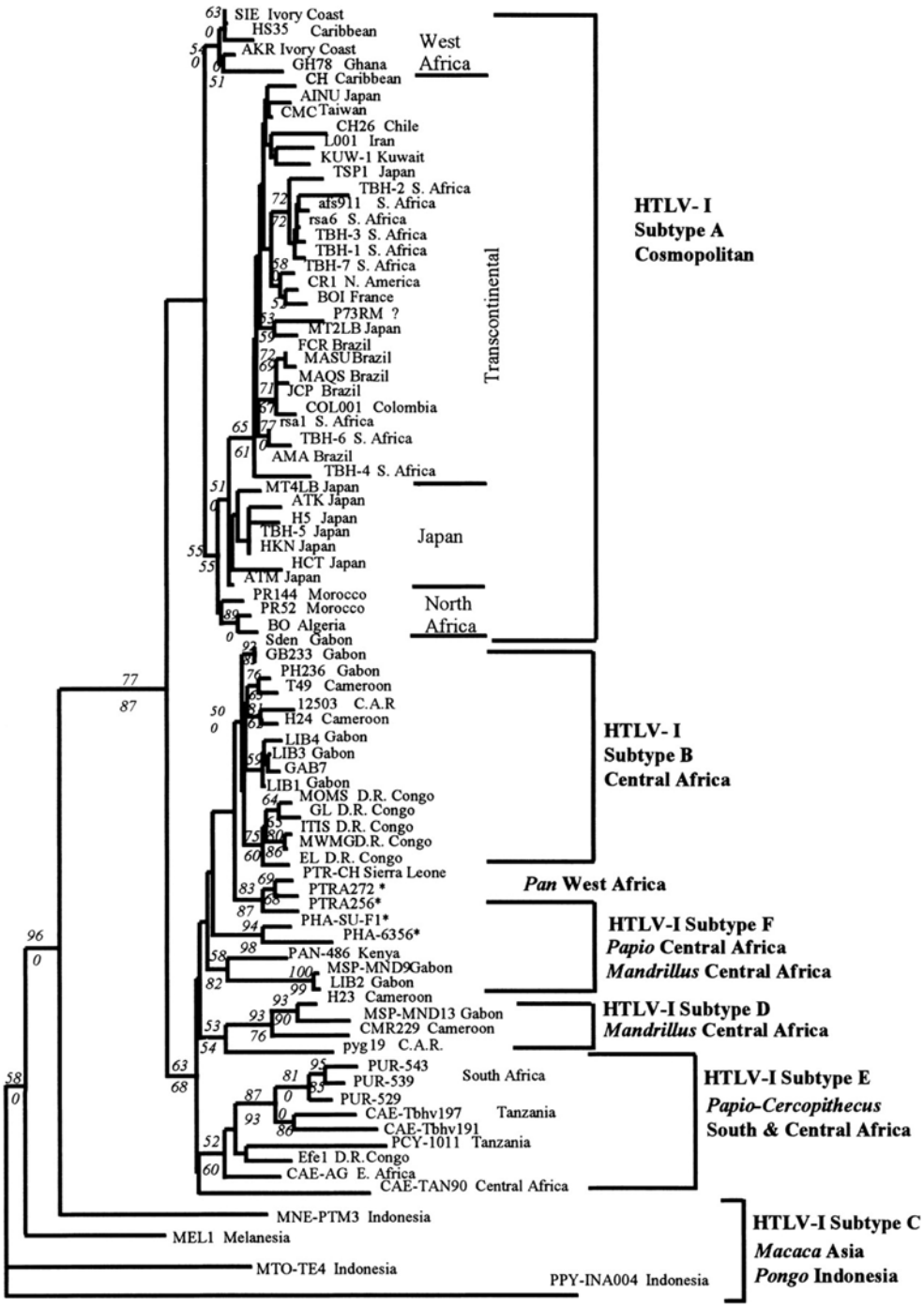

Fig. 45.5 Phylogenetic classification of HTLV-1 based upon the LTR region (per Slattery et al. [70])

population, such as low-risk blood donors or high-risk sex workers and IDUs. Prevalence estimates prior to the 1990s must be viewed with caution because many were based upon screening with early EIAs that suffered from reduced specificity, lacked confirmation by Western blot or other more specific tests, or did not discriminate between HTLV-1 and HTLV-2 cross-reactivity.

The seroprevalence of HTLV-1 is age and sex dependent in most populations, increasing with age and higher in females (Fig. 45.7). There is often localized geographic clustering or clustering within ethnic and social groups due to transmission primarily within families or among individuals with shared risk behaviors. Ongoing mother-to-child transmission and higher sexual transmission rates from males to females may account for the characteristic age and sex distribution. Birth-cohort effects are seen with HTLV-1 and HTLV-2 prevalence and may be due to higher prevalence in past versus current generations attributable to increased

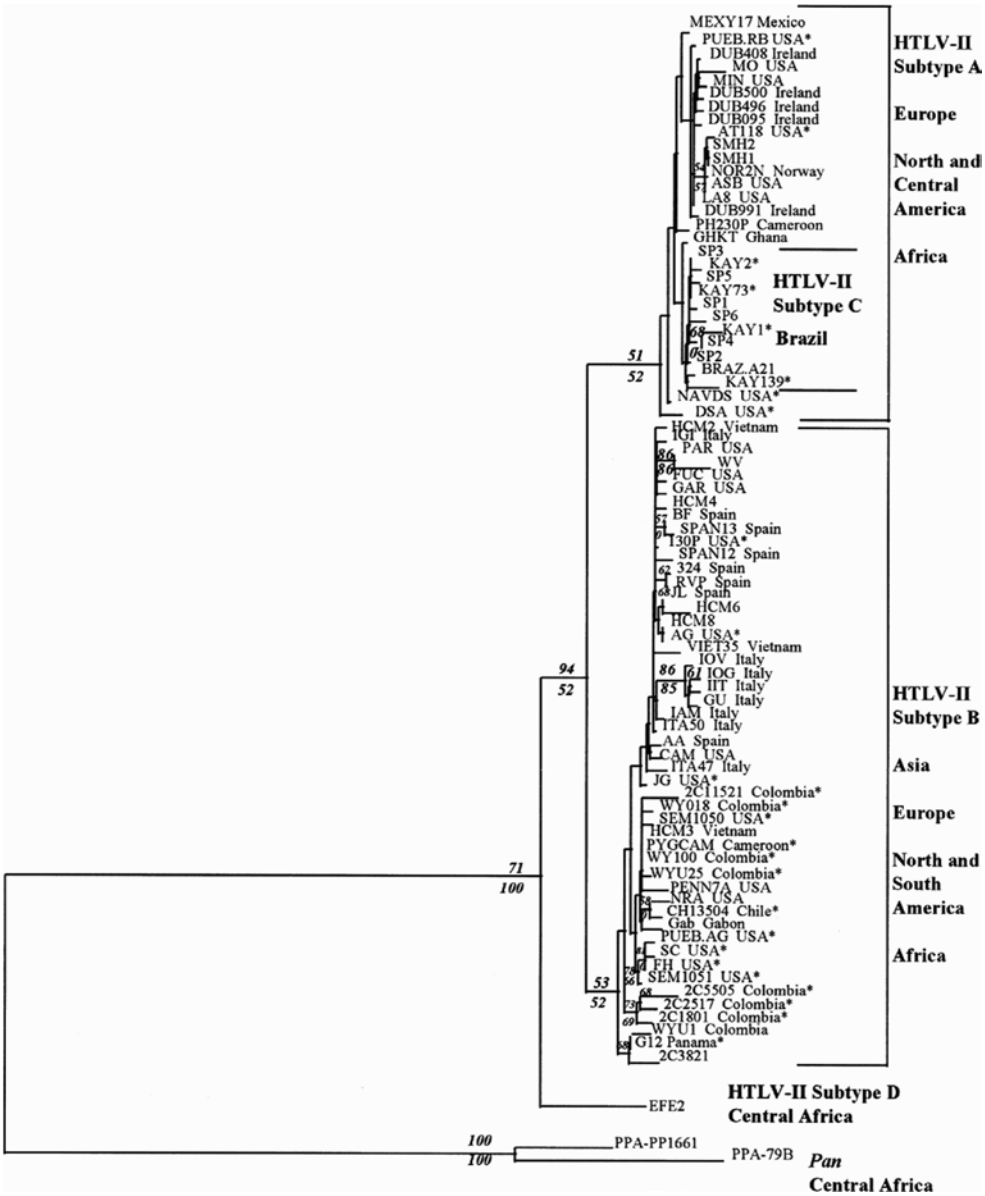

Fig. 45.6 Phylogenetic classification of HTLV-2 based upon the LTR region (per Slattery et al. [70])

condom use, reduced breast-feeding, or reduced injection drug use [56, 102].

Regions with the highest HTLV-1 seroprevalence rates are southwestern Japan, sub-Saharan Africa, islands of the Caribbean basin, and Central and South America [54, 56, 58, 95, 103, 104]. The most recent study (2006–2007) of HTLV-1 in Japan measured seropositivity first-time blood donors and extrapolated the data to fit the general population, resulting in nationwide prevalence estimates of 0.66 % in males and 1.02 % in females [105]. However, pockets of high endemic prevalence in the Japanese islands (up to 36 %) still remain [58]. Several studies in Africa report 1.9 %, 5 %, and 6.6–8.5 % prevalence in Cameroon, Guinea-Bissau, and Gabon, respectively [58, 98, 106]. The Caribbean islands are endemic with HTLV-1, mainly in populations of African slave descent. Prevalence in Jamaica was about 4 % according to a survey in the 1980s [54]. In South America, prevalence in blood donors ranged from 0.14 % in Brazil, 0.73 % in Chile, and 0.07 % in Argentina [58, 60]. In Peru and southern regions of South America, HTLV-1 infection is most prevalent in indigenous people [96]. HTLV-1c subtype is found in Aborigines of northern Australia at a rate of 14 % [58]. Population-based surveys were conducted in two separate villages of a newly endemic region in Mashhad, Iran. One study reported 2.42 and 1.31 % prevalence in men and women, respectively, and the other reported overall prevalence of 2.12 % with no difference between males and females [107, 108]. In both locations seropositivity was significantly associated with age and the subtype was exclusively HTLV-1a.

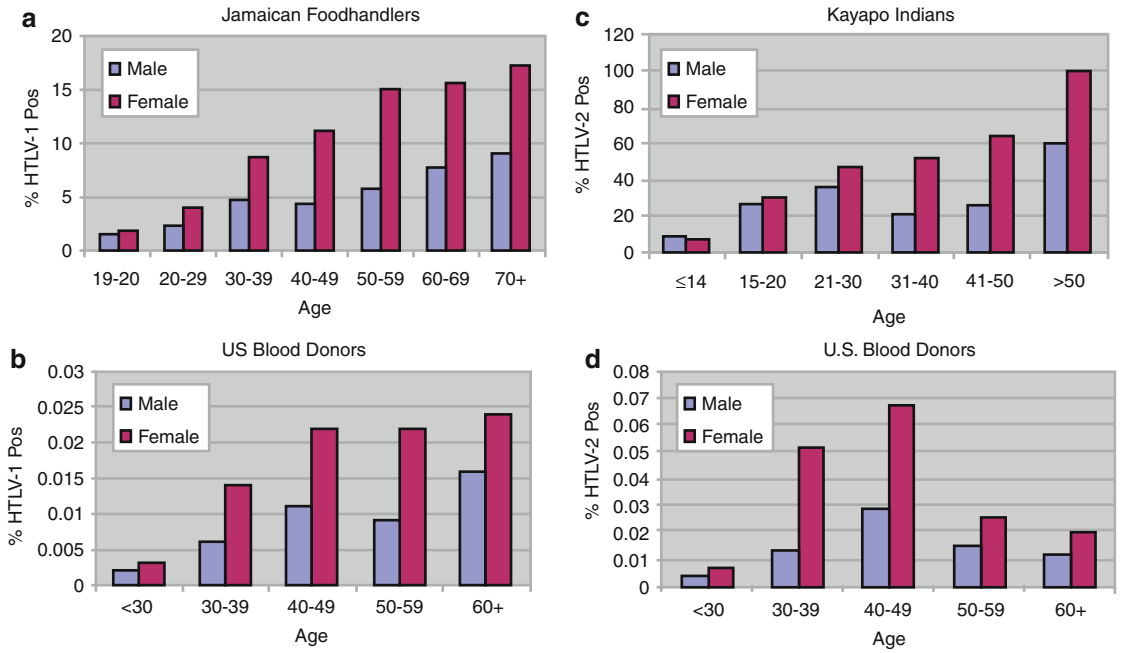

Fig. 45.7 Seroprevalence of HTLV-1 and HTLV-2, illustrating typical age and sex dependence of HTLV-1 among endemic Jamaicans (panel **a**, Murphy et al. [54]) and US blood donors (panel **b**, Murphy et al. [110]). For HTLV-2, a similar age and sex dependence is seen for endemic Kayapo Indians (panel **c**, Murphy *J Infect Dis* 1992) but a probable birth-cohort effect is seen among US blood donors (panel **d**, Murphy et al. [110])

In Europe and North America, the prevalence of HTLV-1 is quite low and is often associated with immigrant populations. The overall prevalence in foreign-born pregnant women in Spain was 0.2 % [109]. Prevalence in French blood donors was reported as 0.0039 % while surveillance data from US blood donors indicated seroprevalence of 0.01 % [110]. HTLV-1 is also found in Native Alaskans in the USA, supporting the association with human migratory patterns [96].

5.3 HTLV-2 Prevalence by Geography and Risk Group

For HTLV-2, there is an age-dependent rise in seroprevalence within endemic Amerindian populations; however, age-specific prevalence among US blood donors peaks between the ages of 40 and 50, consistent with a 1960s–1970s injection drug use-induced birth-cohort effect, plus secondary sexual transmission (Fig. 45.7). HTLV-2 is endemic in many Native American populations in North, Central, and South America as well as the Pygmy tribes (4 % prevalence) in Africa [111, 112]. In Brazil, seroprevalence is highest in the indigenous peoples of the Amazon basin (8–58 %) [111]. A small study of Brazilian blood donors showed a prevalence of 0.006 % [113]. Argentine IDU and urban populations are most frequently infected with subtype 2a, while subtype 2b is detected in Aborigines from Northern Argentina [114]. In Pueblo and Athapaskan Indians in New Mexico, USA, 8 (3.2 %) of 250 persons being treated for sexually transmitted diseases and 15 (3.4 %) of 446 clinic outpatients were seropositive for HTLV-II [115].

In the USA and Europe, HTLV-2 is found at highest prevalence among IDUs. Among 3217 IDUs in 29-drug-treatment centers in the USA, seroprevalence rates of HTLV-2 varied widely by city, ranging from 0.4 % in Atlanta to 17.6 % in Los Angeles [20]. Prevalence is associated with older age, black race, and duration of heroin injection [116]. In Spain and Italy, HTLV-2b subtype is most often detected in IDUs who are coinfected with HTLV-1. The same subtype is also found in Spain and Portugal, while HTLV-2a is most common in North America and Eastern Europe [117].

6 Mechanisms and Routes of Transmission

HTLV-1 and HTLV-2 have several shared routes of transmission: perinatal, primarily through breast milk; sexual; and parenteral, via injection drug use or transfusion of infected cellular blood products. The dominant route of transmission may vary by population. For example, maternal-child and sexual transmission are the norm in endemically infected groups such as Australian Aborigines and Native Amerindian populations while parenteral infection predominately occurs among IDUs [6, 95, 96, 111, 118, 119]. Transmission routes

for HTLV-3 and HTLV-4 have not been established, but in vitro studies of HTLV-3 demonstrate a high affinity for both CD4+ and CD8+ lymphocytes, as seen with HTLV-1 and HTLV-2 [26].

6.1 Maternal-Child Transmission

Transmission from mother to child through breast-feeding is a significant factor in sustaining HTLV-1 and HTLV-2 infection in endemic populations. Provirus from both HTLV-1 and HTLV-2 has been detected in breast milk [120–122]. The risk of vertical transmission is most closely associated with high provirus load in breast milk, the concordance of HLA class I type between mother and infant, and duration of breast-feeding [121, 123–129]. Both high proviral load and antibody titer of the infected mother have been significantly associated with vertical transmission, but only for HTLV-1 [128, 130]. Maternal antibodies readily cross the placenta and can be detected for a time in newborns, but protection from subsequent infection appears to be short-lived, most likely due to the cell-associated nature of HTLV. Ingestion of infected lymphocytes in breast milk is the hypothesized source of infection, and transcytosis of infectious HTLV-1 virions across a tight human epithelial layer, such as occurs in the digestive tract, has been demonstrated in vitro [131]. In addition, evidence of transplacental transmission for both HTLV-1 and HTLV-2 has been documented, but at rates much lower than transmission through breast-feeding [121, 122, 132]. Transplacental infection likely accounts for the small rate of residual mother-to-child infection in infants who are not breast-fed.

6.2 Sexual Transmission

Studies of HTLV-1- or HTLV-2-infected individuals have clearly demonstrated sexual behavior as a risk factor for transmission. While fewer HTLV-2 studies are available, several show evidence of sexual transmission in blood donors, patients at sexually transmitted disease clinics, and Native Amerindian tribes. Case-control and prospective studies of HTLV-discordant couples and seroconversion of negative sexual partners provided the strongest evidence of sexual transmission [71, 133, 134] for both HTLV-1 and HTLV-2. Some studies support higher male-to-female than female-to-male transmission [135, 136], although other studies of discordant partners show more bidirectional infection [134]. The risk of infection also increases with higher proviral load in the transmitter and longer duration of sexual relationship [136]. Cross-sectional studies showing prevalence in both males and females gradually increasing with age support sexual transmission as a predominant source of infection within a community [125, 137–139].

6.3 Blood Transfusion

Transfusion transmission of HTLV-1 and HTLV-2 has been well documented and may still occur in endemic areas where blood products are not routinely screened for HTLV antibody. The first transfusion-transmitted cases of HTLV-1 to be documented in the literature were in a retrospective study of blood donors from the endemic areas of the Japanese islands. Analysis showed that 60 % of recipients who received whole blood later determined to have been positive for HTLV-1 (via testing of routinely stored donor/donation samples) seroconverted within 4–8 weeks of transfusion [140]. Results from this study led to the recommendation that all blood donors be screened for antibody to HTLV. Other studies in Jamaica and another in the USA also confirmed transfusion transmission, albeit with somewhat lower transmission rates. Cold storage of blood and leukoreduction of blood products also lower transmission, presumably due to killing or removal of infected lymphocytes [141–143].

The prevalence of HTLV-1 and HTLV-2 infection in two large US blood bank networks was reported as 9.6 per 100,000 in 2001 and 21.9 per 100,000 in 2000–2009, with the latter showing a decreasing secular trend [144, 145]. Together these surveys covered nearly three-quarters of the US donor population. In the year 2000, the risk of transfusion-transmitted HTLV in the UK, where blood donors are routinely screened for antibody to HTLV-1 and HTLV-2, was reported to range from 0 to 1 per 5,939 recipients, while the most recent data from 2002 to 2006 estimate the risk of an HTLV-1 infectious donation entering the UK blood supply at a very low 0.11 per million donations [146, 147]. In Australia, 0.0003 % of donors were identified as HTLV-1 or HTLV-2 positive between 2000 and 2006 and the modeled residual HTLV transfusion-transmitted infection risk was calculated at substantially less than one per million transfused units [148, 149]. Nucleic acid testing (NAT) has been instituted for other transfusion-transmitted viruses including HIV, HBV, and HCV, but not HTLV, most likely because NAT is performed on plasma and HTLV is primarily lymphocyte associated.

6.4 Injection Drug Use

Both HTLV-1 and HTLV-2 can be transmitted by drug injection, but HTLV-2 has shown a predilection for transmission via this route, especially in the USA where infection in IDUs occurs nationwide and in Europe. Very high prevalence (2–17 %) clusters of either HTLV-1 (2–9 %)- or HTLV-2

(11–25 %)-infected IDUs in the USA can be located on the West and upper East coasts as well as in some Southeastern states [20, 116, 150].

6.5 Other Parenteral Transmission

Other routes of infection are rare, but documented transmission via organ donation and needle-stick injury has occurred. Three recipients of organs from a single HTLV-1-positive donor seroconverted after transplantation and a Japanese health-care worker seroconverted subsequent to a skin puncture with an HTLV-1 contaminated syringe [151]. One HTLV-2-infected blood donor in the USA had no risk factors except occupation as a dentist [143].

7 Pathogenesis and Immunity

7.1 HTLV-1

HTLV-1 exhibits the ability to infect a variety of cell types directly, including T lymphocytes, B lymphocytes, and macrophages, but preferentially targets CD4+ T lymphocytes. In addition, extracellular matrix-mediated transmission and dendritic cell-assisted infection has been described, but how large a role these mechanisms have in establishing persistent infection or in person-to-person transmission is not known [81, 152–154]. Beyond provirus replication at the initial site of infection, it is believed that infection of immune cells occurs within the regional lymphatic system and may result in dissemination of HTLV-1+ cells to reservoirs such as the skin, thymus, liver, spleen, mucosal, and perivascular lymphoid tissue. However, viral replication is not believed to be the primary modulator of persistent infection; instead, clonal proliferation of HTLV-1-infected cells has a significant role [85, 155, 156]. The HTLV Tax proteins have been implicated in the majority of processes leading to persistence and pathogenesis, including cellular activation, transformation, cytokine expression, and inactivation of host tumor-suppressor genes. At initial infection, the immune system mounts a response and most infected individuals develop antibodies within 1–2 months, but periods as long as 18 months prior to seroconversion have been seen in perinatal studies [157]. Antibody usually persists at stable levels throughout life and the majority of HTLV-1 carriers remain asymptomatic, but 2–5 % will develop clinical disease, either ATL or HAM/TSP or rarely both [158]. Why some infected individuals remain asymptomatic while others progress to clinical disease is not well understood and continues to be an area of active research. Recent work has focused on host factors, specifically genetic polymorphisms of HLA alleles and associated immune response. By analyzing HLA alleles in ATL and HAM/TSP familial clusters and the associated HLA allele immune response to HTLV-1, their differential susceptibility to clinical outcome was assessed. Sonoda and colleagues have hypothesized that genetic polymorphisms of HLA alleles determine HTLV-1 clinical outcome, with low immune responder HLA carriers exhibiting no positive immune response and being permissive to HTLV-1 and high immune responder HLA alleles being nonpermissive to HTLV-1 and exacerbating the host inflammatory response. The low responder HLA allele would predispose carriers to ATL and the high responder allele to HAM/TSP [97]. Further studies are needed to elucidate whether specific HLA alleles have predictive potential for exact clinical disease outcomes, analogous to studies that have elucidated the modest genetic contribution to control of HIV infection [159].

7.1.1 ATL Pathogenesis

Adult T-cell leukemia (ATL) develops after many years of chronic infection with HTLV-1 and then in 4 % or less of those infected [160]. The risk of developing ATL appears to be greatest in individuals who acquire HTLV-1 via mother-to-child transmission [161–164]. One hypothesis is that infection of thymocytes early in life leads to the later development of ATL. This theory is supported by in vitro experiments demonstrating that infection of both mature and immature thymocytes resulted in proliferation of HTLV-1-positive thymocytes expressing identical phenotypes [165]. Leukemogenesis is thought to be primarily driven by Tax protein mechanisms that have been well elucidated, including induction of genetic instability and repression of DNA repair mechanisms, transformation and proliferation of primary CD4+ T lymphocytes, and disregulated cytokine production [91, 166, 167]. After Tax has initiated clonal expansion, strong evidence exists that another HTLV-1 protein, HBZ, is critical in maintaining the transformed clonal phenotypes [80, 168–172]. The clones may have functional genomes with the potential for continued expansion or defective genomes that lead to cell senescence [166, 173, 174]. Subsequently a dominant or several dominant clones undergo uncontrolled cell cycling and expansion resulting in clinical manifestation of ATL. Depressed T-cell response and elevated proviral load is a hallmark of ATL. Observation of this pattern in a subpopulation of asymptomatic HTLV-1 carriers suggests that it may be an underlying risk factor [91]. Several studies have correlated antibody level and proviral load with clinical disease outcome as well [175, 176].

7.1.2 HAM/TSP Pathogenesis

HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic progressive inflammatory myelopathy localized predominantly to the thoracic spinal cord. The incidence of HAM/TSP in HTLV-1 carriers is estimated to

be less than 4 % [15, 177]. Epidemiologic evidence suggests that sexual rather than vertical transmission of HTLV-1 is associated with HAM/TSP. This contention is supported by the female predominance of HAM/TSP [178], by sexual activity at an earlier age and higher frequency in HAM/TSP patients compared to controls and by a lack of HTLV seropositivity in the mothers of HAM/TSP patients [48]. Additionally, a study examining fresh, uncultured peripheral blood mononuclear cells for the presence of HTLV-1 tax/rex mRNA by in situ hybridization in HAM/TSP patients and their spouses revealed higher numbers of HTLV-1 mRNA-positive cells in the female spouses of male HAM/TSP patients [179]. The onset of disease is usually gradual and symptoms generally develop when carriers are well into adulthood (40s to 50s), but occasional reports of children with HAM/TSP do occur.

Pathological abnormalities, specifically CD8+ lymphocyte infiltration of brain and spinal cord, have been observed in clinical cases, as has high frequency of cytotoxic T lymphocytes (CTL) with specificity directed against MHC class I-restricted epitopes derived from the Tax protein. Together this is highly suggestive that an immune mechanism involving activated lymphocytes is involved in the development of HAM/TSP. HTLV-1-specific T-cell responses elicit antitumor and antiviral effects in experimental models and are considered to be one of the most important determinants of the disease manifestation, since they are activated in HAM/TSP but not in ATL patients. HAM/TSP patients characteristically have HTLV-1 antibodies or antigens in the blood and cerebrospinal fluid and elevated levels of the lymphokines IL-6, tumor necrosis factor-α, and IL-2. The role of CD4+ T-helper 1 (Th1) cells in CTL response is not as well characterized; however, an association has been seen between HTLV-1 carriers with a specific HLA genotype, lower HTLV-1 proviral loads, and lower rate of progression to HAM/TSP, most likely because of their ability to mount a strong cell-mediated immune response. Control of HTLV-1 infection appears to be by both acquired and innate immunity, and conflict between the two systems may influence disease progression [91, 97, 160, 180–196].

As mentioned above, HLA genotype influences immune response to HTLV-1 infection and has been proposed as a major determinant of HAM/TSP pathogenesis. Bangham and colleagues have shown that the possession of HLA-A*02 and HLA-Cw*08 genotypes was associated with lower proviral load and was protective against HAM/TSP risk in HTLV-1 carriers and that HLA-B*5401 was associated with both a higher provirus load and increased susceptibility to HAM/TSP [197]. In contrast to the theory proposed by Sonoda, above, these authors hypothesized that a strong class I-restricted CTL response to HTLV-1 reduces the proviral load and hence the risk of disease.

7.2 HTLV-2

In contrast to HTLV-1, HTLV-2 clearly does not cause ATL. While HTLV-2 has been linked with HAM/TSP, evidence from cohort studies suggests the risk of acquiring clinical disease is lower than for HTLV-1. These differing pathophysiological outcomes have yet to be fully explained on a molecular, biological, or immunologic basis. Proviral load has been shown to be lower for HTLV-2 than for HTLV-1, different among individuals, but persistent at the same level for many years within the same individual [198]. The reasons for lower HTLV-2 proviral load are not fully understood but may be related to differences between Tax-2 and Tax-1 or between antisense proteins APH-2 and HBZ [74, 75]. In a rabbit model, HBZ and APH-2 may affect proviral load through the later modulation of clonal expansion of HTLV-1- and HTLV-2-infected cells rather than immediately following infection [199]. Finally, a strong CD8+ CTL response directed primarily against Tax-2 does exist in HTLV-2 infection and is inversely correlated with HTLV-2 proviral load, suggesting that differences in immune response could also explain lower pathology with HTLV-2 [200, 201].

8 Patterns of Host Response

8.1 ATL Clinical Manifestations and Treatment

Disease associations of HTLV-1 and HTLV-2 are shown in the Table 45.1. Adult T-cell leukemia/lymphoma (ATL) is a malignancy of peripheral T lymphocytes characterized by monoclonal integration of HTLV-1 provirus in tumor tissue. It may be classified into four forms: acute, chronic, smoldering, and lymphoma types [29]. The acute form of ATL comprises 55–75 % of all ATL [29, 202], with the chronic and the cutaneous forms together comprising the remaining 25 %. Distributions of these subtypes may differ by geographic area. In Japan, ATL typically presents among individuals aged 50–70, while in the Caribbean the typical patient presents at age 40–50.

ATL is almost invariably associated with peripheral and/or central lymphadenopathy. Hepatosplenomegaly is the next most common manifestation, described in approximately 50 % of patients. These findings may be preceded by or occur coincidentally with cutaneous manifestations. Skin lesions are generally described as indolent, nodular, and sometimes indurated. Occasionally patients are reported to present with a more diffuse rash with exfoliation and erythroderma. Pulmonary, CNS, and intestinal involvement are reported less frequently and other extranodal sites are unusual.

Significant anemia and thrombocytopenia are rare early in disease since bone marrow infiltration is unusual. White blood

Table 45.1 Diseases and conditions associated with HTLV-I and HTLV-II infection, with level of evidence for each association

Disease/condition	HTLV-1	HTLV-2
Adult T-cell leukemia/lymphoma	++++	No
HTLV-associated myelopathy	++++	+++
Overall mortality	+++	+++
Cancer mortality	No	++
Infective dermatitis	+++	No
Uveitis	+++	Unknown
Lymphadenopathy	++	++
Pneumonitis	++	++
Polymyositis	++	Unknown
Sjogren's syndrome	++	Unknown
Disseminated strongyloides	++	Unknown

Key: ++++ very strong evidence, +++ strong evidence, ++ possible association, + weak association, *No* evidence does not support association, *Unknown* no evidence in favor or against association

cell (WBC) counts are nearly always elevated and may range into the hundred thousands. Very high WBC counts and elevated serum LDH and calcium are markers of a poorer prognosis. Peripheral blood smears will generally reveal the atypical lymphoid cells with pleomorphic, multilobulated nuclei known as "flower cells." Compared to Sézary cells, nuclear convolutions are more marked in ATL cells. The surface phenotype of ATL cells is usually CD3+/CD4+/CD7−/CD8−/CD25+/CD30+ and terminal deoxynucleotidyl transferase (TdT) negative. Clonally integrated HTLV-1 provirus and clonally rearranged T-cell receptor V-β and V-γ can be detected by inverse PCR of flanking host DNA or southern hybridization [203]. Without treatment acute ATL is invariably rapidly fatal, with pulmonary complications, opportunistic infections, and sepsis emerging as the principal cause of death.

Uncontrolled hypercalcemia also contributes to fatality. Hypercalcemia is described in approximately 30–60 % of patients with ATL and is frequently associated with lytic bone lesions [29, 202]. The pathogenesis of the hypercalcemia may involve Tax-mediated overexpression of parathyroid hormone-related peptide in HTLV-1-infected T cells [204]. More recent evidence suggests that ATL cells overexpress the receptor activator of nuclear factor kappa-B (RANK) ligand gene [205]. Expression of this gene was shown to induce differentiation of human hematopoietic precursor cells into osteoclasts in vitro, in the presence of macrophage colony-stimulating factor (M-CSF).

The chronic and smoldering forms of ATL comprise more infrequent presentations. Monoclonal HTLV-1 integration appears to present in both forms, and thus many of these individuals will eventually transform to acute forms of ATL after many years. Chronic ATL presents with minor cutaneous involvement and persistent lymphocytosis, with fewer cells exhibiting the typical flower-cell morphology. Differentiation of chronic from smoldering forms of ATL seems to be mainly the extent of lymphocytosis and lymphoid cell morphology [5]. In the smoldering form, abnormal lymphocytes comprise <3 % the cells seen on peripheral smear.

The lymphomatous form of ATL may present in the absence of blood or bone marrow involvement in approximately 10–15 % of cases of ATL. T-cell lymphomas typically present with large, firm peripheral lymphadenopathy but may also present as primary CNS or body cavity lymphomas. Lymph node biopsy shows leukemic-type infiltration with medium-sized lobulated lymphoid cells and may closely resemble non-Hodgkin's lymphoma with cells resembling Reed-Sternberg cells [206]. However, the presence of T-cell markers and detection of HTLV-1 proviral sequences, along with HTLV-1 seropositivity, differentiates these tumors. Hypercalcemia is generally absent although eosinophilia has been reported.

The diagnosis of ATL is confirmed by the detection of monoclonally integrated HTLV-1 proviral sequences in leukemic blood or tumor biopsy specimens [207]. A presumptive diagnosis may be made in a patient with serum antibodies to HTLV-1 who has several classic features of ATL including: (1) onset in middle age, (2) presence of abnormal lymphoid cells expressing the T-cell phenotype, (3) peripheral and/or body cavity lymph node enlargement, (4) hypercalcemia with no other explanation, and (4) evidence of clonal T-cell receptor gene rearrangements. Chronic and smoldering forms of ATL may be more difficult to diagnose, but establishment of clonal T-cell receptor gene rearrangements in skin biopsies or blood and detection of HTLV-provirus strongly support the diagnosis.

Treatment for ATL patients is best done in a tertiary care center with expertise in the treatment of HTLV-1-associated malignancies. Median survival with standard lymphoma chemotherapy (e.g., cyclophosphamide, doxorubicin, vincristine, prednisone) is less than 2 years, so innovative treatments are being investigated. Oral zidovudine in combination with high daily doses of interferon alpha has shown promise despite the absence of active HTLV-1 replication in ATL [208]. Recent evidence suggests that arsenic trioxide may induce cell-cycle arrest and apoptosis of ATL cells in vitro and is in consideration as an adjuvant chemotherapeutic agent in combination with interferon alpha [209]. Reports from Japan suggest the use of allogeneic stem cell transplantation following cytoreductive chemotherapy [210, 211]. Waldman and colleagues have attempted immunotherapy using humanized monoclonal antibodies directed against the interleukin-2 and other receptors expressed on ATL cells, with limited success [212, 213].

8.2 HAM/TSP and Other Neurologic Manifestations

Shortly after the discovery of HTLV-1 and its link to ATL, Gessain and colleagues established an association between HTLV-1 and a chronic myelopathy known as tropical spastic

paraparesis or TSP in the Caribbean. This initial report was followed by the recognition of a similar HTLV-1-associated myelopathy, or HAM, in Japan and the acronym HAM/TSP was coined by international consensus [15, 16, 214]. The most prominent feature of HAM/TSP includes chronic inflammatory changes in the spinal cord, most notably a meningomyelitis of the lower thoracic cord, with inflammation localized around capillaries and venules. Hyalinosis of blood vessels, meningeal fibrosis, and glial scars become more prominent in chronic cases, suggesting that treatment to reverse inflammation ought to be implemented early in the course of disease [215]. The occasional cooccurrence in the same patient of ATL in HAM/TSP has been reported [216–219].

HTLV-1 has been causally implicated in HAM/TSP because (1) HTLV-1 has been isolated from the cerebrospinal fluid (CSF) of HAM/TSP patients [220], (2) intrathecal synthesis of HTLV-1 antibodies within the CSF can be detected in some patients [221], (3) viral genome can be detected within involved tissues by polymerase chain reaction (PCR) and by in situ hybridization [222, 223], and (4) HAM/TSP has been shown to develop following blood transfusion to an HTLV-1 seronegative recipient from an HTLV-1-infected donor [224].

HAM/TSP typically develops after years of infection with HTLV-1. Some of the youngest patients reported were still in their first decade of life [225]; however, the majority of individuals present in their fourth or fifth decade. HAM/TSP is gradual in onset and typically presents with leg weakness, spasticity, and low-back pain [226, 227]. Symptoms of urinary frequency or urgency, erectile dysfunction, and sensory neuropathy are common. Weakness and spasticity predominates in the lower versus upper limbs and with progression patients will frequently require a walker or wheelchair. In contrast to multiple sclerosis, cognitive function is spared. HIV vacuolar myelopathy needs to be excluded in cases of HIV and HTLV coinfection.

Although it does not cause ATL, HTLV-2 has been associated with HAM/TSP with a similar presentation, albeit milder in severity and progression, to HTLV-1 cases. As summarized in a recent critical review, there are currently about a dozen known cases of HTLV-2-associated myelopathy [228]. These include four cases from a well-defined prospective cohort of 405 HTLV-2-infected blood donors (cumulative prevalence 1.0 %, 95 % CI 0.3–2.5) followed over more than 10 years [177]. In this same study, six cases of HTLV-1-associated myelopathy were detected among 160 donors (cumulative prevalence 3.7 %, 95 % CI 1.4–8.0), suggesting that the risk of acquiring HAM may be lower in HTLV-2 carriers. Lehky et al. reported another four cases of HAM/TSP in patients with HTLV-2, including four African-American patients, of whom which three were also of Amerindian decent [229]. Magnetic resonance imaging from three of these patients showed white matter disease and HTLV-2 antibodies in the cerebrospinal fluid and serum. HTLV-2 RNA was also detected from a spinal cord biopsy in one patient [229]. These clinical findings along with other case reports of HAM/TSP support a neuropathogenic role for HTLV-2.

In addition to HAM/TSP, HTLV-2 may be associated with several other neurologic disorders, including an ataxic variant of HAM, peripheral neuropathy, and spinocerebellar syndrome. Ataxic HAM shares many of the same signs and symptoms of classic HAM, namely, paraparesis and spasticity, but is distinguished by prominent ataxia, neuropathy, and mental changes. Several cases of ataxic HAM have been reported [230–232]. Finally, some studies have reported an increased incidence of sensory neuropathy in persons infected with HTLV-2 or HIV/HTLV-2 coinfection [15, 42, 177, 233–240]. However, a well-controlled study showed no difference in monofilament sensation between HTLV-2 subjects and uninfected controls, suggesting that confounding by other causes of neuropathy may have been present in earlier reports [241].

Current management of HAM/TSP is suboptimal [242]. Corticosteroids and immunosuppressive agents such as azathioprine may ameliorate disease progression but are unsuitable for chronic use due to adverse effects [243]. Immunotherapy with interferon alpha has produced minimal to moderate results, depending on the degree of inflammation and tissue destruction within the involved tissue [244, 245]. Antiretroviral therapy with zidovudine and 3TC showed initial promise but was ineffective in a controlled trial [242, 246]. Symptomatic therapy for spasticity and bowel and bladder dysfunction is important.

8.3 Autoimmune and Infectious Diseases

A number of rheumatologic syndromes have been linked to HTLV-1 (and possibly HTLV-2) infection. However, with the exception of HTLV-1 uveitis, epidemiologic proof of association between HTLV-1 and autoimmune diseases is weak. In the example of HTLV-associated uveitis [247], HTLV viral sequences can be detected in vitreous fluid in conjunction with higher numbers of HTLV-1-infected T lymphocytes compared with the peripheral blood [248]. High levels of HTLV-1 proviral load have also been associated with Sjogren's syndrome and keratoconjunctivitis sicca [249, 250]. Concurrent autoimmune-mediated disorders, including polymyositis, Graves' disease, arthritis, and HAM/TSP, have been described in several case series.

Infectious diseases and opportunistic infections occur most frequently in the context of acute ATL. These include *Pneumocystis jiroveci* (formerly *Pneumocystis carinii*) infection, pulmonary aspergillosis, cytomegalovirus (CMV) pneumonitis, disseminated herpes zoster, infection

with *Cryptococcus neoformans*, *Mycobacterium avium-intracellulare* infection, and hyperinfection syndrome with *Strongyloides stercoralis*. Trimethoprim/sulfamethoxazole prophylaxis against *P. jiroveci* infection should be administered to patients with acute forms of ATL and possibly to patients with chronic ATL. Patients with prior tuberculin reactivity or residency in countries with high rates of tuberculosis should be considered for isoniazid (INH) prophylaxis.

Some HTLV-1- and HTLV-2-infected carriers without ATL also appear to have immune deficiency associated with an increased risk for certain infectious disease complications, including strongyloidiasis and *Pneumocystis* pneumonia. Other infections associated with HTLV-1/2 infection (single case reports) include Norwegian scabies, disseminated molluscum contagiosum, and extrapulmonary histoplasmosis. Finally, staphylococcal and streptococcal skin infections are common in the infectious dermatitis syndrome [251] described in Jamaica. Interestingly, infectious dermatitis occurs in Jamaica, Brazil, and South Africa but not in Japan, suggesting either socioeconomic or genetic cofactors [252–255].

HTLV-2 has been associated with an increased incidence of pneumonia, bronchitis, tuberculosis, bladder or kidney infection, and abscess. While several studies have found that respiratory disorders were significantly higher among persons infected with HTLV-2, particularly pneumonia and bronchitis [256–259], the pathological mechanism has yet to be determined. HTLV-2 has also been associated with increased incidence of tuberculosis (TB) in studies involving IDU [260] and blood donors [256, 261].

HTLV-2 subjects were similar to uninfected controls in both pulmonary function and production and function of antipneumococcal antibody in response to pneumococcal vaccination, although baseline levels of antipneumococcal antibody were increased [262, 263]. Murphy and colleagues hypothesized that increased incidence of pneumonia and acute bronchitis in HTLV-2 could be the result of transient pneumonitis or bronchiolitis with or without concomitant respiratory infections [258]. An inflammatory pneumonitis has been demonstrated in HTLV-1 patients, in which bronchoalveolar lavage fluid has been found to contain cytokines and other inflammatory markers. Recently, bronchiectasis has been associated with HTLV-1 infection among Australian Aborigines [264].

Skin and soft tissue infections are common among injection drug users, a population in which HTLV-2 is prevalent. The incidence of abscess and the risk factors associated with its occurrence among IDU has received limited investigation. A cross-sectional study found that HTLV-2-seropositive IDU were more vulnerable to skin and soft tissue abscess than were seronegative IDU [259]. However, the findings of this study may have been confounded by factors related to both abscess and HTLV-2 seropositivity, such as sex, race, and frequency of drug injection. Subsequent case-control studies with better control for possible confounding variables did not find a significant association between abscess and HTLV-2 infection [265, 266].

8.4 Other Cancers and Mortality

A prospective cohort study of 138 HTLV-1, 358 HTLV-2, and 759 seronegative former blood donors reported that HTLV-2 was associated with both increased cancer mortality and all-cause mortality [34]. No specific cancer diagnosis predominated. The same study found a nearly twofold increased HTLV-1 mortality that did not reach statistical significance, consistent with increased mortality seen with HTLV-1 and HIV-2 coinfection in West Africa [267]. Another large prospective study also found increased all-cause mortality, but not increased cancer mortality in HTLV-1 patients [36]. Ongoing follow-up of large cohorts will be needed to confirm these findings and determine which causes of death are linked with HTLV-1 and HTVL-2.

8.5 Clinical Laboratory Abnormalities

A number of abnormalities in clinical laboratory tests have been reported with HTLV infection. Abnormal lymphocytes, characterized by poly-lobulated and convoluted nuclei, may be observed in small number among healthy HTLV-1 seropositives but are also characteristic of the leukemia associated with ATL [268]. Lymphocyte and platelet counts are slightly increased among healthy HTLV-1 and HTLV-2 carriers, and red blood cell mean corpuscular volume may be slightly increased in HTLV-2 carriers [269–271].

8.6 HTLV-2 and HIV Coinfection

Since HTLV-2 is prevalent among IDUs in the USA and Europe, coinfection with HIV-1 is common and therefore research on HIV-1/HTLV-2 coinfection is particularly relevant. Recently, investigation has focused on HTLV-2 as a potential modifier of HIV disease progression [272]. Earlier studies on HIV-1/HTLV coinfection did not differentiate between HTLV-1 and HTLV-2. More recent findings have been contradictory; some studies suggest that HTLV-2 coinfection accelerates HIV disease progression and other studies demonstrate no significant effect. Many of the studies had limitations however, including cross-sectional design and small numbers of coinfected patients. Addressing many of the limitations of prior studies, the best analysis on HIV-1/HTLV-2 coinfection included pooled longitudinal data on 370 HIV-infected IDUs from four cohort studies [273]. Dates

of HIV seroconversion for each subject were clearly defined, thereby allowing for the prospective monitoring of clinical AIDS development or AIDS-related mortality in IDUs with and without HTLV-2 coinfection. The rates of decline in CD4 cell percent were similar in singly HIV-1-infected and HIV-1/HTLV-2-coinfected IDUs, and the study concluded that overall, HTLV-2 coinfection did not independently affect HIV disease progression.

Conversely, data concerning the effect of HIV-1 coinfection on HTLV-2 viral load have been limited. One study found no significant difference in viral load between HTLV-2-infected and HIV-1/HTLV-2-coinfected individuals, and similarly, no correlation was seen between HTLV-2 viral load and CD4+ or CD8+ counts in HIV-1-coinfected individuals [274]. Another focus of current research has been on the effects of highly active antiretroviral therapy (HAART) on HIV-1/HTLV-2 coinfection. Preliminary data suggests that HAART may have a paradoxical affect of raising HTLV-2 proviral load. Two separate case series found a similar and marked increase in HTLV-2 proviral load after initiating treatment, which was then followed by a decrease in viral load over time; however, the degree of decline in HTLV-2 proviral load differed between the two studies [262, 275].

8.7 Unconfirmed Disease Associations

HTLV-2 infection has also been diagnosed in certain rare hematologic malignancies, including atypical hairy cell leukemia [17, 276], B-lymphocytic chronic lymphocytic leukemia [277], large granular lymphocytic leukemia [278], and mycosis fungoides [279]. Systematic surveys have not identified any association of these lymphoid malignancies with HTLV-2, suggesting that these patients are coincidentally infected [280]. Finally, an initial report of an HTLV-2 association with chronic fatigue syndrome was not confirmed by a more definitive study [281, 282].

9 Control and Prevention

9.1 Preventing Mother-to-Child Transmission

The efficacy of substituting infant formula for breast-feeding in the prevention of mother-to-child transmission from an HTLV-1-infected mother has been well demonstrated. In late 2010, Japan initiated a nationwide public health intervention to prevent vertical HTLV-1 transmission by HTLV-1 testing of pregnant women and substitution of infant formula for breast-feeding [129]. This followed a successful demonstration program in Nagasaki Prefecture beginning in 1987, which revealed a marked reduction of HTLV-1 transmission from 20.3 to 2.5 % [283]. The same study also demonstrated that breast-feeding for less than 6 months still carried a 7 % risk of transmission, so is not to be recommended. An interventional study has also demonstrated the efficacy of bottle-feeding for prevention of mother-to-child HTLV-1 infection in Brazil [284]. This program was careful to provide infant formula and training in its hygienic use to the mothers. However, experience with bottle-feeding as part of HIV prevention programs lends a note of caution to this approach in low-income countries due to the risk of gastrointestinal infection in infants where adequate hygiene for the preparation of bottle-feeding is not available [130]. This suggests that the risks and benefits of bottle-feeding interventions need to be evaluated on a country-by-country basis before implementation. In addition, human breast milk donated for consumption by other infants should be tested for HTLV, as pasteurization would likely also eliminate the risk of transmitting HTLV infection [285].

9.2 Preventing Sexual Transmission

Although formal clinical trials have not been accomplished and would be ethically questionable, observational data support the efficacy of routine condom use in the prevention of sexual transmission of HTLV-1 and HTLV-2 in serodiscordant couples [134, 136, 286]. The use of latex condoms should also be recommended to HTLV-1 and HTLV-2-infected persons with multiple sexual partners and those engaged in sex work, as these precautions will also prevent HIV and other sexually transmitted diseases in addition to HTLV. The more widespread use of condoms in programs designed to prevent HIV transmission may have had the secondary effect of preventing sexual transmission of HTLV-1 in countries where both are prevalent.

Testing for HTLV-1 has also been integrated into programs for assisted reproduction, with legal requirements in many US jurisdictions [287]. The appropriate counseling of persons found to be HTLV-1 seropositive in this setting should include the performance of confirmatory testing as detailed above. Advances in reproductive technology may also allow the safe use of sperm from HTLV-1-infected men [288].

9.3 Preventing Transmission by Blood Transfusion and Parenteral Exposures

Screening for HTLV-1 and HTLV-2 infection in blood donors is mandated in many countries throughout the world. The efficacy of this approach is clear in countries with high prevalence and routine HTLV antibody screening of all blood donations has likely prevented thousands of HTLV infections. The "residual risk" of HTLV infection, namely, the risk of transmission from an HTLV infection not detected by serologic screening,

has been shown to be less than one infection per million transfused blood units in the USA and Australia and slightly higher (5 per million units) in Brazil [60, 149, 289]. Most of this risk is due to recent infections during the "window period" before antibodies have developed, although some are due to false-negative screening tests. Routine screening of blood donors also provides useful data on prevalence and secular trends of HTLV infection in endemic countries [105]. However, the cost-benefit ratio is less favorable in countries with very low HTLV prevalence, where false-positive screening tests also pose the risk of false notification of donors regarding HTLV infection [55, 61]. In some settings with very low HTLV incidence, testing blood donors only at their first blood donation may be a viable strategy; a similar approach has recently been adopted for *T. cruzi* testing in the US blood donors.

Although specific studies have not been undertaken, universal precautions for the prevention of parenterally transmitted viruses should be effective in the prevention of HTLV-1 and HTLV-2 infection. Similarly, the efficacy of needle exchange programs in prevention of HTLV infection among IDUs has not been explicitly demonstrated, but it is likely that such programs would be effective since HTLV is considered to be less transmissible by small parenteral exposures than HIV or hepatitis C virus.

9.4 Vaccine Development

The development of a vaccine for HTLV-1 has received relatively little research attention and even less commercial interest, especially compared to vaccine development for HIV-1. An effective HTLV-1 vaccine should elicit both humoral response and cell-mediated immunity. As summarized in a recent review [290], approaches to HTLV-1 vaccine development have included: heat-inactivated HTLV-1, Env glycoproteins produced in *Escherichia coli,* DNA vaccines encoding *tax* or *env* genes (in combination with RVV-env or RVV-env+gag), synthetic peptide derived from *env* gp46 and Tax, complex chimeric synthetic multivalent peptide vaccines, and recombinant vaccinia viruses expressing Gag and/or Env proteins. Despite limited success in eliciting protective responses in animal models, none of these vaccines achieved final development, mainly due to partial or short-lived response, safety concerns, and perhaps also a lack of interest from an industrial partner.

9.5 Clinical Management of HTLV-1 and HTLV-2 Seropositives

Evaluation and treatment of asymptomatic HTLV-1 and HTLV-2 carriers are the most frequently encountered clinical situations and generally follow HTLV diagnostic screening of asymptomatic blood, tissue, sperm, or oocyte donors. The first step is to confirm HTLV infection, either by review of positive screening EIA and confirmatory tests performed by a reputable testing laboratory or by submission of another specimen. False-positive results are common using EIA alone in low-risk patients, and they may be relieved of a burdensome diagnosis by the simple performance of a confirmatory assay. Typing of the infection as HTLV-1 or HTLV-2 is important because of the different disease outcomes associated with the two viral types. This can be done either by type-specific Western blot or immunoblot, differential antibody titration on IFA, or PCR. A clinical history regarding risk factors for HTLV infection is important in establishing the pretest probability of infection and can be helpful in typing the infection. Familial or sexual contact with people from HTLV-1 endemic areas favors that infection, while a history of injection drug use or sex with an IDU is more consistent with HTLV-2 infection. Patients with "indeterminate" WB results are also unlikely to be infected if they have no risk factors for HTLV infection [291]. Population-based screening for HTLV infection (except for blood donors) is generally not indicated because of the low penetrance of disease and lack of effective therapies.

According to CDC guidelines, persons with confirmed HTLV-1 or HTLV-2 infection should be informed that they are infected with HTLV [292]. They should be told that HTLV is not the AIDS virus, that it does not cause AIDS, and that AIDS is caused by a different virus called HIV. They should be told that HTLV-1 and HTLV-2 are lifelong infections. They should be given information regarding modes and efficiency of transmission, disease associations, and the probability of developing disease. In particular, persons infected with HTLV-1 or HTLV-2 should be advised to: (1) share the information with their physician; (2) refrain from donating blood, semen, body organs, or other tissues; (3) refrain from sharing needles or syringes with anyone; (4) refrain from breast-feeding infants; and (5) consider the use of latex condoms to prevent sexual transmission.

If the HTLV-positive person is in a mutually monogamous sexual relationship, testing of the sex partner should be recommended to help formulate specific counseling advice. If the sex partner is also positive, no further recommendations are indicated. If the sex partner is negative, the couple should be advised that the use of latex condoms can help prevent transmission of HTLV-1 or HTLV-2 to the negative partner, male or female. Male-infected and female-noninfected couples desiring pregnancy should be made aware of the finite risk of sexual transmission of HTLV-1 or HTLV-2 during attempts at pregnancy and of the small risk for vertical transmission from mother to infant unrelated to breast-feeding. Such couples might be advised to use latex condoms at all times except during the fertile period while they are attempting pregnancy. The use of latex condoms is

strongly recommended for HTLV-1-positive persons with multiple sex partners or otherwise engaging in non-mutually monogamous sexual relationships. These persons should be reminded of the risk of acquiring other sexually transmitted infections, including HIV.

Asymptomatic seropositive patients should be followed up by their primary care or infectious disease physician, with annual to biennial return visits. Medical history should elicit symptoms of leukemia, lymphoma, or neurologic disease. Physical examination is directed at the lymph nodes, neurologic system, and skin in order to detect manifestations of ATL, HAM/TSP, or HTLV dermatitis. Laboratory evaluation may be limited to a complete blood count. Whereas small increases in the absolute lymphocyte and platelet counts and alteration in red blood cell mean corpuscular volume have been described in prospective studies of HTLV-1 and HTLV-2 carriers, there is no indication that these findings have clinical significance [271, 293]. More important is to rule out subclinical leukemia by a normal lymphocyte count and absence of "flower-cell" morphology. In general, asymptomatic carriers or those with nonspecific symptoms should be reassured by reminding them of the low penetrance of hematologic and neurologic disease.

There is also substantial psychological and social morbidity associated with chronic HTLV infection [294–296]. While some authors have suggested a causal role for HTLV in major depression [297], others concluded that physical symptoms related to HTLV infection were more likely mediators of self-reported anxiety and depression [298]. The treating physician needs to screen for psychological effects of chronic HTLV infection and consider referral for counseling and/or psychiatric evaluation should these symptoms be severe.

10 Unresolved Problems

10.1 Epidemiologic Puzzles

Despite almost 30 years of epidemiologic research, several puzzles remain regarding the geographic dispersion of HTLV-1. Its origins in Africa appear to be well founded, with multiple isolates having been identified, differing in molecular sequence according to geography but closely resembling isolates from simian species in the same regions. Similar evidence supports the Melanesian origin of the HTLV-1c variant. Beyond that, the picture is more complicated. While it seems clear that HTLV-1 was transported with human populations during the slave trade to the Caribbean and the Americas, defining specific linkages between regional variants in the New World and their source populations in Africa has been challenging. The search has been made more difficult by the relative lack of variation within the so-called cosmopolitan HTLV-1 subtype. Recently, scientists have identified foci of HTLV-1 in isolated South American populations that appear to map closely with African strains [299]. The high HTLV-1 prevalence in Kyushu and Shikoku in southwestern Japan, in contrast to absent or very low prevalence in neighboring China and Korea [300, 301], remains a puzzle. Initial hints of substantial HTLV-1 prevalence in Mongolia have not been borne out by further research [302]. The hypothesis that HTLV-1 was introduced into southern Japan by Portuguese traders, who also traded with Africa and included Africans among the crew, was initially dismissed but might bear further scrutiny. Finally, the presence of isolated pockets of HTLV-1 such as those among populations in Mashhad, Iran, suggests historical infections and/or human migrations still to be explained [108].

A similarly complicated picture applies to the geographic dispersion of HTLV-2, although the details differ. There is internal consistency with the finding of HTLV-2b among African Pygmies who live in proximity with simian species who harbor closely related STLV-2 viruses. Recent data from Central African Bakola Pygmies confirms high HTLV-2b prevalence (2.5 %; 27/105) [112]. Similarly, HTLV-2b is found in native Amerindians, although New World monkeys have not been found to be infected with STLV. What is puzzling is the extreme molecular homology between HTLV-2b viruses from isolated South American Indians and Bakola Pygmies from Central Africa. These findings are difficult to reconcile with contemporary models of human migrations whereby Amerindians migrated from Asia more than 40,000 years before the present. Thus, there remains an apparent paradox in HTLV-2 evolution: an ancient African origin of HTLV-2a, 2b, and 2d and migration to the New World with human peoples or, less likely, a more modern introduction into both Pygmies and Amerindians.

A second, more modern pandemic of HTLV-2 can be found among IDUs in North America and Europe. Among North American IDUs, HTLV-2a predominates, and it is likely that the virus was introduced from Amerindian populations within the last hundred years and amplified by the parenteral transmission route, which is more efficient than sexual or mother-to-child transmission. Adding an additional twist, IDUs in Europe are more likely to harbor HTLV-2b than HTLV-2a. Whereas one would assume that European IDUs received HTLV-2 from their North American IDU counterparts, the real story may be more complicated, with several Amerindian-to-IDU transmissions and separate amplifications in concert with more modern geographic dispersion among IDUs [303]. Such a picture is supported by the mix of HTLV-2a and HTLV-2b among blood donors in North America, most of whom were infected via IDU or secondary sexual transmission. However, the precise Amerindian population of origin and the timing of this introduction remain unknown and would require much larger studies including both IDUs and Amerindians from a broad geographic area in North America.

Finally, the recent discovery of HTLV-3 and HTLV-4 among Pygmies in Central Africa opens new ground for epidemiologic research. As of now, there are several identified HTLV-3 isolates but only a single HTLV-4 isolate. Given the close proximity to simian species and predominance of hunting and bushmeat consumption, it is likely that these viruses represent relatively recent simian-to-human transmissions. A detailed study of a large extended family failed to reveal evidence of human-to-human transmission of HTLV-3 [100]. Thus, until a greater number of infected humans are identified, it remains speculative that either of these viruses has established a true human reservoir of infection. Likewise, demonstrating any disease outcomes associated with HTLV-3 and HTLV-4 will require larger epidemiologic studies. In this context, the study of blood donor populations in Cameroon and the Democratic Republic of Congo may be revealing.

10.2 Etiologies of ATL and HAM/TSP

Despite more than three decades of research, the etiologies of ATL and HAM/TSP remain poorly defined. It is clear that the virus, by stimulating lymphocytic proliferation, favors the emergence of potentially malignant genome mutations in lymphoid tissue. While the majority of past research has focused upon the *tax* gene because of its ability to transactivate both HTLV and human genes proximal to the integration site, the past several years have seen a new focus on the HBZ gene pathway. Most research on HTLV-1 malignant transformation has focused on in vitro models but has included some animal work, such as the generation of mice transgenic for the *tax* gene. A major obstacle for translational and epidemiologic studies has been the relatively low incidence of malignancy among chronically infected humans together with the long latency between retroviral infection and the occurrence of ATL. Thus, prospective cohort studies have been able to capture only a relatively small number of incident ATL cases among well-defined study populations. The recent study by Birmann, Hisada et al. overcame this limitation by assembling cases of incident ATL and matched HTLV-1-infected controls from across multiple prospective cohorts [304]. Another large multicenter study from Japan showed that HTLV-1 proviral load and a family history of ATL were significant risk factors for development of ATL among 1218 previously healthy HTLV-1 carriers [44]. During follow-up, 14 participants (proviral load 4.17–28.58 copies/100 PBMCs) progressed to ATL, but no cases occurred among those with a baseline proviral load lower than approximately 4 copies/100 PBMCs. Other promising approaches could involve case-control designs and genomic analyses of biological samples from ATL cases and HTLV-1-infected healthy carriers. The HTLV-1 and ATL model may also be seen as a promising one for future studies of the genomics of cancer.

Similarly, HAM/TSP occurs in only a small percentage of those infected with HTLV-1 and HTLV-2 and has an insidious onset. Encouragingly, virological and immunologic studies have yielded several adverse prognostic markers including high proviral load and both deleterious and protective HLA haplotypes. However, future research into the etiology of this immunologic-based neuropathic disease will require the prospective study of large, well-characterized cohorts of HTLV-infected subjects. For both ATL and HAM/TSP, the potential impact of transmission route on disease occurrence requires further study. The preferential occurrence of ATL following mother-to-child transmission, and HAM/TSP following sexual or parenteral transmission, may yield clues to the different pathophysiological pathways of these two disease outcomes.

Finally, effective treatments for both ATL and HAM/TSP are sorely lacking and suffer from their status as orphan diseases occurring predominately in low- and middle-income countries. The recent establishment of a clinical trials network for the ATL is an encouraging sign [305], and a similar working group for the treatment of HAM/TSP has recently been established under the auspices of the International Retrovirology Association [43]. These working groups are attempting to select drug targets that are effective, practical, and affordable in the low- and middle-income country setting. They will then design simple clinical trials and attempt to fund and implement these trials in endemic countries.

10.3 Directions for Future Epidemiology Research

Future epidemiologic research in HTLV-1 and HTLV-2 should focus upon monitoring the prevalence and incidence of infection in endemic populations and the adaptation from the HIV field of population-based prevention strategies that may be effective for HTLV. Secular trends of HTLV-1 and HTLV-2 prevalence in well-defined populations such as blood donors should be monitored as sentinels for infection in broader populations. Such studies have already demonstrated declining prevalence due to birth-cohort effects in both the USA and Japan but have yet to be tied to the effectiveness of a specific intervention. The blood donor population presents the advantage of routine screening of a segment of the general population, albeit of relatively low risk, and allows the collation of data over time. Specific puzzles in HTLV epidemiology may be addressed by larger-scale, geographically based prevalence studies with sequencing of both HTLV isolates and human population genomic markers in order to determine past transmission and human migrations.

While it is clear that condom use and interruption of breast-feeding are effective in preventing sexual and mother-to-child HTLV transmission, respectively,

additional epidemiologic studies of these prevention strategies are warranted. Specifically, it will be important to determine in several endemic countries whether the relatively low burden of HTLV-associated disease prevented is worth the potential risk of infant mortality due to the substitution of infant formula for breast-feeding. Modeling and simulation studies may be useful to understand the potential impact of various intervention strategies in endemic countries. For example, concurrent implementation of HIV prevention may be expected to have a significant effect upon HTLV-1 prevalence in dually endemic countries such as Brazil. Changes in ATL and HAM/TSP incident should also be monitored in response to prevention interventions aimed at HTLV-1 and HTLV-2.

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Polyomaviruses: Progressive Multifocal Leukoencephalopathy and Other Diseases

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1 Introduction

Polyomaviruses are small, nonenveloped, double-stranded DNA viruses, which are widely distributed in nature. Viruses of this family infect many species including humans, monkeys, cattle, rabbits, rodents, and birds. The viruses are species specific and they do not naturally infect any species other than their natural hosts. Primary infections with human polyomaviruses generally occur in childhood and are not associated with serious illness, but the viruses persist in the infected host. Most of the diseases associated with human polyomaviruses occur later in life in immunosuppressed groups, probably as a result of recrudescence of active infection brought about by the immune deficiency of the host. Human illnesses associated with these viruses include progressive multifocal leukoencephalopathy (PML) which is caused by JC polyomavirus (JCPyV) and is a frequent complication in individuals with HIV/AIDS, nephropathy in renal transplant recipients, and hemorrhagic cystitis in bone marrow transplant recipients, both caused by BK polyomavirus (BKPyV) and Merkel cell carcinoma, which is associated with the recently recognized Merkel cell polyomavirus (MCPyV).

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2 Historical Background

The first polyomaviruses were isolated in the 1950s from rodents. The mouse polyomavirus (MPyV) was so named because of its ability to produce many (Greek poly) types of tumors (Greek oma) in experimentally inoculated mice. The first primate polyomavirus to be identified was simian virus 40 (SV40) in 1960 from primary kidney cultures of the rhesus macaque [1]. SV40 was oncogenic for experimentally inoculated hamsters [2, 3]. MPyV and SV40 have been extensively used in laboratory studies of virus-induced cancer, and these studies have led to a detailed understanding of cellular transformation, integration of the viral genome in the cellular chromosomes, cell cycle regulation, and oncogenes and tumor suppressor genes. In vitro studies of replication of SV40 origin-containing DNA have helped understand eukaryotic chromosomal DNA replication. The genomes of MPyV and SV40 were among the first to be physically mapped and also among the first that were completely sequenced. Daniel Nathans was a corecipient of the Nobel Prize in 1978 for the study of the structure of the SV40 DNA by restriction endonuclease enzymes.

The first recognized contact of humans with polyomaviruses was with SV40, which was a contaminant of the inactivated commercially available polio vaccines and of the experimental live polio vaccines [4]. The virus pools for these vaccines were grown in rhesus kidney cultures, many of which were contaminated with the indigenous SV40. The presence of SV40 in the rhesus kidney cultures was not recognized because the virus was not cytopathic for these cultures and was identified only after these cultures were inoculated into African green monkey kidney cultures where SV40 produced a cytopathic effect. It is estimated that almost 100 million people in the USA, about 62 % of the population, had received the inactivated polio vaccine during the time (1956–1960) when some of the vaccine lots were contaminated with SV40 [4]. But the proportion of the vaccine lots that was contaminated, or the amount of live SV40 in the contaminated lots, is not known. The formalin that was used to inactivate polioviruses

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also inactivated SV40, but to a lesser extent. It has been established that a proportion of the inactivated polio vaccine lots contained live SV40, in small amounts, when they were administered to people by the subcutaneous route. Individuals who received contaminated oral live polio vaccines in the early trials received a larger amount of SV40 by the oral route. Inactivated adenovirus vaccines, which were administered to military personnel, were also contaminated with SV40. Whether this exposure of humans to live SV40 has led to the establishment of SV40 as a human infection or to any SV40-induced illnesses and cancers is a matter of debate. These data are summarized in Sect. 8 of this chapter.

The identification of the first human polyomaviruses was reported in 1971. JCPyV was recovered in primary human fetal glial cell cultures after inoculation of brain extracts from a patient with PML [5], and BKPyV was isolated after inoculation of urine from a renal transplant recipient into Vero cell, a continuous cell line derived from African green monkey [6]. No additional human polyomaviruses were recovered until 2007 when the genomes of two new polyomaviruses, KIPyV and WUPyV, were independently detected in respiratory tract secretions of children by use of molecular techniques [7, 8]. In 2008, Merkel cell polyomavirus (MCPyV) was identified by employing mass sequencing of a messenger RNA library of Merkel tumor cells and bioinformatic analysis to identify nonhuman sequences which have homology to the genomes of known infectious agents [9]. MCPyV is commonly found on healthy skin. Three additional human polyomaviruses, HPyV 6 and HPyV 7 in 2010 and HPyV 10 in 2012, were identified from skin swabs by using the rolling circle amplification (RCA) technique to isolate circular DNA viral genomes [10, 11]. Another new polyomavirus, trichodysplasia spinulosa virus (TSPyV), was recovered, also using the RCA technique, from the facial tissue of a heart transplant patient who was suffering from a rare skin disorder, trichodysplasia spinulosa, which only occurs in immunosuppressed individuals [12]. In 2011, a new polyomavirus, HPyV 9, was recovered from the urine and blood of a renal transplant recipient using generic PCR [13]. HPyV 9 is closely related to the African green monkey-derived lymphotropic polyomavirus (recently renamed the B-lymphotropic polyomavirus (LPyV), which was isolated in 1979 [14]. A new polyomavirus, Malawi polyomavirus (MWPyV), was identified by pyrosequencing purified virus-like particles recovered from the stool of a child in Malawi [15]. This virus is genetically highly related to HPyV10 and is likely the same viral species. Additional polyomaviruses have recently been detected in stool samples from children and adults with diarrhea [16–18]. The rapid increase in the number of human polyomaviruses beginning in 2007 is undoubtedly related to the use of molecular methods to identify viral genomes in place of cell culture techniques previously used to identify infectious agents.

The evolutionary relationships between human polyomaviruses based on a maximum-likelihood analysis of VP1 amino acid sequences are shown in Fig. 46.1, which also include select polyomaviruses of nonhuman origin [19]. A recent taxonomic revision of the species has divided them into three genera [20]. The *Avipolyomaviruses* comprise the species that infect birds. They form a distinct lineage not closely related to human polyomaviruses. The biology of the *Avipolyomaviruses* is markedly different from that of the mammalian polyomaviruses. Compared to asymptomatic infection, which is characteristic of mammalian polyomaviruses in immunocompetent hosts, the *Avipolyomaviruses* are frequently associated with acute fatal disease. A second genus, the *Wukipolyomaviruses*, includes only human polyomaviruses. They form a distinct lineage very distantly related to the other human polyomaviruses, and the genus at present contains no known polyomaviruses from other host species. The viruses in this genus are not known to cause disease and knowledge of their natural history is limited. All the remaining known human and animal polyomaviruses are currently grouped in the genus *Orthopolyomavirus*, which comprises many distinct lineages. Many lineages of the polyomavirus family contain viruses from humans as well as from nonhuman primates. For example, JCPyV and BKPyV are closely related to one another and to simian viruses SV40 and SA12; MCPyV is most closely related to a chimpanzee polyomavirus and TSPyV to an orangutan polyomavirus. The closest relative of HPyV 9 is the lymphotropic polyomavirus (LPyV) of African green monkeys. This phylogeny argues against a strict co-divergence of polyomaviruses, with their respective hosts, as previously believed [21, 22], and favors cross-species transmission events in the evolution of human polyomaviruses [23, 24].

The human polyomaviruses, illnesses associated with them, and the body sites from which they are recovered most frequently are listed in Table 46.1. In the following sections, JCPyV, BKPyV, and MCPyV are described in detail. Information on the other human polyomaviruses is summarized in Sect. 8.

3 Methodology Involved in Epidemiologic Analyses

3.1 Mortality Data

Polyomavirus infections are not associated with mortality except in immunosuppressed populations. BKPyV-associated nephropathy was first identified in 1995 and is not currently a reportable disease. Mortality data for PML in the USA between 1979 and 2005 are available from the National Center for Health Statistics, Centers for Disease Control and Prevention [25]. During this time period, the

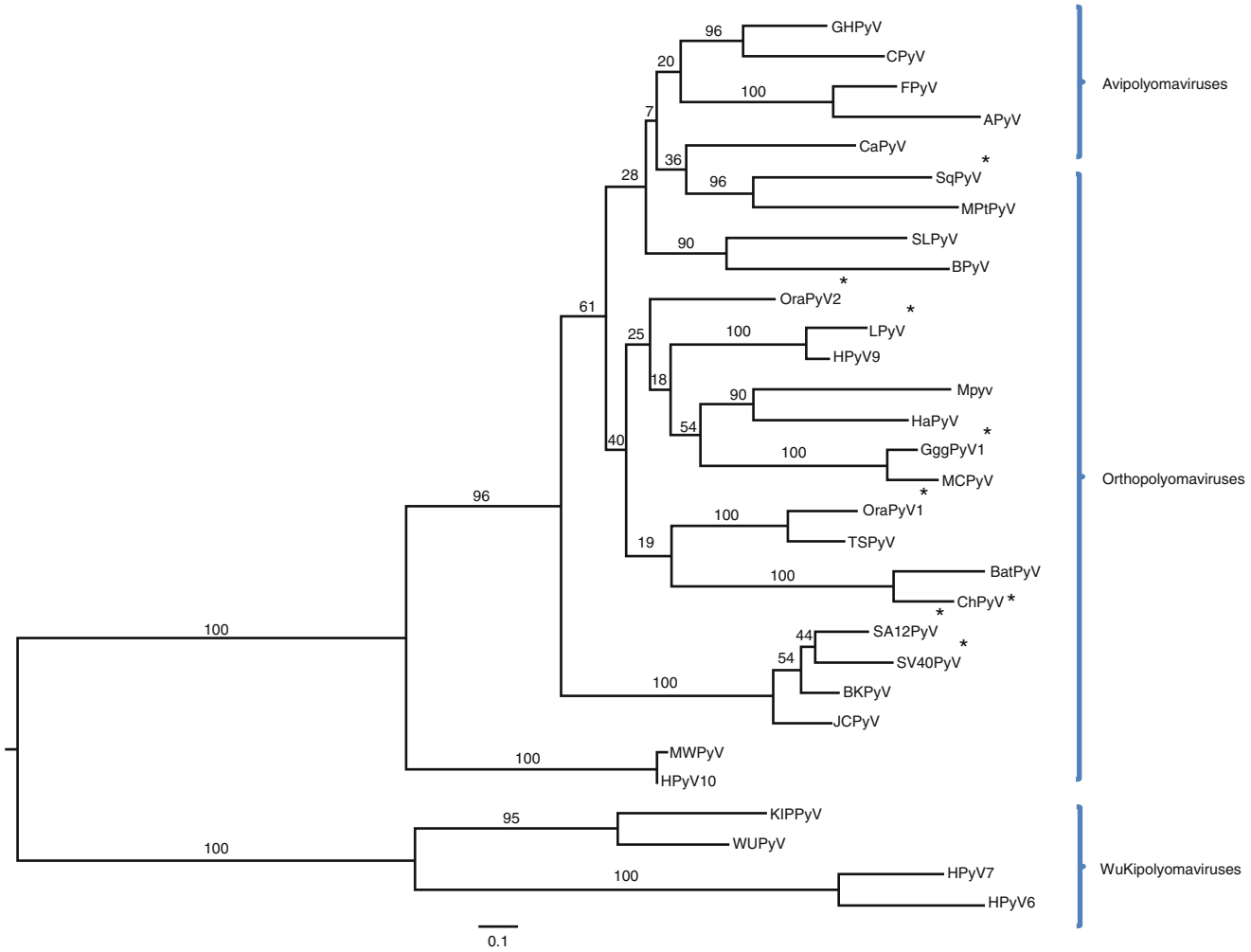

Fig. 46.1 Phylogeny of polyomaviruses. Midpoint-rooted phylogenetic tree of VP1 sequences of polyomaviruses. Phylogenetic analysis was performed using a maximum-likelihood method implemented in PhyML [16]. Bootstrap values are indicated in the branches, as percentage of 100 pseudo-replicates. The VP1 sequences used in this analysis are *BKPyV* (NC_001538), *JCPyV* (NC_001699), *WUPyV* (NC_009539), *MCPyV* (NC_010277), *HPyV6* (NC_014406), *HPyV7* (NC_014407), *TSPyV* (NC_014361), *HPyV9* (NC_015150), *HPyV10* (JX262162), *MWPyV* (JQ898291), *KIPyV* (NC_009238), *SV10PyV* (NC_001669), *OraPyV1* (NC_013439), *OraPyV2* (FN356901), *ChPyV* (NC_014743), *GggPyV* (HQ385752), *SA12PyV* (NC_007611), *LPyV* (NC_004763), *SqPyV* (NC_009951), *BatPyV* (JQ958886), *BPyV* (NC_001442), *SLPyV* (NC_013796), *HaPyV* (AJ006015), *MPyV* (NC_001515), *MPtPyV* (NC_001505), *APyV* (NC_004764), *CaPyV* (GU345044), *CPyV* (NC_007922), *FPyV* (NC_007923), and *GHPyV* (NC_004800). The human polyomaviruses are underlined and in bold font. The nonhuman primate polyomaviruses are marked with an asterisk. Viruses within the *Avipolyomaviruses*, *Orthopolyomaviruses*, and *Wukipolyomaviruses* genera are indicated by brackets

Table 46.1 Human polyomaviruses

Virus	Year of report	Common site of recovery	Associated illness	Reference
JCPyV	1971	Urine and PML[a] brain	PML	[5]
BKPyV	1971	Urine	Nephropathy and hemorrhagic cystitis	[6]
KIPyV	2007	Upper respiratory tract	Not known	[7]
WUPyV	2007	Upper respiratory tract	Not known	[8]
MCPyV	2008	Merkel cell carcinoma, skin	Merkel cell carcinoma	[9]
TSPyV	2010	Skin	Trichodysplasia spinulosa	[12]
HPyV6	2010	Skin	Not known	[10]
HPyV7	2010	Skin	Not known	[10]
HPyV9	2011	Urine and blood	Not known	[11]
HPyV10	2012	Skin	Not known	[13]
MWPyV	2012	Stool	Not known	[15]

[a]*PML* progressive multifocal leukoencephalopathy

average annual age-adjusted PML death rate was 1.07 per 1 million persons. The rate was much higher in males compared to females (1.81 vs 0.35) and among Blacks compared to Whites (2.03 vs 0.97). The rates varied by age group with the highest rate observed among persons 35–49 years of age at death (2.72). The PML rates were low in the earliest years and steadily increased to peak in the mid-1990s. The rise in the PML death rate is largely attributable to the increasing incidence of HIV after 1984, and over time, the demographic characteristics of persons dying from PML have closely mirrored the changing demographics profile of the AIDS epidemic. PML death rates decreased dramatically between 1992–1995 and 2002–2005 from 2.76 to 0.66. This was mostly attributable to a decrease among males, among Whites, and among persons 20–34 years of age and 35–49 years of age. The proportion of HIV-associated deaths with a PML diagnosis on the death certificate decreased after 1996, from a high of 1.5 % in 1996 to a low of 0.9 % in 2003 and 1.02 % in 2005. The most likely explanation for this decline in the PML death rate is the introduction of highly active antiretroviral therapy (HAART) in 1996. Similar declines in incidence of PML in HIV-positive patients were reported in the only available national, population-based study, which was conducted in Denmark [26]. In the large EuroSIDA cohort, the incidence of PML among HIV-positive patients decreased from 10 to 1 case per 1,000 person-years between 1995 and 2001 [27].

Although no mortality data for MCPyV-associated Merkel cell carcinoma (MCC) are available, the virus has been detected in at least 80 % of Merkel cell cancers in several case series and, as detection methods improve, the proportion of virus-associated cases is likely to rise. Population-based statistics have been reported for MCC in the period 1978–2006 using the Danish Cancer Registry [28]. From 1978 to 1984, only a few incident cases (<20) were reported. MCC incidence increased progressively between 1985 and 1994. Between 1994 and 2006, MCC incidence was stable at 2.2 cases per million person-years. MCC incidence was very low among those younger than 60 years (on average 0.19 cases per million persons-years) and increased to 11.5, 19.5, and 50.3 cases per million persons-years among individuals who were 70–79 years old, 80–90 years old, and 90 years or older, respectively. Five years after MCC diagnosis, 55 % of patients with localized disease, as compared to 84 % of the patients with nonlocalized disease, had died.

3.2 Morbidity Data

Primary polyomavirus infections may cause mild nondescript illnesses which are not well characterized.

3.3 Serological Surveys

Serological surveys of human polyomaviruses have shown that, in general, initial exposure to these viruses occurs in early childhood and continues to a greater or lesser extent throughout life, with a very high proportion of individuals eventually showing serological evidence of infection. However, the age-seroprevalence profiles for individual viruses also differ in some respects and suggest that there may be virus-specific differences in the route and efficiency of transmission or in environmental exposure. An age-seroprevalence study undertaken in England in 1991 using hemagglutination inhibition (HI) assays for BKPyV and JCPyV showed very different patterns for the two viruses [29]. Overall, 81 % of sera tested positive for BKPyV, and the seroprevalence varied significantly by age. Sixty-four percent of children in the youngest age group (1–4 years) were positive, and the figure rose to 91 % at 5–9 years. After 40 years of age, the prevalence of detectable antibody slowly declined to 68 % at 60–69 years of age. By contrast, the overall seroprevalence to JCPyV was only 35 %, but it also varied significantly with age. Only 11 % of children <5 years of age and 14 % 5–9 years of age were JCPyV seropositive, but the seroprevalence thereafter rose throughout life to reach a peak prevalence of 50 % at 60–69 years of age. In the same study, age-seroprevalence to SV40 was measured by neutralization assay. The antibody prevalence to SV40 remained at 1.3–5 % throughout all age groups and titers were low. There was a significant positive association between the presence of antibody to SV40 and antibody to both BKPyV and JCPyV, and also to the titer of BKPyV antibody, suggesting possible cross-reactivity between SV40 and the human polyomaviruses. In fact, competitive inhibition assays using viruslike particle (VLP) ELISA have shown that nearly all the SV40 reactivity in human serum can be explained by cross-reacting BKPyV or, less commonly, JCPyV antibodies [30–32].

A serological survey of Italians attending outpatient clinics between 2005 and 2008 using VLP ELISA assays showed a similar age-seroprevalence profile but a higher overall seroprevalence compared to that measured by HI assays, consistent with the greater sensitivity of the former assay method (Fig. 46.2) [33]. For JCPyV, seroprevalence increased steadily with age, from 0.5 % for children <10 years of age, to 50 % for young adults 10–19 years of age, to a peak seroprevalence of 80 % in adults >70 years of age. BKPyV seroprevalence was already 62 % for children <10 years of age, peaked at 79 % for those in the second decade of life, remained stable through the third and fourth decades of life, and began to gradually decline starting with adults 40–49 years of age (64 %), finally reaching its nadir of 55 % in adults >70 years of age.

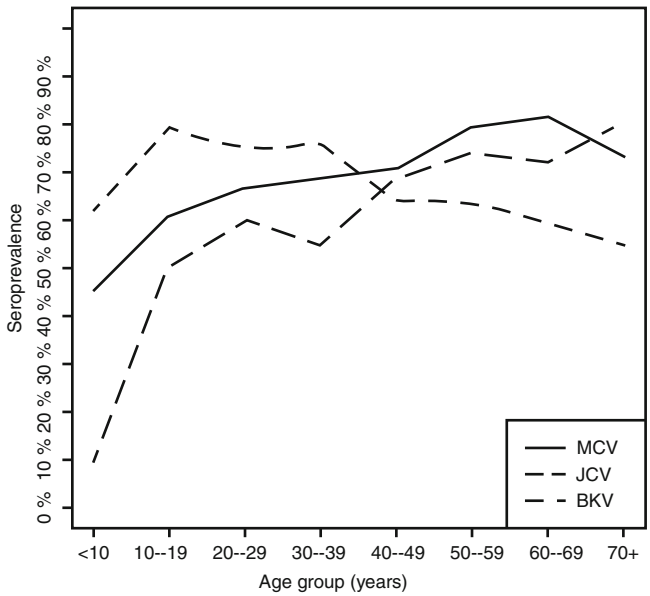

Fig. 46.2 Age-specific seroprevalence of Merkel cell polyomavirus, *BK virus*, and *JC virus* among 945 individuals recruited from hospital-based general and subspecialty outpatient clinics. Serum samples were tested at a 1:200 dilution in viruslike particle (VLP)-based ELISAs. The distribution of reactivities of serum samples from children less than 10 years of age was used to set cutoff points for seropositivity, and results are displayed as the percent positive in 10-year age groups (Copyright © American Society for Microbiology, Viscidi et al. [33])

Substantially similar age-seroprevalence profiles for BKPyV and JCPyV have been reported by others using VLP ELISA assays [34, 35]. In the study using Italian serum samples, MCPyV age-seroprevalence was also measured. In children <10 years of age, 45 % were MCPyV seropositive. Seroprevalence rose to 60 % by 10–19 years of age and peaked at 81 % in adults 60–69 years of age. There was a slight fall off in adults >70 years of age (73 %). MCPyV titers were positively associated with age, while BKPyV titers declined with age, and there was a null association of JCPyV titers with age. The reasons for these changes in the level of virus-specific antibody with age are unknown but may be due to differences in endogenous or exogenous sources of antigenic stimulation.

An age-seroprevalence survey for KIPyV and WUPyV revealed profiles that share many of the features of the profiles for the other human polyomaviruses, namely, evidence that primary infection occurs commonly in children and seroprevalence remains high or rises in adult life [36]. Among children 1–5 years of age, the seroprevalence to both WUPyV and KIPyV was 45 %. For KIPyV, a peak seroprevalence of 61 % was recorded in children 1–15 years of age and gradually declined to 50 % in adults >70 years of age. For WUPyV, seroprevalence rose slowly throughout adulthood, peaking at 71 % in 50–60-year-olds and remaining at this level in adults >70 years of age.

3.4 Laboratory Methods

3.4.1 Virological Methods

Virus isolation has not been routinely used for laboratory diagnosis of polyomavirus infections. As discussed below, two forms of the viral genomes of BKPyV and JCPyV exist, which are designated archetype and rearranged based on the DNA sequence of the regulatory regions. The archetype form of each virus is thought to be the transmissible form that is capable of establishing a persistent infection in the host, while the rearranged form is characteristic of disease-associated virus. The archetype form cannot be isolated in cell culture. Rearranged variants of BKPyV and JCPyV are able to grow in cell cultures of renal proximal tubule epithelial (RPTE) cells and human fetal glial cells, respectively [37, 38], but the availability of simple and highly sensitive methods for viral protein and DNA detection make cell culture an unattractive and rarely used method for polyomavirus diagnosis. None of the newly discovered human polyomaviruses have been successfully cultivated. Viral proteins can be detected in disease tissue by immunohistochemical methods. Because these techniques are highly specialized and labor intensive, they are used mostly for research purposes. Additionally, well-characterized reagents are not commercially widely available.

The standard and most commonly used method for virus detection is based on polymerase chain reaction (PCR). PCR, developed in 1983, is a method to amplify a few copies of a target DNA sequence by several orders of magnitude. The method relies on thermal cycling, consisting of repeated cycles of heating and cooling for melting and enzymatic replication of DNA. The reaction relies on short DNA fragments called primers, complementary to a target DNA sequence, to initiate the enzymatic activity of a heat-stable DNA polymerase, most commonly a bacterial *Thermus aquaticus* (Taq) polymerase. DNA generated in the reaction is itself a template for replication allowing for a chain reaction and exponential amplification of target DNA, and thus the name polymerase chain reaction. When the technique was first developed, DNA products of the reaction were measured by gel electrophoresis. A significant advance in the technology has been the development of quantitative real-time PCR. The key feature of this method is that the amplified DNA is detected as the reaction progresses in real time. In addition to providing quantization of the number of target DNA copies in the starting sample, the method provides much greater precision than the older method of measuring the end products of PCR. Another technical advance has been the introduction of fluorophore-containing probes, such as TaqMan, to measure the amount of amplified product in real time. TaqMan probes consist of a fluorophore attached to the 5′-end of a probe and a quencher at the 3′-end. As long as the fluorophore and the quencher are in proximity, quenching

inhibits any fluorescence signals. TaqMan probes are designed such that they anneal within a DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesizes a nascent DNA strand, the 5′–3′ exonuclease activity of the polymerase degrades the probe. Degradation of the probe releases the fluorophore, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Fluorescence detected in the real-time PCR reaction is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. TaqMan PCR assays have become the preferred method for detection of polyomavirus DNA in clinical specimens, and several laboratories have developed assays for BKPyV, JCPyV, and MCPyV, as well as the other recently discovered human polyomaviruses [39–44].

No polyomavirus PCR assays are FDA approved. However, the clinical need for the diagnosis of BKPyV-associated nephropathy has spurred the development and clinical validation of several TaqMan assays for BKPyV. Consistent with the expected performance of TaqMan assays, the published assays have shown linear ranges of several $\log_{10}$ orders of magnitude, detection limits of 100–1,000 copies per ml or less, and very good intra- and inter-assay precision. A quantitative real-time PCR assay for a target in the VP2 gene of BKPyV, the Simplexa BKPyV assay (Focus Diagnostics), has been developed and shown to quantify DNA linearly over a range of 2.7–10.4 Log_{10} copies/ml, with a limit of detection of about 3000 copies/ml in plasma and urine [45]. Intra-assay precision yielded coefficients of variation (CV) ranging from 0.3 to 3.9 % and inter-assay precision yielded CVs of 1.1–7.8 %. By comparison to a reference laboratory real-time PCR, the Simplexa BKPyV assay had a sensitivity of 100 % and a specificity of 85 %. The assay showed no cross-reactions in samples containing JCPyV, HSV1, HSV2, enterovirus, adenovirus, HBV, HPV, EBV, and CMV. An evaluation of the Artus BK Virus RG PCR test (QIAGEN), which targets a conserved region of the VP2 and VP3 genes, showed a linear range of quantification from 2.28 to 8.29 $\log_{10}$ DNA copies/mL and a limit of detection of 2.00 $\log_{10}$ copies/mL [46]. The precision of the assay was highly reproducible among runs with coefficients of variance (CV) ranging from 0.2 to 7.0 %. Too few samples were tested to determine accurately the sensitivity of the assay. No biological false positives were found with other pathogens potentially found in human urine and plasma. Genotyping analysis using large T antigen sequences demonstrated that 90 % of the positive samples were BKPyV subtype I. In the absence of standardization, comparisons among assays are difficult and many factors can contribute to differences, including sample type, DNA extraction and purification method, preparation used to generate standard curves, amount of input template, choice of primers and probe, and PCR amplification conditions. In a comparison of seven TaqMan real-time PCR assays for BKPyV, the most significant source of error among individual specimens was primer or probe mismatch due to subtype-associated polymorphisms, primarily among subtype III and IV isolates [47]. The importance of genetic variation in circulating BKPyV genotypes as a source of error in the performance of BKPyV TaqMan PCR assays has also been described by other investigators [40, 48]. The greatest limitation to the clinical application of BKPyV PCR assays is the lack of a robust definition of clinical relevance since the inherently high sensitivity of PCR assays can lead to positive results that may not inform clinical practice. The use of PCR in management of BKPyV nephropathy is discussed in Sect. 8. TaqMan PCR assays for JCPyV, MCPyV, KIPyV, and WUPyV have been published, but none of the assays have been standardized and no commercially produced reagents are available.

3.4.2 Serological Methods

Antibodies to JCPyV and BKPyV capsid protein can be measured by hemagglutination inhibition (HI) assays, but ELISA techniques have largely replaced them. Because JCPyV and BKPyV are difficult to cultivate and MCPyV and the other recently identified human polyomaviruses have not been cultivated, recombinant proteins are used as antigens. For some viruses, it has been possible to produce viruslike particles by the expression of the major capsid protein VP1 in eukaryotic expression systems [32]. Viruslike particles are formed by the spontaneous self-assembly of the VP1 protein into a capsid that morphologically resembles a virion and displays conformationally correct epitopes on its surface. Alternatively, the VP1 protein can be expressed in *E. coli* as a glutathione-S-transferase (GST) fusion protein [49]. For ELISA, the antigens are immobilized on plastic microtiter plates, allowed to react with serum, and bound antibodies are detected with enzyme-labeled antihuman immunoglobulin following the addition of an appropriate substrate. An alternative method involves immobilization of GST-VP1 fusion proteins to polystyrene beads containing fluorescent dye (Luminex technology). After incubation of the beads with serum, bound antibody is reacted with an antihuman immunoglobulin labeled with a different fluorophore from that in the bead. The reaction mixture is measured in a flow cytometry device that allows simultaneous detection of the antigen-loaded bead and antigen-antibody complexes bound to the bead. Since over 100 beads with different fluorescent intensities are available, up to 100 antigens can be employed in a single reaction, allowing for high-throughput detection of multiple antibody specificities in a single test. No direct comparisons of the performance of VP1 fusion proteins versus viruslike particles as serological reagents have been done, but the fact that VLPs more closely resemble authentic capsids suggests that they may be superior in displaying native conformational epitopes.

The high titers of antibody elicited by polyomavirus infections suggest that ELISA assays should be very sensitive, but because primary infections are asymptomatic and rarely diagnosed, it has not been possible to establish the true sensitivity of serological measures of exposure. The specificity of serological assays for BKPyV and JCPyV antibodies has been demonstrated by competitive inhibition assays which have shown that reactivity can be completely inhibited by preincubation of sera with the homologous antigen and the reactivity is not inhibited by heterologous antigen [31] Competitive inhibition assays have also demonstrated that cross-reacting antibodies elicited primarily by infection with BKPyV explain the low level of human seroreactivity to the macaque polyomavirus, SV40 [50]. Since the other human polyomaviruses are genetically more distantly related to each other than BKPyV is to JCPyV, it is likely that seroreactivity to these viruses is also highly species specific. An interesting but unexplained biological response to JCPyV and BKPyV infection is the negative correlation in the level of antibodies to the viruses. Thus, individuals who react strongly to JCPyV tend to react more weakly to BKPyV and vice versa.

The only commercially available serological assay for a human polyomavirus is the Focus Diagnostics JCPyV serology assay. The assay is performed in two steps, a screening standard microtiter plate VLP-based ELISA followed by a confirmatory step in which the serum is preincubated with JCPyV VLPs and retested in the ELISA assay. Serum samples that are positive in the ELISA and inhibited by >40 % in the confirmatory step are scored as JCPyV antibody positive. Using serum samples from natalizumab-treated multiple sclerosis patients, the JCPyV seropositivity rate was 53.6 % [51]. The sensitivity of the assay to identify those infected with JCPyV was estimated to be 97.5 % based on the percentage of serum samples from urinary JCPyV DNA-positive patients that tested positive for anti-JCPyV antibodies. Diagnostic specificity (false-positive rate) could not be established because most JCPyV infections are asymptomatic. The intra-assay precision for low and high positive controls and negative controls was 5.7, 2.9, and 3.7 %, respectively, and inter-assay precision for the respective controls was 9.7, 8.7, and 7.0 %. Robustness, as measured by performance across different laboratories, showed a concordance among laboratories of 95–98 %. The use of the assay for clinical management of multiple sclerosis patients eligible to received natalizumab is discussed in Sect. 9.1.

3.4.3 Cytological Methods

Polyomavirus-associated nephropathy and hemorrhagic cystitis are frequently accompanied by the presence of cytologically abnormal cells, referred to as decoy cells, in the urine that can be visualized by Papanicolaou stain (Fig. 46.3). They are virally infected renal tubular or uroepithelial cells characterized by an irregular shape and enlarged nuclei with basophilic inclusions [52]. Quantitative urine cytology is one modality for screening for BKPyV reactivation and diagnosis of BKPyV-associated nephropathy in renal transplant recipients. While the method has very high negative predictive value, the positive predictive value is low because urinary shedding of polyomaviruses is common in healthy persons, it cannot distinguish different types of polyomaviruses, and urinary shedding of cytomegalovirus is also associated with decoy cells. Additionally, special skill is required to interpret urinary cytology.

4 Biological Characteristics of Polyomaviruses

4.1 Physical Properties, Genomic Organization, and Function of Viral Proteins

Polyomavirus particles measure about 44 nm in diameter and have a buoyant density in CsCl of 1.34 g/cm^3. The virion is a nonenveloped, icosahedral capsid composed of three virus-encoded proteins, VP1, VP2, and VP3. The major capsid protein, VP1, accounts for more than 70 % of the virion mass and has a molecular weight of 39–44 kDa. The capsid encloses a single molecule of circular double-stranded DNA that is in complex with cellular histone proteins. The viral DNA genome is ~5,000 bp and is organized into three functional regions, the early and late coding regions and a regulatory region (Fig. 46.4).

The early region encodes two viral regulatory proteins, the large T and small t antigens (T-Ag and t-Ag). T-Ag possesses multiple enzymatic activities and has the ability to bind DNA and a number of cellular proteins. T-Ag regulates the production of early mRNA, initiates viral DNA replication, and activates late gene transcription. Among the cellular proteins that interact with T-Ag are two proteins important for regulation of cell growth, the tumor suppressor proteins, retinoblastoma susceptibility protein (Rb) and p53. The interaction of T-Ag with these two proteins plays a central role in the ability of polyomaviruses to transform and immortalize cells in vitro and to induce tumors in animals. The t-Ag protein may serve an ancillary role to T-Ag activity in cell transformation. The t-Ag is known to inactivate protein phosphatase 2A leading to increased cyclins D1 and A, and downregulation of p27. The cyclins and p27 are involved in control of cell cycle progression. The role of the t-Ag in the viral life cycle is not fully defined, but it is known to be dispensable for the lytic cycle of polyomaviruses in cultured cells. Alternately spliced T-Ag transcript have been described for JCPyV and BKPyV, and further research may reveal similar transcripts in other human polyomaviruses. The functions

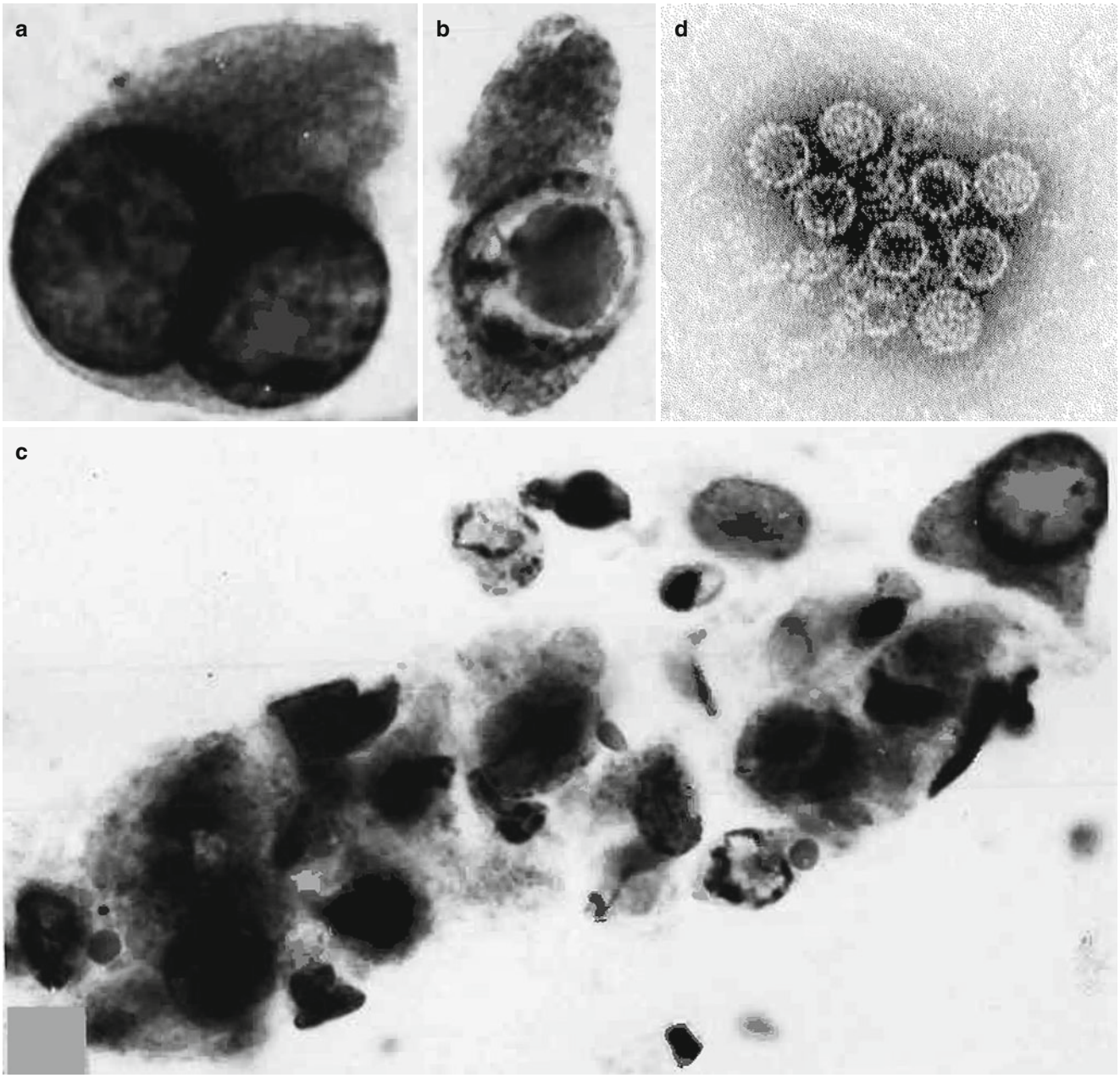

Fig. 46.3 BKPyV urinary cytopathology and viruria. Urinary epithelial cells in a renal transplant recipient; (**a**, **b**) cells with enlarged nuclei, (**c**) cast formation of virus-infected epithelial cells. (**d**) Electron micrograph of virions of BKPyV in urine; virus particles are aggregated with an antiserum (From Ref. [49] with permission)

of the proteins encoded by these alternately spliced transcripts have not been well defined. The effect of polyomavirus infection, and particularly the early region proteins, on the cell transcriptome has been investigated using global gene expression techniques. Studies of primary kidney epithelial cells infected with BKPyV [53] and of primary human fetal glial cells infected with JCPyV [54] have shown upregulation of numerous genes involved in cell cycle and proliferation, including genes involved in apoptosis, signal transduction, transcriptional regulation, DNA damage repair, protein ubiquitination and folding, RNA processing, and cellular trafficking and structure.

The late coding region contains the genetic information for the major structural protein, VP1, and the two minor structural proteins VP2 and VP3. The sequence of VP3 is contained entirely within that of VP2. Based on the crystal structure of SV40 [55], the human polyomavirus capsids are predicted to contain 360 molecules of VP1 arranged in 72 pentameric subunits. Each pentamer associates with a single VP2 or VP3 molecule to form the individual capsomeres.

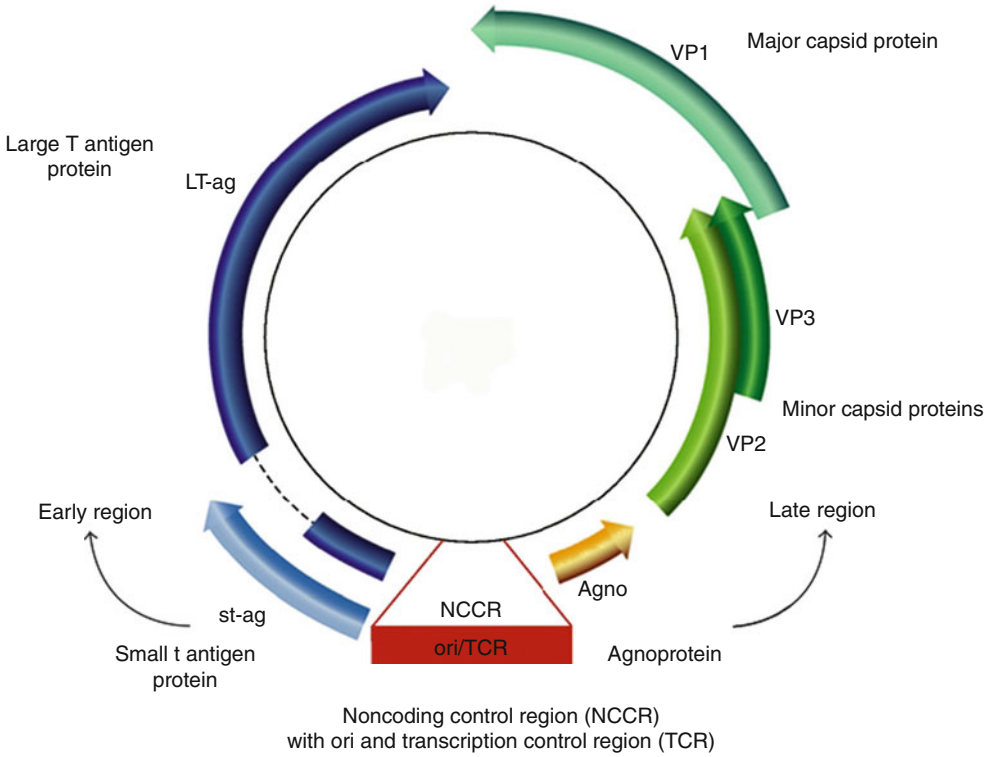

Fig. 46.4 Simplified representative human polyomavirus genome. Polyomavirus genomes are circular double-stranded DNA ~5,000 bp in length. The noncoding control region (*NCCR*) is ~300–400 bp in length and contains the origin of replication (*ori*) and promoter and enhancer elements. Genes are transcribed in opposite directions from opposite strand of DNA. The early region transcribes the large T antigen and small t antigen. The late region transcribes three viral structural proteins, the *VP1* major capsid protein and the *VP2* and *VP3* minor capsid proteins. BKPyV and JCPyV, but not the other human polyomaviruses, also transcribe an accessory protein, agnoprotein

The late region of BKPyV and JCPyV, but not of the other known human polyomaviruses, encodes an agnoprotein. The JCPyV agnoprotein appears to play a role in viral DNA replication and transcription and perhaps also in the dysregulation of cell cycle control and DNA repair [56]. JCPyV agnoprotein may also play a role in nuclear and cellular release of the virus [57, 58]. BKPyV agnoprotein has been shown to interact with α-SNAP, a molecule involved in disassembly of vesicles during secretion; however, the functional relevance of the interaction is unclear [59].

The viral noncoding regulatory region (NCRR) spans 300–500 bp and is located between the early and late coding regions. The NCRR contains the DNA replication origin, the TATA box, T-Ag binding sites, cellular transcription factor binding sites, and the promoters and enhancers for transcription of early and late genes. Hypervariability of the regulatory region is a typical feature found in polyomaviruses, particularly for BKPyV and JCPyV. Classification schemes for regulatory region variants of JCPyV and BKPyV divide them into archetypal variants, which are characteristic of viruses found in urine, and rearranged or tandem repeat variants, which are associated with disease [60–63]. The archetypal regulatory region is divided into defined blocks of sequence that are duplicated or deleted in the rearranged variants. In BKPyV, the archetype virus NCCR is divided into five blocks of sequence designated O, for the origin of replication, P, Q, R, and S. The JCPyV archetype regulatory region is similarly divided into blocks of sequence designated a, b, c, d, e, and f, with the origin of replication located between block a and the early genes. The regulatory region of archetypal variants is organized into a linear series of promoter and enhancer sequences. Rearranged variants contain partial or complete duplications and deletions of the various sequence elements. The sequence variability in the NCRR among BKPyV and JCPyV variants is believed to confer selective growth advantages to these viruses in their hosts [61, 64].

4.2 Infectious Life Cycle of Polyomaviruses

The life cycle of polyomaviruses is initiated by the adsorption of virions to the cell surface. This process requires interaction with cell surface sialic acids. For BKPyV, an N-linked glycoprotein with α(2,3)-linked sialic acid serves as the receptor [65], and for JCPyV, an N-linked glycoprotein containing terminal α(2,6)-linked sialic acid is used [66]. The proteinaceous component of the cellular receptor for BKPyV is unknown; however, the serotonin receptor $5HT_{2A}$ was identified as the putative receptor for JCPyV [67]. Polyomaviruses enter the cytoplasm by endocytosis. BKPyV enters by caveolae-mediated endocytosis [68] and JCPyV by clathrin-dependent endocytosis [69]. Polyomaviruses are next transported to the endoplasmic reticulum (ER). Within the ER, the capsid is destabilized by the disruption of disulfide bonds, allowing

virions to be translocated across the ER membrane into the cytoplasm. Capsid stability is further disrupted by the low calcium concentration in the cytosol, exposing nuclear localization signals. The partially disassembled virions enter the nucleus through nuclear pores. Within the nucleus, virions are fully uncoated and associate with nuclear domain 10 (ND10) bodies. Most likely ND10 bodies are used as a scaffold for viral DNA replication and assembly of new progeny virions. The viral minichromosome is replicated and transcribed in the nucleus. DNA replication is initiated by the large T antigen through interactions with host proteins. Replication proceeds bidirectionally. In addition to its direct role in viral DNA replication, the large T antigen also binds to cellular proteins to induce signals to drive quiescent cells toward S phase. Dividing host cells are enriched for the host molecular machinery necessary for efficient viral DNA replication. In the nucleus, the viral genome serves as a template for the host RNA polymerase II transcriptional machinery. The restriction of viral tropism at the cellular level is believed to be governed in part at the transcriptional level. Regulation of transcription is dependent on sequences in the NCRR and the availability of host transcription factors. Unlike other human DNA viruses, such as herpesviruses, polyomaviruses do not encode for transcriptional activating proteins. The transcription factors, NF-κB, Tst-1, and NF-1 have been demonstrated to contribute to the neurotropism of JCPyV [70, 71]. Interestingly, the NCRR of BKPyV contains an estrogen response element, which mediates an increase in viral promoter activity upon hormone stimulation [72]. Thus, the elevated estrogen levels during pregnancy might contribute to viral reactivation and explain the increase in BKPyV shedding observed in pregnant women. Following viral transcription, there is a switch to DNA replication. While early transcription is believed to be a determinant of viral cell tropism, DNA replication is a determinant of species specificity. Polyomavirus T-Ag interacts with host cell DNA polymerases in a species-specific manner [73]. Once the viral genome has been replicated, T-Ag mediates repression of early gene transcription and stimulates transcription of late genes. Expression and subsequent nuclear localization of the viral structural proteins VP1, VP2, and VP3 leads to assembly of virion capsids. Assembly occurs predominantly in the nucleus. Newly packaged virions are thought to be released by either lytic rupture of the host cell or secretion from the plasma membrane.

5 Descriptive Epidemiology

5.1 Incidence and Prevalence

The incidence rates of primary polyomavirus infections in humans are unknown because the infections are asymptomatic and rarely diagnosed. Estimates of the prevalence of polyomavirus infection are based on serological surveys and good estimates are only available for JCPyV, BKPyV, and MCPyV, as described in Sect. 3.3.

5.2 Geographic Distribution

Polyomaviruses are widespread in human populations. However, geographic and ethnic differences exist in the distribution of genetic subtypes of BKPyV and JCPyV. BKPyV is divided into four major subtypes, I, II, III, and IV. Subtype I is the most prevalent, followed by subtype IV, with subtypes II and III occurring less frequently. Subtype I has been subdivided further into the three subgroups Ia, Ib, and Ic [74]. A regional distribution of these subgroups is also apparent: subgroup Ic is prevalent in Japan, Ib is widespread in European countries [75, 76], and Ia is prevalent in Africa [77]. Subtype Ib has recently been further subdivided into Ib-1 and Ib-2 [77]. With the exception of Japan, BKPyV subtype IV was found to be widely prevalent in East Asia [78]; however, prevalence of this subtype has likely been underestimated in other regions [77]. Six subgroups of subtype IV have also been identified, each showing a close relationship to population geography [79]. All JCPyV strains belong to a single serotype. Based on genetic variations, three JCPyV "superclusters" (A, B, and C) are distinguishable [80]. Type A is distributed throughout Europe, type B throughout Asia and Africa with a minor subtype found in Europe, and type C in West Africa [81]. Types A and B have each been split further into a number of "subordinated lineages" and "sublineages" [81, 82].

5.3 Occurrence in Special Epidemiologic Setting

Almost all of the illnesses associated with JCPyV and BKPyV infections occur in conditions associated with immunodeficiency of the host, but the two viruses have different tropisms and react differently to the immunosuppressing event or regimen. In healthy individuals previously infected with JCPyV, virus recrudescence as evidenced by viruria occurs in about 20–30 % of the people and increases in older age [83]. Infection with HIV does not affect this rate of JCPyV viruria [84]. In contrast, BKPyV recrudescence and viruria are markedly elevated by HIV infection, for instance, from 5 to 22 % in one study [84]. JCPyV is a neurotropic virus causing PML, whereas BKPyV is largely a urinary tract virus responsible for pathological lesions in the kidney, urinary bladder, and ureter. HIV infection has resulted in a tenfold increase in the incidence of PML, whereas increase in BKPyV-induced pathology is largely determined by the nature of drugs and the intensity of the

immunosuppressive regimens in transplant recipients. Despite these generalizations, there are some instances where JCPyV is responsible for cases of nephropathy [85, 86] and BKPyV for isolated cases of encephalitis in normal [87], or HIV-infected [88] individuals and in a bone marrow transplant recipient [89]. The pathology related to MCPyV, Merkel cell cancer, occurs in older (>50 years of age), fair-skinned individuals with a history of sun or ultraviolet light exposure and in persons with T cell immune suppression, such as HIV-infected patients and organ transplant recipients [90, 91]. Merkel cell cancer incidence is increased in individuals with a prior diagnosis of other skin cancers or chronic lymphocytic leukemia or lymphoma [28, 92].

6 Mechanisms and Routes of Transmission

The best-studied human polyomaviruses are BKPyV and JCPyV. Infection occurs at an early age in human populations, although the steadily increasing seroprevalence of JCPyV throughout life suggests exposure may also occur at a later age in some individuals. The absence of clinical symptoms during primary polyomavirus infection has made the identification of the site of entry difficult. The most likely site is either the respiratory tract or the gastrointestinal tract. In support of an oral route of transmission, BKPyV has been detected in saliva and has been shown to replicate in salivary gland cells [93]. JCPyV has been identified in human tonsil stromal cells and in B lymphocytes contained in tonsils and has been shown to productively infect tonsillar stromal cells in culture [94]. However, JCPyV has not been detected in saliva or oropharyngeal secretions [95, 96]. BKPyV, JCPyV, and other human polyomaviruses have been detected in urban sewage, which also supports the possibility of oral-fecal transmission [97, 98]. Human polyomaviruses have been shown to be stable at pH 5 and for prolonged periods of time in sewage at room temperature [99]. These findings support the possibility of transmission through diverse environmental exposures. Although mouse polyomavirus can be acquired vertically through transplacental transmission, there is little virological or serological evidence to support in utero transmission of human polyomaviruses [100, 101]. In addition to in utero passage across the placenta, vertical or perinatal transmission of viruses can occur during the birthing process or soon after birth. Using a postnatal rise in the IgG level or the transient appearance of an IgM or IgA response in newborns as evidence of infection, one small study reported BKPyV and JCPyV infection of newborns in 21 and 16 % of pregnancies, respectively [102]. Transmission within families has been shown to be the principal mode of spread of JCPyV [103, 104]. In fact, intrafamilial transmission is so common that genetic variation of the JCPyV genome has been used as a marker for the migration of human populations [80, 105]. Molecular evolutionary analysis of JCPyV genetic variants has demonstrated two distinct viral lineages, both arising from the ancestral African type, in support of the model of human dispersal out of Africa [106]. These analyses have also identified a split of the Asian lineage of JCPyV into two main branches, one diffusing in Japan and Americas, the other in Southeast Asia. Although intrafamilial spread of BKPyV is also common, analysis of BKPyV genotypes in Japanese-Americans living in southern California supports substantial transmission outside the family as well [107].

7 Pathogenesis and Immunity

7.1 Pathogenesis

After initial replication at the site of entry, polyomaviruses spread hematogenously to other organs. This stage of infection is clinically silent and presumably the host immune response rapidly controls viral replication before any tissue damage can occur. There is no evidence that polyomaviruses establish a latent state of infection. They most likely persist as infectious virus in cells where a low virion output is minimally injurious to the host and the infection is contained by still not fully understood immunological mechanisms. The principal site of persistence of BKPyV and JCPyV is the kidney, as demonstrated by periodic amplification of low-level infection and urinary shedding of virus. Immunostaining has shown that the sites of viral replication are the epithelial cells of the kidney, ureter, and bladder. In a study of healthy blood donors, asymptomatic urinary shedding of BKPyV and JCPyV was observed in 7 and 19 %, respectively, of 400 subjects [34]. The shedding of JCPyV was more commonly observed among individuals who were seropositive only for JCPyV, compared with individuals who were seropositive for both BKPyV and JCPyV, suggesting limited cross-protection from BKPyV immunity. Because of the lack of longitudinal studies, it is unknown if urinary shedding is persistent or intermittent. Additionally, it is unknown whether a subset of infected individuals shed virus for long time periods or the majority of infected persons will shed virus at some point in time. Viremia is rare in healthy persons. Among the 400 blood donors, neither JCPyV nor BKPyV were detected in plasma [34]. However, another report described detection of JCPyV viremia in approximately 2 % of normal individuals [108]. Whether JCPyV or BKPyV establish persistence in tissues other than kidney is controversial. JCPyV has been detected by PCR in B lymphocytes from tonsillar tissue and bone marrow; however, in vitro studies have failed to show that the virus infects and replicates in B cells, but rather the virus remains cell

surface associated. JCPyV has also been detected in stromal tonsillar cells and has been shown to infect these cells in culture [109]. In addition to its presence in kidney, BKPyV has been detected by PCR in peripheral blood, but the cell type harboring the virus has not been determined. Once JCPyV reaches the central nervous system, it replicates vigorously in oligodendrocytes, leading to PML. Two models have been proposed for brain infection with JCPyV. One model proposes that primary infection occurs in tonsillar tissue with subsequent trafficking of virus to bone marrow and kidney. Upon immunosuppression, the virus is amplified and mobilized from the bone marrow and crosses the blood-brain barrier. An alternative model proposes that the brain may serve as a site of low-level persistence, and with immunosuppression, the virus present in the brain is amplified. This model is based on studies which have shown detection of JCPyV DNA in brain of healthy persons [110–112]. The pathogenesis of MCPyV bears many similarities to that of JCPyV and BKPyV, but a significant difference is evidence for infection of cutaneous tissue, which is not observed for the latter two viruses. MCPyV DNA has been detected in skin samples from 40 to 95 % of healthy persons [10, 113, 114]. The virus has also been detected in respiratory tract secretions [115, 116]. Additionally, MCPyV has been detected in a high proportion of swab samples of environmental surfaces [117]. The mode of transmission of MCPyV is unknown but cutaneous, fecal-oral, or respiratory routes are all possibilities. Primary infection appears to be asymptomatic.

7.2 Immunity

Polyomaviruses induce robust humoral immune responses to viral capsids, with high levels of immunoglobulin G detectable in serum of many individuals by ELISA assays. When viral recrudescence occurs in association with disease, antibody levels generally rise as the viral load increases [118, 119]. There is no evidence for antibody-mediated protection against disease. Once a persistent viral infection is established, the principal mechanism of immune-mediated control of viral replication is believed to be cellular immunity. This paradigm likely applies to polyomaviruses because recrudescence of viral replication and virus-associated disease occurs in immunosuppressed individuals. There is circumstantial evidence to support a protective role of cellular immunity in polyomavirus-associated disease. Several studies have shown that BKPyV-specific T cell responses are associated with recovery from BKPyV infection in renal transplant recipients, while low or undetectable responses correlate with viral persistence [120–124]. Similarly, JCPyV-specific T cell responses are associated with clinically quiescent PML, while patients without detectable responses fail to control their neurological disease [125, 126]. The presence of MCPyV-specific CD8+ T cells in Merkel cell tumors is associated with improved survival [127].

8 Patterns of Host Response

8.1 Clinical Manifestations

8.1.1 Progressive Multifocal Leukoencephalopathy (PML)

PML is a fatal subacute demyelinating disease of the central nervous system that occurs as a complication of a wide variety of conditions of T cell deficiency. These T cell deficiencies include lymphoproliferative disorders such as Hodgkin's disease, chronic lymphocytic leukemia, and lymphosarcoma; chronic diseases such as sarcoidosis and tuberculosis; primary immunodeficiency diseases; prolonged immunosuppressive therapy as, for example, in organ transplant recipients and in patients with rheumatoid arthritis, systemic lupus erythematosus, and myositis. Pathologically, the PML is characterized by foci of demyelination in the white matter of the central nervous system. Oligodendrocytes, which are the myelin-producing cells in the brain, have enlarged nuclei containing inclusion bodies, and they surround the areas of demyelination (Fig. 46.5). Astrocytes, sometimes of greatly increased size and with bizarre nuclear changes, are found in the area of demyelination. The disease was described under many names until 1958 when it was recognized as a single entity [128]. In 1961 Richardson, noting the nuclear changes in oligodendrocytes, the lack of inflammatory response in many cases, and the relationship to immunodeficiency, suggested that the disease may be a result of lytic infection of

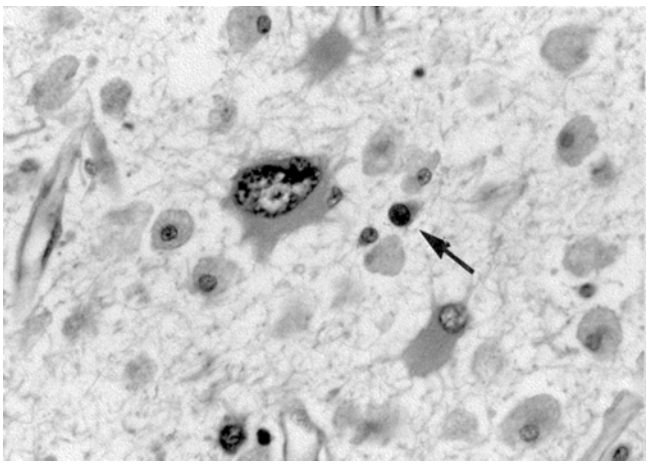

Fig. 46.5 Progressive multifocal leukoencephalopathy: cytopathology of PML. Bizarre giant astrocytes and oligodendrocytes with enlarged nuclei (*arrow*) (Courtesy of Dr G Zu Rhein, Madison, Wisconsin, from Ref. [49] with permission)

oligodendrocytes with an opportunistic virus [129]. Polyomavirus particles were visualized in the nuclei of the affected oligodendrocytes in 1965 [130, 131], and the causative virus, JCPyV, was isolated in 1971, after inoculation of extracts of a PML brain in primary human fetal glial cell cultures [5].

The advent of AIDS in the 1980s markedly changed the incidence and the age distribution of PML cases [132–134]. Deaths due to PML increased fourfold between 1979 and 1987. PML was recognized as an AIDS-defining illness in 1987 and is reported in as many as 5 % of AIDS cases with neurological abnormalities [135] and in 0.72 % of all AIDS cases reported to the CDC between 1981 and 1990 [134]. A marked increase in the number of PML cases from 0.2 per million persons in 1984 to 3.3 per million persons in 1994 is ascribed to AIDS [136]. It has been estimated that 55–85 % of current cases occur in HIV-positive individuals with AIDS [137]. Previously, PML was a disease occurring in the fifth and sixth decades of life. As most cases of AIDS occur in the 20–49-year age group, the age distribution of PML cases has shifted to younger ages. PML in patients with primary immunodeficiency diseases and in renal transplant recipients is a disease of young children and young and middle-aged adults. Cases of PML in the older patients are most likely the result of recrudescence of a persistent low-level JCPyV infection. In the younger patient, it is possible that unchecked primary JCPyV infection may lead to PML.

In early 2005, the administration of monoclonal antibodies was recognized as a new risk factor for PML when two patients with multiple sclerosis and one patient with Crohn's disease developed PML in association with the administration of natalizumab, a monoclonal antibody to α4 integrin that prevents entry of inflammatory cells into brain and other tissues [138–140]. The estimated incidence of PML was roughly one in 1,000 for all patients enrolled in trials of natalizumab and one in 300 for patients undergoing treatment with the drug for 2 or more years. More recently, PML has also been documented in patients taking efalizumab for psoriasis, and rituximab for a variety of disorders [141]. The incidence and pathogenesis of PML following immunomodulatory drugs have been discussed [142–144]. Bloomgren and colleagues [144] collected data from many sources to identify risk factors for PML in patients with multiple sclerosis who were treated with natalizumab. There were 212 confirmed cases of PML among 99,571 natalizumab-treated multiple sclerosis patients worldwide. Using different subsets of data, they estimated that the lowest risk was among those who were negative for JCPyV antibodies before the treatment (0.09 per thousand) and the highest risk (11.1 per thousand) for those who were JCPyV antibody positive before treatment, had received immunosuppression prior to natalizumab treatment, and had also a long period (25–48 months) of natalizumab therapy. In contrast to the high mortality rate of PML in AIDS patients, only 52 of 242 reported cases of PML caused by natalizumab died (https://medinfo.biogenidec.com). However, survivors have varying levels of disability ranging from severe to mild.

Clinical and Pathological Features

Clinically, PML has an insidious onset and may occur at any time in the course of the underlying illness. The signs and symptoms point to a multifocal involvement of the brain. Impaired speech and vision and mental deterioration are common early features of the disease. The patient remains afebrile and headache is uncommon. Interestingly, careful monitoring of natalizumab-treated patients with magnetic resonance imaging (MRI) has led to the report of 3 patients with "asymptomatic PML" [145–147].

As a rule, the disease is progressive, resulting in death within 3–6 months after onset [148]. Paralysis of limbs, cortical blindness, and sensory abnormalities occur in later stages. A few patients may survive for years with stabilization of the condition and even apparent remission. A longer survival time is thought to be associated with a more marked inflammatory response in the brain. Patients who have JCPyV-specific CD8+ cytotoxic T lymphocytes appear to survive longer than patients without such cells [149]. The introduction of highly active antiretroviral therapy (HAART) for HIV/AIDS has had only a modest effect on the incidence of PML, which may be related to the fact that it can present at relatively higher CD4 counts than some other opportunistic infections [150, 151]. HAART has, however, led to an improvement in the prognosis of PML in HIV/AIDS patients [152].

The diagnosis of PML can be conclusively established by pathological examination of a biopsy or at postmortem. Macroscopically, the brain shows foci of demyelination that may vary widely in size and may become confluent and necrotic in the advanced stages of disease. The lesions are most frequent in the subcortical white matter. The cerebrum is almost always affected. Microscopically, the presence of enlarged oligodendrocyte nuclei around the foci of demyelination is diagnostic. These altered nuclei contain abundant amounts of JCPyV particles, antigen, and DNA. JCPyV particles or antigen are not found in normal brains or in non-diseased areas of PML brains. Noninvasive techniques, particularly nuclear magnetic resonance imaging (MRI) of the brain, provide an effective means for the diagnosis of PML. MRI scans of the head are nearly always abnormal in association with PML. The typical MRI abnormalities are localized to the subcortical white matter with increased T2 signal and little contrast enhancement after gadolinium administration [153]. Cerebrospinal fluid (CSF) analysis typically shows minimal pleocytosis, less than 20 cells per microliter, and only modestly elevated protein, usually amounts less than 100 mg per deciliter. PCR analysis of CSF for JCPyV DNA is the best

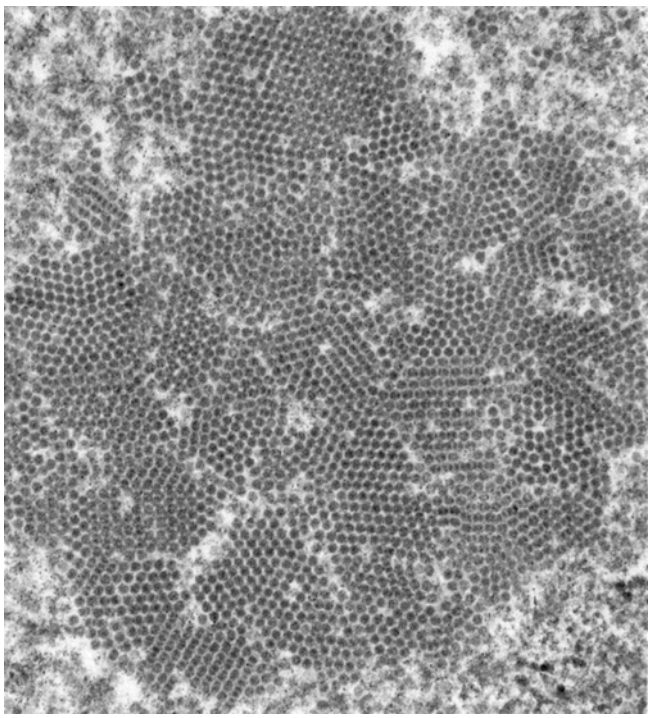

Fig. 46.6 Virions in the nucleus. Crystalloid arrangement of JCPyV virus particles in an infected oligodendrocyte nucleus (from patient JC) (Courtesy of Dr G, Zu Rhein, Madison, Wisconsin, from Ref. [49] with permission)

noninvasive test for the confirmation of PML. Its sensitivity is ~80 %, and it is highly specific [154].

Virological Aspects

The nuclei of the altered oligodendrocytes in the affected area contain abundant amounts of JCPyV particles that are often found in dense crystalline arrays (Fig. 46.6). Old "burnt-out" lesions of PML may be devoid of oligodendrocytes and viral particles. In situ hybridization or immunoperoxidase staining readily detects the virus in affected tissue. The virus is also found in cerebrospinal fluid [155] and in small amounts at extraneural sites [156]. JCPyV DNAs from PML cases show specific changes in their regulatory regions. JCPyV DNA from urines of normal individuals display an "archetypal" regulatory region [157]. PML isolates as a rule show a duplication of domain A and a deletion of domain B of the regulatory region [158]. Such rearrangements are not seen in JCPyV DNAs from healthy individuals, and it is very likely that they occur in the course of a prolonged JCPyV multiplication in the brain and that they increase the ability of JCPyV to grow in the central nervous system.

Pathogenesis of PML

The key pathogenetic event in PML is the cytocidal infection of oligodendrocytes with JCPyV. The destruction of oligodendrocytes results in demyelination, because these cells are responsible for the formation and maintenance of myelin. Neurons are not affected in PML. The widespread and multifocal distribution of discrete foci of demyelination is suggestive of a hematogenous spread of the virus to the brain. The JCPyV genome is present in B lymphocytes following infection with JCPyV, and it has been suggested that in the immunocompromised host, infected B lymphocytes transport the virus to the brain and initiate PML [159]. An alternative explanation is that the virus is already seeded in the brain at the time of primary infection and that a low-level persistent infection is amplified during immunosuppression to initiate PML [160]. PML occurs on the background of severely depressed cellular immunity, and lymphocytes of patients with PML have a decreased ability to respond to JCPyV antigen [161]. This may allow higher JCPyV multiplication and facilitate virus entry into the brain.

Why natalizumab, a monoclonal antibody to alpha4 integrin that prevents entry of inflammatory cells into the brain, would uniquely predispose persons to the development of PML remains unknown. The drug could increase the risk of PML by mobilizing persistently infected B lymphocytes from the bone marrow and spleen. Alternatively, the drug may impair the cellular immune response to JCPyV by preventing the entry of virus-specific cytotoxic T lymphocytes into the brain or sites of viral persistence.

Treatment of PML

There is no specific antiviral drug against JCPyV with proven efficacy in the treatment of PML. Although cidofovir has in vitro antiviral activity for nonhuman polyomaviruses and has been used to treat PML, a recent analysis of pooled raw data from multiple studies, including 370 HIV-associated PML patients, showed that cidofovir did not influence PML-related mortality or residual disability [162]. The best treatment is the restoration of the immune system, although this carries the risk of immune reconstitution syndrome (IRIS). IRIS is a term used to describe a group of clinical syndromes associated with immune reconstitution following HAART therapy for HIV patients. The manifestations are diverse, including fever and worsening of the clinical manifestations of an underlying opportunistic infection. The diagnosis requires distinguishing between progression of the initial opportunistic infection, development of a new opportunistic infection, unrelated organ dysfunction, and drug toxicity. The influence of IRIS on patient survival is variable and can be favorable or unfavorable [163, 164]. Treatment of PML caused by natalizumab involves discontinuation of the drug and in some cases its removal from the bloodstream by plasmapheresis/immunoadsorption. IRIS can occur in patients with natalizumab-associated PML, particularly after removal of the drug [165].

8.1.2 JCPyV Granule Cell Neuronopathy

Neurons are not productively infected in PML, but productive infection of neurons with JCPyV is seen in this rare disease, which may occur alongside PML [166] or without concurrent PML [167].

8.1.3 Nephropathy and Hemorrhagic Cystitis

BK Virus-Associated Nephropathy (BKVAN)

Although BKPyV was recognized as a virus infection of the urinary tract from the time of its isolation in 1971, its harmful effect on the kidney, BKVAN, was not evident until the 1990s. BKVAN is an important cause of progressive graft dysfunction and graft loss in patients with renal allografts [168]. The expanding use of tacrolimus and mycophenolate mofetil in immunosuppressive regiments to control graft versus host disease may have increased the incidence of BKVAN. The intensity of immunosuppression appears to be a more important factor in disease than the specific agent. BKVAN is the most common virus-induced pathology affecting renal allografts with an incidence of ~8 % and resulting in graft loss in the range of 10 % to greater than 80 % of the individuals who have nephropathy [169]. Histological features of BKVAN include epithelial cells containing intranuclear inclusion bodies characteristic of BKPyV replication, necrosis of cells of the tubules and collecting ducts, and varying degrees of interstitial inflammation. The majority of cases occur in the first year posttransplantation. Depending on the extent of virus-induced renal injury, patients present with varying degrees of allograft dysfunction. Serum creatinine can vary from normal in early-stage disease to a marked increase in late-stage disease with extensive renal damage. Systemic signs of infection such as fatigue and fever are absent. Host factors, such as age greater than 50 years, male gender, white ethnicity, and diabetes mellitus, and allograft factors, such as number of human leukocyte antigen (HLA) mismatches between donor and recipient, tubular injury due to drug toxicity and proinflammatory state of the graft brought on by surgical injury, warm ischemia, and reperfusion during implantation, can also increase the risk of BKVAN [170]. The nephropathy almost always occurs in the transplanted kidney. A less than optimal BKPyV-specific T cell response may underlie BKVAN [171]. JCPyV infection is a rare cause of nephropathy [86].

The definitive diagnosis of BKVAN requires a renal biopsy showing polyomavirus-induced cytopathic changes in tubular or glomerular epithelial cells. However, because BKVAN can be focal in distribution, a negative biopsy result does not rule out BKVAN with certainty. A consensus conference has proposed a histological staging scheme for BKVAN [172]. Stage A, early disease, is characterized by small numbers of tubular epithelial cells showing signs of viral replication with intranuclear inclusion bodies. In stage B, florid disease, the above findings are accompanied by signs of tubular injury and interstitial inflammation. Stage C, the late sclerosing stage, is characterized by tubular atrophy and diffuse interstitial fibrosis.

Noninvasive diagnostic techniques can be used to screen for BKPyV replication and to make an early diagnosis of BKVAN. Polyomavirus inclusion bearing "decoy cells" are easily detected in urine with a Papanicolaou-stained cytology preparation. The positive predictive value of the detection of decoy cells for BKVAN is low, but cytological monitoring of the renal transplant patients would identify those at risk of BKVAN. Similarly, the finding of high copy numbers of BKPyV DNA by PCR is of some clinical value for the diagnosis of BKVAN. In contrast, testing for BKPyV DNA in plasma appears to be more promising as a noninvasive diagnostic test for BKVAN. In one study, PCR assays for BKPyV DNA in plasma had a sensitivity of 100 % and a specificity of 88 % for detecting biopsy confirmed BKVAN [169]. A high level of BKPyV viremia was closely associated with concurrent BKVAN, probably because the viremia was the result of a high level of virus multiplication in the kidney.

Hemorrhagic Cystitis

Bone marrow transplant recipients undergo heavy immunosuppressive treatment in preparation of transplant from a donor. A transient hemorrhagic cystitis that occurs in the first few days after transplantation is thought to be a result of toxicity of the immunosuppressive drugs. A late-onset hemorrhagic cystitis (2–12 weeks post transplant) which occurs in some recipients is of a longer duration (longer than 7 days). BKPyV viruria is strongly associated with the late-onset disease. In one study where BKPyV viruria, as estimated by an ELISA assay, was related to 16 of 18 well-characterized cases, BKPyV was detected in 55 % of the urines collected during the cystitis episodes and 8–11 % of specimens collected in cystitis-free periods [173]. The onset and termination of BKPyV viruria often coincided with the onset and termination of the disease. BKPyV viruria levels in patients with hemorrhagic cystitis are much higher than those in the viruria in asymptomatic BMT patients [174]. BKPyV viruria in BMT recipients may also be associated with gross or microscopic hematuria without evidence of clinical cystitis [175]. In a study of hemorrhagic cystitis in 26 children receiving stem cell transplantation, BKPyV viruria was found in 21 children, adenovirus viruria in 4 children, and JCPyV viruria in a single child [176]. The intensity of the conditioning regimen and allogeneic transplant are additional risk factors for BKPyV-associated hemorrhagic cystitis [177]. The disease may also occur in HIV-infected individuals who have not received a transplant [178] or in individuals who have received transplants other than bone marrow.

8.1.4 Relation of JCPyV and BKPyV to Human Cancer

Polyomaviruses are oncogenic for experimental animals and many can transform human cells. Therefore, a variety of human tumors have been investigated for possible polyomavirus etiology. Tumor tissues or tumor-derived cells have been

examined for viral particles, viral T antigens, viral genomes, and viral transcripts. For most of the positive studies, the copy number per cell, when estimated, has been much less than one, and supporting evidence for the presence of virus (e. g., detection of virus in tumor cell by in situ hybridization, presence of viral transcripts) has been lacking. Other studies of the same tumor types have been negative. Therefore, it has been difficult to decide if the positive studies represent virus involvement in tumor formation, or detection of a persistent infection in rare cells present in tumor tissue, or laboratory artifacts. In addition to the studies of viral sequences, sera from cancer cases and controls have been screened for antibodies to viral capsid and T antigens. The International Agency for Research on Cancer (IARC) has reviewed extensively the data on the carcinogenicity for humans of JCPyV, BKPyV, and MCPyV (IARC vol104). The Agency examines both the evidence in experimental animals and in humans. In analyses of the human evidence, it puts more emphasis on prospective and case-control studies than on case reports and case series. The evidence IARC has reviewed and its conclusions are summarized below.

JCPyV

It has been suggested that brain tumors that coexist with PML may have a JCPyV etiology [179, 180]. In patients without PML, JCPyV DNA and T antigen have been reported in an oligoastrocytoma [181], and JCPyV DNA has been amplified with nested PCR from a xanthoastrocytoma [182]. Several investigators have reported JCPyV sequences from brain tumors [183–185], but other studies of similar brain tumors have been negative [186–188]. JCPyV sequences have also been reported from leukemia and lymphoma [189] and from colon tumors [190].

The IARC (vol 104) classified JCPyV as possibly carcinogenic to humans (group 2B) [191].

BKPyV

BKPyV sequences have been reported from a wide variety of human tumors which include pancreatic B-islet adenomas [192], brain tumors of several histological types [193], osteosarcoma [194], Kaposi sarcoma [195], kidney carcinoma [196], childhood neuroblastomas [197], and prostate carcinoma [198]. Other studies of brain tumors have been negative [186–188].

In a patient with both a kidney and pancreas transplantation, the recipient developed BKPyV-produced interstitial nephritis as well as a carcinoma of the bladder with widespread metastases. High levels of BKPyV T antigen were detected in the primary tumor as well as in the metastatic carcinoma [199]. This patient may represent the carcinogenic potential of BKPyV in a severely immunosuppressed individual. But BKPyV is not a common cause of bladder carcinomas. In another study, BKPyV sequences were not detected in tissues of transitional cell carcinoma of the bladder [200].

The IARC (vol 104) classified BKPyV as possibly carcinogenic for humans (group 2B) [191].

8.1.5 Merkel Cell Carcinoma

Merkel cell cancer (MCC) is an aggressive neuroendocrine cancer of the skin. The presence of electron dense neurosecretory granules in the tumor cells allowed for classification as a neuroendocrine tumor, and since Merkel cells are the only cutaneous cells which form such granules, it was postulated that these carcinomas derive from Merkel cells. However, alternative hypotheses suggesting a role for skin stem cells of epidermal lineage or immature B lymphocytes have also been proposed [201, 202]. The incidence of MCC is about 1,500 cases per year in the USA, and has been increasing in the USA, with an estimated annual percentage change of 8 % during the period from 1986 to 2001 [203]. Whether the increase is due to a true rise in the incidence of MCC or improved diagnosis is unknown. The cancer presents as a rapidly growing, painless, firm, non-tender, dome-shaped red skin nodule, most commonly involving the head and neck, less commonly the extremities, and rarely the trunk. At presentation the disease is usually localized but it can spread to regional lymph nodes and metastasize widely. In one series, the median survival was 40 months for patients with localized disease and 13 months in those with distant metastases [204]. Exposure to sunlight and ultraviolet (UV) light are recognized risk factors for MCC, based on its anatomic predilection, geographic distribution, association with white fair-skinned individuals, and association with other UV light-related skin cancers. Immunosuppression is an additional risk factor since the disease has an unusually high prevalence in organ transplant recipients, HIV-infected patients, and those with B cell malignancies [90, 205–208]. However, the vast majority (>90 %) of MCC patients have no clinically apparent immune dysfunction [92]. The primary treatment modality for localized disease is surgical excision. MCC is a radiosensitive malignancy and adjuvant radiotherapy has been advocated for local and regional disease control [209]. Because involvement of regional lymph nodes is common in MCC at presentation, prophylactic lymph node dissection is sometimes employed in treatment of localized disease [210, 211]. The prognosis for patients with distant metastases is very poor. Various chemotherapeutic regimens have been tried with limited success [212, 213].

In 2008 a novel polyomavirus was detected in Merkel cell cancer tissue using next-generation high-throughput sequencing of cDNA and analysis of the sequence data for nonhuman sequences with homology to known viral sequences in public databases [9]. Two transcripts were identified with partial homology to lymphotropic polyomavirus (LPyV). A complete polyomavirus genome was sequenced from tissue DNA and shown to differ from previously known human polyomaviruses and given the name Merkel cell polyomavirus (MCPyV).

The virus was detected by Southern blot in 8 (80 %) of 10 cases and, in 6 tumors, the restriction digestion pattern was consistent with monoclonal integration into the host genome. Subsequent studies have generally supported the estimate for the proportion of Merkel cell cancers containing MCPyV DNA [214–217]. Determination of viral load in MCC tissue by quantitative PCR has shown copy numbers ranging from 0.05 to 173 copies per cell [218, 219]. Of note, although MCPyV DNA has also been detected in normal skin and other non-MCC tissues, the viral copy number per cell equivalent of DNA in these samples is several orders of magnitude lower than that in MCC tissues which have high copy numbers. In nearly all tumor-derived MCPyV genomes sequenced, missense mutations or deletions in the early region result in the expression of truncated large T antigen proteins [218, 220]. The large T antigen proteins retain domains capable of binding pRB, but lose their helicase domains and p53 binding domains. Although clearly lacking all the functions of an intact large T antigen, the truncated form of the protein has been shown to be essential for the survival of MCPyV-positive tumor cells in vitro and for their sustained proliferation [221, 222]. None of the reported mutations affect expression of an intact small t antigen oncoprotein. Seroprevalence studies show that MCPyV, like other human polyomaviruses, is highly prevalent in human populations and that exposure occurs during childhood. Precisely when MCPyV infects Merkel cells and what events need to occur for development of MCPyV-associated tumors are unknown.

Human Carcinogenicity of MCPyV

The paradigm of small DNA tumor virus-induced carcinogenesis envisions a necessary role for viral oncoproteins in activation of transduction pathways and interference in tumor suppressor pathways in order to induce cells to enter the cell cycle and proliferate. These functions are essential for the viral life cycle, which depends on an actively dividing cell to provide the host machinery for viral replication. However, in a cell which is not permissive for a lytic viral infection, the induction of cell division can cause oncogenic transformation of the cell and lead to a cancer. Transformation caused by MPyV, SV40, JCPyV, and BKPyV has been extensively studied and reviewed in the literature [223–227]. Transformation requires functions of the large T antigen, small t antigen, and in some cases proteins encoded by alternately spliced early region transcripts. Although the precise mechanism of transformation differs among the polyomaviruses, the ability of large T antigen to bind p53, the retinoblastoma protein (pRb), and heat shock protein 70 and that of small t antigen to bind protein phosphatase 2A (PP2A) appears to be essential.

The mechanism of MCPyV-induced cell transformation is still poorly understood. In most MCPyV-positive MCC tumor tissues, the virus is integrated into the host chromosome [9, 228], a signature feature of virus-induced tumors since it allows maintenance of the viral genome in dividing cancer cells. Integration sites within the host genome are unique for each patient and the break points within the viral genome are also unique, but they occur mainly in the second exon of the large T antigen [9, 228]. There is no evidence that integration occurs in cellular genes implicated in carcinogenesis. A requirement for oncogenic viral proteins for maintenance of MCC cells was demonstrated by growth arrest and cell death of MCPyV-positive cell lines in vitro when all early region transcripts were inhibited by siRNA knockdown [221]. Selective knockdown of MCPyV small t antigen alone has also been shown to retard growth of virus-positive cell in vitro [229]. MCPyV small t antigen, unlike that of SV40 and MPyV, is sufficient to cause transformation in rodent cell assays and is not dependent on interactions with PP2A [229]. Perhaps uniquely, MCPyV small t antigen perturbs the PI3K-Akt-mTOR pathway, which is important for cap-dependent translation, by maintaining hyperphosphorylation of the eIF4E binding protein via an unknown mechanism [229]. MCPyV large T antigen and small t antigen expression have been detected by immunohistochemistry in the majority of MVPyV-positive MCC tumors [218, 229]. Serum antibody directed against a shared portion of early region proteins can be detected in up to 40 % of MCC cases, while these antibodies are present in <1 % of virus-positive healthy controls [230]. Humoral immune responses to viral oncoproteins are also seen in patients with papillomavirus-associated cervical cancer and head and neck cancers and are tumor-specific markers [231, 232].

The IARC (vol 104) on the basis of the strong mechanistic evidence for its carcinogenicity classified MCPyV as "probably carcinogenic for humans" (Group 2A) [191].

8.1.6 Other Human Polyomaviruses

In addition to JCPyV, BKPyV, and MCPyV, described above, eight other polyomaviruses have been recovered from humans.

KIPyV and WUPyV Polyomaviruses

The genomes of these two viruses were recovered and identified from respiratory secretions of individuals with mild respiratory illness [7, 8]. They are closely related, sharing 65–70 % amino acid identity with each other as compared to only 15–50 % identity with JCPyV and BKPyV. The antibody prevalence to these viruses in the general population is high, 55–90 % for KIPyV and 69–98 % for WUPyV virus [36, 233, 234]. Although these viruses were detected in respiratory secretions, subsequent studies have failed to identify a role for KIPyV or WUPyV in respiratory tract disease in healthy children or immunocompromised children [235, 236]. At present the viruses are not associated with any specific illness.

Human Polyomavirus 6 (HPyV 6), 7(HPyV 7), and 10 (HPyV 10)

HPyV 6, HPyV 7, and HPyV 10 were detected in human skin swabs using the rolling circle amplification technique [10, 11]. At present there is no illness associated with these viruses. Due to the lack of serological reagents, the prevalence of infection in human populations is unknown. Likewise, the natural history of infection with these cutaneous human polyomaviruses is also unknown, although they are suspected to share many features of the natural history of MCPyV.

Trichodysplasia Spinulosa-Associated Virus (TSV)

Trichodysplasia spinulosa (TS) is a rare disease of the skin which occurs in recipients of solid organs on immunosuppressive therapy and in patients with lymphocytic leukemia [237–239]. The patient develops follicular papules and keratin spines (spicules) on the face, often accompanied by alopecia of the eyebrows and of the eyelashes. Pathologically, the hair follicles are markedly distended and show abnormal maturation. The inner root sheath cells of the follicles are highly proliferative and contain polyomavirus-like particles, as seen by electron microscopy [237].

TSV genome was isolated from plucked spicules of a TS patient [12] and has also been detected in lesions of other TS patients [240]. Phylogenetically, TSV is tightly linked to Bornean orangutan polyomavirus, and among human polyomaviruses, it is closest to MCPyV [240] . It is a common human infection, with antibody prevalence of 80 % in adults and 89 % in renal transplant recipients [240, 241]. The presence of TSV in high copy numbers in the pathological lesions of TS suggests that the virus may be etiologically related to TS.

Human Polyomavirus 9 (HPyV 9)

Scuda et al. [13] identified a human polyomavirus, HPyV 9, from blood and urine of renal transplant recipients, which is closely related to a virus reported in the 1970s from African Green monkey, now named B-lymphotropic polyomavirus (LPyV) [14, 242]. A virus nearly identical to HPyV 9 has also been recovered from the skin surface of a patient with Merkel cell carcinoma [243]. The virus was shed for >20 months from this patient and was also found on the skin of his spouse. Using a capsomere L1 ELISA, an HPyV 9 seroprevalence of 47 % was determined in healthy adults and adolescents and 20 % in a group of children [244]. In another study using a viruslike particle-based ELISA, the seroprevalence of HPyV 9 among adults was approximately 33 % and that among children 1–7 years of age was 10 % [245]. These studies suggest that exposure to this virus is less common than that to the other human polyomaviruses and that infections occur at all ages. Both studies demonstrated cross-reactivity between LPyV and HPyV 9, and thus substantiated the hypothesis put forth in 1981 that human seroreactivity to LPyV, which was discovered in African green monkey cells, might signify the existence of a human virus closely related to LPyV [242].

Malawi Polyomavirus (MWPyV) and HPyV10

A novel polyomavirus was discovered in the stool of a healthy child from Malawi using shotgun pyrosequencing of purified viruslike particles recovered in feces, and the virus was named Malawi polyomavirus (MWPyV) [15]. The virus was also detected in stool samples from the United States suggesting it has a wide geographic distribution. At present it is unknown whether the virus causes infection in humans or is an environmental contaminant present in stool. There is no known illness associated with the virus. It shares 99 % homology with HPyV10, a virus detected in skin specimens from a patient with a rare genetic disorder known as warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome, and may be the same viral species [11].

8.1.7 SV40 and Human Disease

The possibility that millions of people in the USA may have been exposed to live SV40 between 1956 and 1960 because SV40 was a contaminant of the polio vaccines and the adenovirus vaccines, combined with the demonstration that SV40 was oncogenic in laboratory animals, sparked many investigations to determine if the virus caused any harm in humans. The review of the circumstances of the human exposure to SV40 and of the early studies concluded that while there were no immediate dire consequences following SV40 exposure, continued surveillance was necessary to detect neurological disease and cancers which may occur at a low frequency and after a long interval [4].

Beginning in 1992 several laboratories, employing PCR technology, reported the detection of SV40 sequences in pediatric brain tumors [246], mesothelioma [247], osteosarcoma [248], non-Hodgkin lymphoma [249, 250], and other tumors. The proportion of tumors reported to be positive for SV40 was high (20–60 %), but the SV40 copy number per cell in the virus-positive tumors, when estimated, was quite small, much lower than one copy per cell. The authors of these studies suggested that SV40 was circulating in the human population and it may have contributed to the development of these tumors. Other studies of the same tumors for SV40 sequences were essentially negative for mesothelioma [251], brain tumors [187, 252], and non-Hodgkin lymphoma [253, 254]. In a blinded study, the SV40 PCR assays were evaluated in nine participating laboratories; SV40 sequences were found at about the same low frequency in mesothelioma, normal lung, and negative controls [255]. In a careful study, Lopez-Rios et al. [256] definitively identified contamination with common laboratory plasmids that contain SV40 sequences as the reason for false-positive data in SV40 PCR in their own laboratory and also identified a previously published study from another laboratory [257] where plasmid

contamination may have led to false-positive data. Despite more than 50 reports of SV40 genomic sequences in tumors, firm evidence of SV40 genomes or of SV40 transcripts in tumors is lacking.

It has been known since the 1960s that a small proportion of human sera have low antibody titers to SV40 [258]. After the discovery of human polyomaviruses JCPyV and BKPyV in 1971, this reactivity to SV40 has been shown to be a result of cross-reactivity with antibodies to JCPyV and BKPyV [30, 32]. In case-control studies of mesothelioma, brain tumors, and lymphomas SV40, seroreactivity in cases was similar to that in controls [251, 259–261].

The risks of mesothelioma, brain tumor, and non-Hodgkin lymphoma have also been compared in epidemiologic studies where populations exposed to potentially contaminated polio or adenovirus vaccines were compared to those not exposed to these vaccines [262–264]. These studies revealed no evidence that the risk of these cancers was greater in groups more likely to have received contaminated vaccine.

A working group at the International Agency for Research on Cancer concluded that "in view of the strongly negative human data on infection and carcinogenesis, SV40 is "not classifiable as to its carcinogenicity to humans" (Group 3) [191].

8.2 Diagnosis

All of the recognized illnesses associated with JCPyV and BKPyV infections are caused by infection and lysis of specific cells by the viruses. As described in previous sections, characteristic cytological and pathological features and demonstration of the viruses from affected tissues by PCR allow diagnosis of the diseases. For MCPyV-associated Merkel cell carcinoma, the diagnosis can be made by histology showing cells with the characteristic monoclonal antibody staining pattern of Merkel cells, the presence of electron dense neurosecretory granules and the detection of MCPyV by PCR [265, 266].

9 Control and Prevention Based on Epidemiologic Data

9.1 PML

A large majority of cases of PML occur in HIV-infected individuals. Preventing HIV by a vaccine or by drugs or by change in behavior would have a major impact on the incidence of PML. Treatment of HIV-infected individuals who also have PML by highly active antiretroviral treatment (HAART) has been reported to be beneficial for PML patients [152, 267]. With the continued improvement in antiretroviral therapy, the progression of HIV infection to AIDS is less rapid and less frequent, and many more infected individuals now lead a near-normal life. They are less likely to develop PML.

Because years may elapse between infection with HIV and the onset of PML, it would be useful to find predictors of PML in HIV-infected patients. In a study of 28 HIV-infected PML patients for whom specimens were available up to 12 years (median 6.4 years) before the diagnosis of PML, JCPyV viruria, viremia, and antibody status were examined for their predictive value [268]. The frequency of viruria in HIV-infected individuals who developed PML was similar to that in individuals who did not develop PML, but persistent viruria was more common in cases than controls. No cases had detectable viremia prior to diagnosis. In a study of prediagnostic serum viral markers among 83 HIV-infected PML cases with samples collected every 6 months up to 2.5 years before diagnosis, an increase in JCPyV IgG levels was observed in cases compared to controls in the 6-month window preceding diagnosis [119]. There was no statistically significant difference in plasma JCPyV viremia between cases (17 %) and controls (12 %). The viremia was low titered in both cases and controls.

9.2 BKVAN

In contrast to PML in HIV-infected individuals where no predictors of PML were identified, BKVAN can be predicted in renal transplant recipients. Screening of recipients by urinary cytology and BKPyV viruria will identify patients who are at risk for BKVAN, and presence and level of BKPyV viremia implies impending or concurrent BKVAN. This allows the clinician to change or reduce the immunosuppressive regimen and may lead to patient recovery.

It has been suggested that BKVAN in the transplanted kidney is a result of deficient BKPyV-specific T cell immunity, which allows the recrudescent virus to infect and destroy the transplant [171]. It may be possible to bolster BKPyV-specific immunity for all transplant patients by appropriate immunization.

9.3 Merkel Cell Carcinoma

MCPyV infects many individuals early in life, and it is not known why in a few instances the infection leads to carcinoma. The risk factors for Merkel cell carcinoma are not known well enough to identify protective measures.

9.4 Vaccines

Almost all the pathogenic effects of human polyomaviruses are related to recrudescence of the virus which remains persistent at a very low level after primary infection. It may be

possible to prevent the primary infections by prophylactic vaccines. However, the vaccines would have to be administered in early childhood and may need to be repeated in subsequent years. At present, there are no candidate prophylactic vaccines against any of the human polyomaviruses. It may be possible to boost immunity in patients who have been previously infected and are at risk for disease, such as prospective recipients of renal transplants. Whether a vaccine should target humoral or cellular immune responses is unknown. In general, cellular immunity is required to clear an established infection. Some hope has been raised by the success achieved at preventing herpes zoster (shingles), a disease caused by the reactivation of varicella zoster virus, with a vaccine that elicits antiviral cellular immunity. However, the genetic mismatch between the recipient and kidney donor may limit the development of a similar approach for BKVAN. Development of therapeutic vaccines for the treatment of cancer is an active area of experimental and clinical research. Intratumoral MCPyV-specific CD4+ and CD8+ T cells have been detected in Merkel tumors, and the presence of intratumoral CD8+ lymphocytes is associated with improved survival [127, 269]. Experimental DNA vaccines that elicit cellular immune responses to MCPyV large T antigen have shown efficacy in animal models with MCPyV large T antigen expressing tumors [270, 271].

10 Unresolved Problems

10.1 Pathogenesis of PML

It is not clear if PML is caused by recrudescence of a persistent low-level JCPyV infection which is already seeded in the brain during primary infection or if the virus in the urinary tract or other sites of persistence is amplified and crosses the blood-brain barrier to infect the brain. JCPyV recovered from PML brains shows specific changes in the regulatory region of the genome, which presumably enable the virus to multiply in the brain. It is not known if these changes occur in the virus prior to the establishment of a lytic infection in the central nervous system or if the changes occur in the course of prolonged multiplication of the virus in the brain.

10.2 Is MCPyV Infection Etiologically Linked to Merkel Cell Carcinoma?

A minority of Merkel cell carcinomas is virus negative. Does that represent a disease etiologically distinct from virus-positive Merkel cell carcinoma and with different risk factors? If they are the same disease, what is the role of MCPyV in the formation of the tumor? The virus-tumor association should be evaluated for its strength, consistency, specificity, plausibility, and temporality [272].

10.3 Pathogenesis of MCPyV-Associated Cancer

In nearly all MCPyV-positive MCC tumors, the early region open reading frame contains mutations leading to a predicted truncated form of the large T antigen protein, which eliminates the p53 binding region [228, 273, 274]. Since mutations in the p53 gene are uncommon in MCC [275], how the virus causes cancer without affecting p53 function remains unclear.

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Part IV

Other Transmissible Agents

Prion Diseases

47

Ermias D. Belay and Jason C. Bartz

1 Introduction

Prion diseases, also known as transmissible spongiform encephalopathies, are a group of invariably fatal neurodegenerative diseases that affect both humans and animals. They are caused by an unconventional agent termed a prion. Strong evidence indicates that their etiology and pathogenesis involve modification of a host-encoded normal cellular protein known as the prion protein (PrP^C) [1, 2]. Unusual characteristics of prion diseases include their occurrence sporadically without any apparent environmental source of infection in some species, genetically in association with specific prion protein gene mutations in humans, and their transmissibility either within or across different species [2]. In most prion diseases, characteristic neuropathologic features include widespread neuronal loss, spongiform lesions, and astrogliosis, corresponding with accumulation of the agent in different parts of the brain. The presence of the abnormal prions can be demonstrated in the brain and often in other tissues of humans and animals affected by prion diseases [3–6]. Several laboratory tests such as immunohistochemistry and biochemical tests are used to determine the presence of infecting prions in tissue specimens [4, 6, 7]. Acquired forms of prion diseases have very long incubation periods often lasting for years and sometimes even decades [8–10].

Prion diseases of humans include kuru, Creutzfeldt-Jakob disease (CJD), variant CJD, Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI) (Table 47.1) [2, 11].

Kuru was first described in the 1950s as a fatal ataxic neurologic disease among the Fore tribe of the highlands of Papua New Guinea [9, 12–15]. In 1959, Hadlow made the crucial observation that the neuropathology of kuru was similar to scrapie, raising the possibility that, similar to scrapie, kuru might also be transmissible [16, 17]. However, transmissibility was not confirmed until the 1960s when Gajdusek and colleagues successfully transmitted kuru by intracerebral inoculation of brain tissue from deceased patients into chimpanzees [18–23]. This was the first instance of human prion disease to be successfully transmitted to experimental animals.

Kuru is the first epidemic human prion disease to be thoroughly investigated. Since the 1950s, over 2,700 kuru cases have been documented [9, 24]. Strong epidemiologic evidence suggests that the disease spread among the Fore people by ritualistic cannibalism. In ancient Fore culture, giving respect for the dead involved a mourning ritual with plastering of brain tissue all over the body, including mucous membranes and consumption of the decedent's body parts. Relatives who died of kuru were honored with this practice exposing surviving family members to infectious brain tissue which might have amplified the kuru epidemic [25, 26]. After the ritualistic practice ended in the late 1950s, the number of new cases dramatically declined and no persons born after 1959 developed the disease. Likely incubation periods of the most recent seven male cases reported by Collinge et al. ranged from 39 to 56 years, although the longest incubation period may have been up to 7 years longer [9].

Prion diseases of animals include scrapie in sheep, goats, and mouflon; bovine spongiform encephalopathy (BSE) in cattle; feline spongiform encephalopathy in domestic and zoo cats; ungulate spongiform encephalopathy in exotic zoo ruminants; chronic wasting disease (CWD) in deer, elk, and

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Table 47.1 Animal and human prion diseases

Type of prion disease	Affected host	Year first described or identified	Mode of disease transmission/occurrence
Animal prion diseases			
Scrapie	Sheep and goats	1730s	Contaminated environment, possibly direct contact, oral
Bovine spongiform encephalopathy	Cattle	1986	Contaminated feed, oral
Chronic wasting disease	Deer, elk, and moose	1967	Contaminated environment, direct animal contact, oral
Transmissible mink encephalopathy[a]	Mink	1947	Contaminated feed, oral
Feline spongiform encephalopathy[b]	Domestic and wild cats	1990	BSE-contaminated feed, oral
Ungulate spongiform encepahlopathy[b]	Exotic ruminants (e.g. kudu, nyala)	1986	BSE-contaminated feed, oral
Human prion diseases			
Kuru		1950s	Ritualistic cannibalism involving brain tissue ingestion, oral
Sporadic CJD		1920s	Unknown
Iatrogenic CJD		1974[c]	Via pituitary hormones, dura and cornea grafts, neurosurgical equipment
Variant CJD		1996	Consumption of BSE-contaminated cattle products, human blood products
Familial CJD		1924	Prion protein gene germline mutations, inherited
Gerstmann-Sträussler-Scheinker syndrome		1936	Prion protein gene germline mutations, inherited
Fatal familial insomnia		1986	Prion protein gene germline mutations, inherited

CJD Creutzfeldt-Jakob disease
[a]The last known outbreak of transmissible mink encephalopathy occurred in 1981 in Wisconsin
[b]The known feline and ungulate spongiform encephalopathies are believed to have resulted from BSE transmission
[c]The first report of iatrogenic CJD was in 1974 in a recipient of cornea obtained from a CJD decedent; human pituitary growth hormone-associated CJD was first reported in 1985 and dura mater graft-associated CJD in 1987

moose; and transmissible mink encephalopathy (TME) in farmed mink (Table 47.1) [27].

Epidemiologic evidence indicates that feline and ungulate spongiform encephalopathies were caused by transmission of the BSE agent via consumption of BSE-contaminated feed [28]. Although strong evidence is lacking, speculations persist that scrapie in sheep was the original source of prion diseases in other animals, such as BSE and CWD.

TME occurred in outbreaks among farmed mink primarily in the United States but also in Canada, Finland, Germany, and Russia [28]. TME outbreak investigations suggested that the disease was causally linked with consumption of scrapie-contaminated meat [28]. The last known outbreak of TME occurred in 1985 in Wisconsin. An investigation of this outbreak indicated that the mink on that particular farm were fed only downer cattle, igniting much speculation about the existence of a spontaneous prion disease in cattle even before BSE was identified in the United Kingdom. Non-epizootic cases of TME have not been reported.

2 Etiologic Agent of Prion Diseases

Before the 1980s, prion diseases were widely believed to be caused by "slow viruses" despite the fact that no viral particles or disease-specific nucleic acids were identified in association with scrapie transmission in laboratory animals [29, 30]. The scrapie agent, which was most widely studied at the time, could not be grown in cell culture. Its successful transmission to laboratory mice in 1961 greatly facilitated research efforts to understand the nature of the agent [1]. Because of the long incubation period associated with scrapie, transmission to wild-type mice was tedious and in many instances required a year to complete. Development of transgenic mice greatly facilitated prion disease research by allowing closer modeling of the diseases and reducing the incubation period in the experimental models [31, 32].

Two distinctive properties of the scrapie agent led to the suspicion that the agent was devoid of nucleic acids and, thus, may not be a virus but primarily composed of a protein. These properties included: (1) resistance of the scrapie agent to procedures, such as treatment with ultraviolet light and ionizing radiation, that normally inactivate other microorganisms, including viruses, and (2) the reduction of scrapie infectivity by procedures that denature or degrade proteins [1]. The concept that the scrapie agent might replicate in the absence of nucleic acids or might just be a protein was postulated as early as the 1960s by Alper and colleagues, Pattison and Jones, and Griffith [30, 33, 34]. In 1982, Prusiner and colleagues described the successful enrichment of a hydrophobic protein, the presence of which was required for scrapie transmission in laboratory animals [1, 35, 36]. Prusiner

introduced the term "prion" to describe this protein by borrowing and mixing the first few letters from the descriptive phrase "*pro*teinaceous *in*fectious" particle [1]. Since then, additional evidence has accumulated indicating that prions may be acting alone in causing prion diseases. However, the critical steps in the production, propagation, and pathogenesis of this infectious protein remain unclear. As a result, the study of prions has become an important, relatively new area of biomedical research. Some critics of the prion hypothesis still believe that nucleic acids undetected by current methods may play a crucial role in the pathogenesis of prion diseases [37–40]. Prions seem to be almost entirely composed of proteins with a glucose moiety attached to them. Creation of prion infectivity by modifying the conformation of synthetic or recombinant polypeptides has led credence to the view that prions may entirely consist of proteins with no nucleic acid genome [41, 42]. How a protein-only agent would confer strain specificity to prions causing different diseases in different species has been a topic of debate for many years. Studies have suggested that strain specificity may be enciphered in the different physical properties of prions and their varying protein conformations.

2.1 Cellular Prion Protein

Soon after the nature of infecting prions was described, researchers discovered the similarity of the abnormal prions with that of a normal protein found as a structural component of cell membranes [43–45]. This discovery marked a turning point in our understanding of prion diseases and partially explained the absence of inflammatory infiltrates in pathologic specimens of infected tissues and the lack of humoral response in several prion diseases. PrP^{C} is usually found as a monomeric GPI-linked glycoprotein on the cell membrane and is soluble in mild detergents. In humans, PrP^{C} is encoded by the prion protein gene located on the short arm of chromosome 20 [43, 44]. Similar to other proteins, PrP^{C} is produced in the endoplasmic reticulum and transits through the Golgi apparatus to the cell surface.

PrP^{C} is expressed in large quantities in neurons, but it is also expressed in lymphoid tissues and other organs and tissues in relatively smaller quantities. Several possible functions have been proposed for PrP^{C} [46]. Its location in parts of the cell membrane specializing in signaling was believed to indicate that it may be involved in signal transduction. Other studies have hypothesized that PrP^{C} interacts with other membrane proteins to provide neuroprotective functions, although more direct evidence is lacking. Another perhaps more widely studied proposed function of PrP^{C} is binding copper and involvement in its metabolism [46]. PrP^{C} readily binds copper in physiologic concentrations, and its overexpression has been shown to promote cellular uptake of copper, indicating that it may play a significant role in copper homeostasis [46].

Prions appear to be composed largely or entirely of the abnormal protein designated as PrP^{Sc}. The underlying pathophysiologic mechanism in the occurrence of prion diseases involves the biochemical conversion of PrP^{C} into the pathogenic PrP^{Sc}. This conversion occurs by a poorly defined post-translational autocatalytic process, possibly requiring the aid of cofactors such as proteins or nucleic acids. It appears that PrP^{Sc} reproduces itself by recruiting PrP^{C} and stimulating its conversion into the disease-causing isoform [47–51]. This conformational change confers protease-resistant properties to PrP^{C} and a three-dimensional structure distinguishable from the infecting prions [49, 52]. The initial instigating PrP^{Sc} molecule may originate from exogenous sources or within the brain from somatic or germ line prion protein gene mutations. Knockout mice devoid of the prion protein gene are resistant to scrapie infection, indicating that the production of PrP^{C} is required for the generation and propagation of PrP^{Sc} [53]. During its conversion, PrP^{Sc} acquires more beta-sheet structure that renders it resistant to proteolytic enzymes, conventional disinfectants, and standard sterilization methods. A higher proportion of the tertiary structure of PrP^{C}, on the other hand, is composed of alpha helices which make it more sensitive to denaturation by proteinase-K treatment [54, 55]. Removal of the neuroprotective functions of PrP^{C} as more of it becomes converted to the pathogenic PrP^{Sc} and accumulation of PrP^{Sc} in neurons have been suggested as major contributory factors in the underlying pathogenesis of prion diseases and widespread neuronal death.

3 Creutzfeldt-Jakob Disease

CJD is the most common form of prion disease in humans and has been reported in all continents of the world. It was first recognized in Europe in the early 1920s and bears the name of two German neurologists, Hans Gerhard Creutzfeldt and Alfons Maria Jakob, who separately reported patients with rapidly progressive neurodegenerative diseases [56]. At least two of the patients initially reported by Jakob had the typical neuropathologic features that have since been recognized as the hallmark of CJD.

The clinical manifestation of CJD is usually characterized by the onset of dementia, ataxia, or behavioral abnormalities. As the disease progresses, patients commonly develop dysarthria, movement disorders such as gait abnormalities, myoclonus, and tremors. These neurologic deficits are rapidly progressive and patients often develop akinetic mutism towards the terminal stages of the illness, usually over a period of weeks to several months [57–62]. CJD is invariably fatal with median illness duration of about 4 months. Over 50 % of patients die within 6 months and about 80 % within 1 year of disease onset [57]. The presence of a characteristic electroencephalogram (EEG) finding of triphasic, periodic sharp wave complexes can be demonstrated with multiple

testing in approximately 75 % of patients [63–65]. Elevated levels of 14-3-3 proteins in the cerebrospinal fluid (CSF) can also be found in most CJD patients [66–71]. Elevated CSF 14-3-3 is a marker for rapid neuronal death and, in the appropriate clinical context, can often help in making a premortem diagnosis of CJD. This test is nonspecific, however, and may be elevated in other neurologic conditions that result in rapid neuronal death. A similar CSF test that detects tau proteins can also aid in the premortem diagnosis of CJD. Elevated tau protein levels in combination with a positive 14-3-3 test may increase the sensitivity and specificity of a CJD diagnosis [71]. Magnetic resonance imaging (MRI) findings showing high intensity in the basal ganglia and cortical regions of the brain have been correlated with a CJD diagnosis [64, 72–79]. The characteristic EEG and MRI findings and elevated levels of 14-3-3 in the appropriate clinical context are used as diagnostic criteria for CJD [78].

In the United States, the median age of CJD patients at the time of death is 68 years with approximately 70 % of cases occurring between 55 and 75 years of age (Fig. 47.1) [80–82]. Overall, CJD has been reported in many countries with an annual incidence ranging from one to two cases per million populations, including in the United States (Fig. 47.2). However, the incidence increases with age and approaches five cases per million populations for those ≥65 years of age. A decline in CJD incidence in persons ≥80 years of age has been consistently reported in many developed countries which may be due to ascertainment bias in this older age group. In the United States, epidemiologic studies have consistently shown that the incidence of CJD is at least 2.5 times lower in blacks than whites [81–83]. The precise reason for this wide discrepancy is not well understood, but under diagnosis among black populations is unlikely to entirely explain the striking difference in CJD incidence. The age-adjusted incidence for males in the United States is slightly higher than that for females, particularly among those ≥60 years of age [83, 84]. CJD has been shown to occur in three different forms: sporadic, iatrogenic, and familial forms.

A definitive diagnosis of CJD can only be made by histopathologic or immunodiagnostic testing of brain tissue obtained at autopsy or biopsy. Histopathologic examination of brain tissue demonstrates the hallmark triad of spongiform lesions, neuronal loss, and astrogliosis. Since more specific diagnostic tests have been developed, histopathology alone is rarely used to confirm the diagnosis of CJD. Immunodiagnostic assays, such as immunohistochemistry and Western blot testing that show the presence of PrP^{Sc}, are widely used to confirm the CJD diagnosis. Recently, a new test termed real-time quaking-induced conversion (RTQuIC) was developed to detect prions in peripheral tissues or fluids such as the CSF, blood, or urine [85]. The detection method used in this test is based on that used in protein misfolding cyclic amplification. RTQuIC can detect minute amounts of prions and has been shown to be 80% sensitive and over 90% specific in confirming a CJD diagnosis from CSF specimens. In addition, the presence of infecting prions has been demonstrated in the olfactory epithelium of CJD patients [86]. Because these tissues are accessible from the nasal cavity, specimen collection by deep nasal swab and subsequent testing by RTQuIC has shown promising results as a non-invasive, rapid, antemortem, diagnostic assay for CJD.

Currently, no effective treatment exists for CJD or any other prion disease. Clinical management of patients is primarily supportive to ameliorate some of the aggravating symptoms of the disease [87]. Many compounds and drugs (e.g., pentosan polysulfate, quinacrine, and doxycycline) have been investigated as possible therapeutic modalities in CJD patients and in animal models [88–97]. Although some compounds have been shown to be effective antiprion agents in in vitro experiments, they were only successful in prolonging the incubation period and survival times in animal models. Prolongation of survival or no effect has been reported in human patients treated with some of the drugs that have already been licensed for indications other than CJD treatment. Limited randomized trials are underway to evaluate the usefulness of some of these drugs. Development of disease-specific treatment regimens is complicated by the fact that widespread neuronal damage has already occurred by the time signs and symptoms appear. Therefore, the ideal antiprion treatment should not only inhibit the propagation of infecting prions but also reverse neuronal damage or degeneration that may have already resulted in cognitive impairment and physical disability. Such a drug or a compound should also be able to readily pass the blood-brain barrier. Lack of a reliable antemortem test poses a challenge in diagnosing patients as early as possible so that they could be enrolled in clinical trials to initiate investigational treatment in time to alter the course of the disease.

3.1 Sporadic CJD

Sporadic CJD accounts for approximately 85 % of cases and occurs in the absence of outbreaks with no known environmental source of infection. Decades of research has not identified a specific source of infection for sporadic CJD patients. Spontaneous generation of the pathogenic prions was hypothesized as a cause for sporadic CJD, possibly resulting from age-related random somatic mutations or errors during prion protein gene expression. The surprisingly stable and uniform incidence of sporadic CJD in time and space and the absence of recognizable transmission patterns to account for a substantial number of the cases were the strongest arguments favoring the spontaneous occurrence of sporadic CJD. Not all researchers are convinced about the spontaneous occurrence of sporadic CJD, and many studies have been

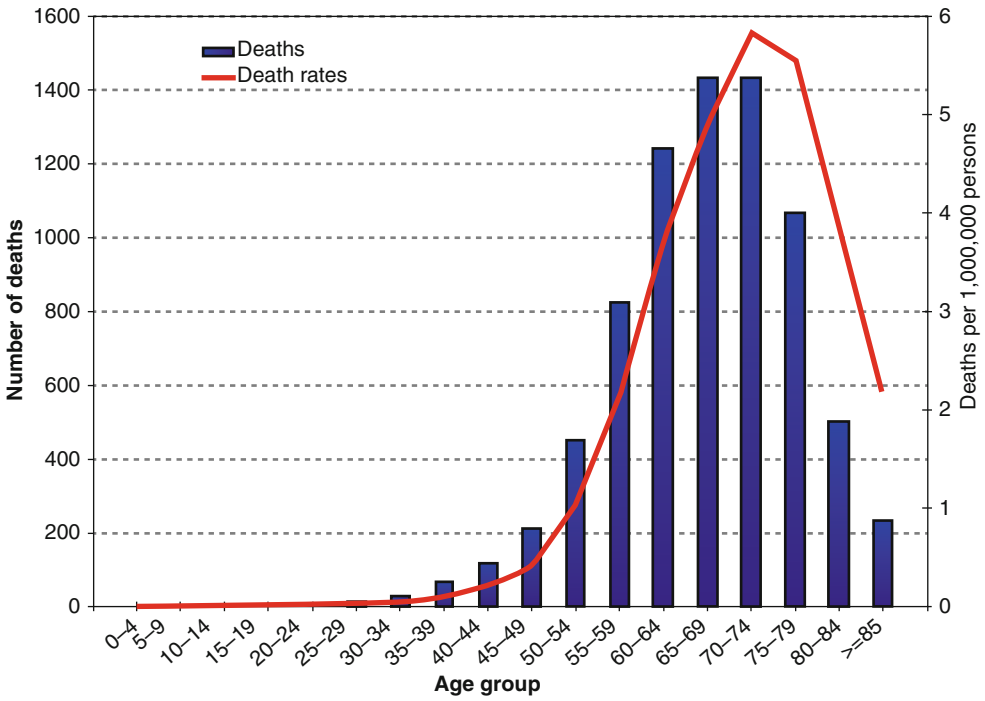

Fig. 47.1 Creutzfeldt-Jakob disease deaths and death rates by age group, the United States, 1979–2008. Deaths obtained from the multiple cause-of-death data for 1979–1998 are based on ICD-9 codes, and those beginning in 1999 are based on ICD-10 codes with available computerized literal death certificate data. Death information was also obtained from other surveillance mechanisms; includes familial prion disease

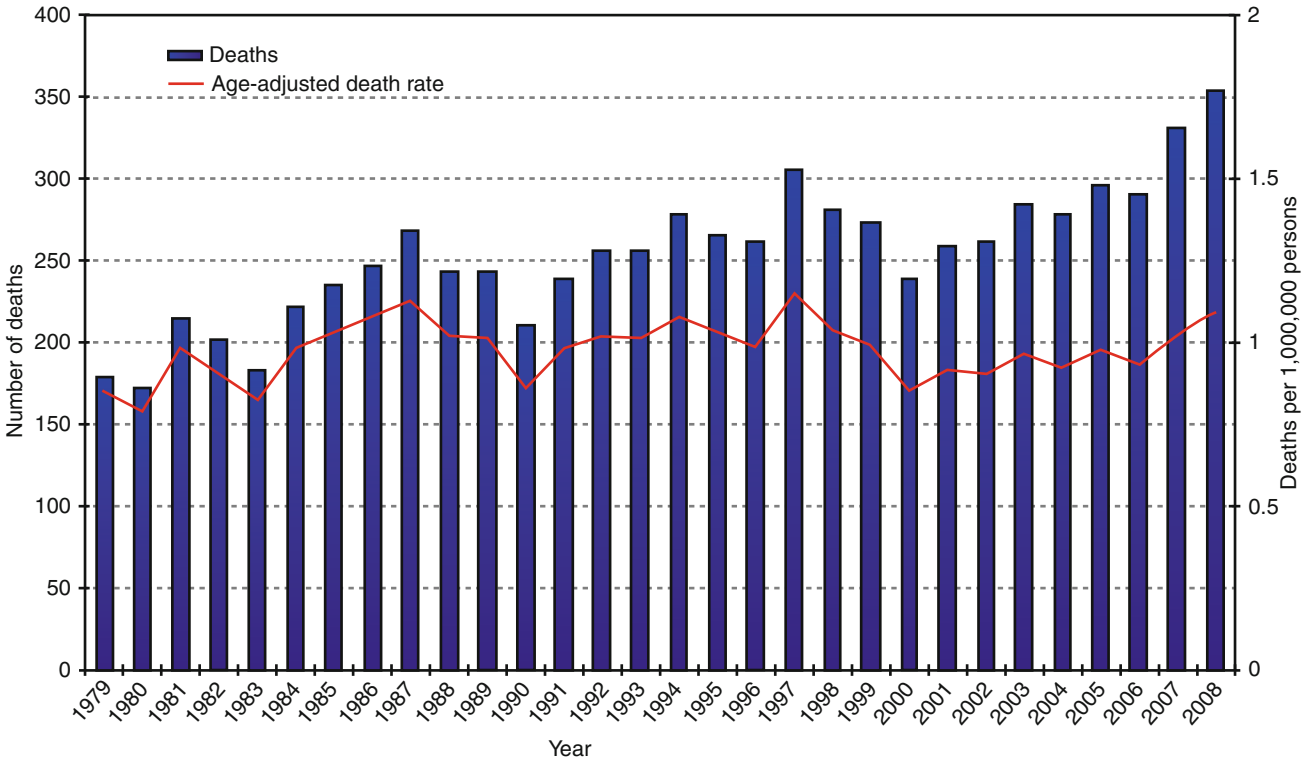

Fig. 47.2 Creutzfeldt-Jakob disease deaths and age-adjusted death rates, the United States, 1979–2008. Deaths obtained from the multiple cause-of-death data for 1979–1998 are based on ICD-9 codes, and those beginning in 1999 are based on ICD-10 codes with available computerized literal death certificate data. Death information was also obtained from other surveillance mechanisms; includes familial prion disease. Rates are adjusted to the US standard 2000 projected population

Table 47.2 Characteristics of subtypes of sporadic Creutzfeldt-Jakob disease (CJD) and sporadic fatal insomnia (sFI)

Subtype	Number of patients ($n=609$)	Percent	Clinicopathologic features
MM1/MV1	352	57.8	Typical CJD clinical and neuropathologic manifestations, typical EEG, rapidly progressive disease.
VV2	90	14.8	Commonly ataxia at onset and late dementia, typical EEG rare, short duration, subcortical pathology, plaque-like deposits
MV2	83	13.6	Similar to VV2 but long duration and presence of kuru-type amyloid plaques in cerebellum
MM2	52	8.5	Progressive dementia, typical EEG rare, long duration, cortical pathology, coarse spongiosis
VV1	25	4.1	Usually young age at onset, typical EEG rare, severe pathology in cerebral cortex with relative sparing of cerebellum, faint synaptic prion staining
MM2 (sFI)	7	1.1	Similar to FFI but without prion protein gene mutations

Data used in the table were obtained from the National Prion Disease Pathology Surveillance Center, Cleveland, OH
M Methionine, *V* Valine

conducted to search for possible sources of infection and risk factors for the disease [98–102].

Possible environmental sources of infection for sporadic CJD have been explored using multiple case-control studies [100–109]. Consumption of animal products, including the brain and other organs; receipt of blood and blood products; occupational exposures; and exposures via surgical procedures were evaluated in these studies. Because of the long incubation period of CJD, obtaining a reliable history of such exposures many years in the past may not be easy. In addition, exposure histories are usually obtained from next-of-kin who may not be familiar with the timing and extent of the exposures. Types of controls used (community or hospitalized) and the timing of selection and interview of the controls could also bias findings of case-control studies [109]. In a study that combined data from Denmark and Sweden, researchers attempted to minimize these biases specifically for evaluating surgical exposures by obtaining information about surgical procedures for both cases and controls from existing hospital records [102]. The study indicated that any major surgery conducted ≥20 years before CJD onset was significantly more common in cases than both matched and unmatched controls. A much larger European study using community controls showed a similar association of history of surgery with the risk of sporadic CJD, albeit using different data collection methodologies [101]. A similar study in Australia also showed that surgical procedures were significantly associated with the development of sporadic CJD [110]. No significant difference in the frequency of surgical procedures was identified among sporadic CJD cases compared with controls in a study performed in Japan [111]. These studies raised the possibility that a certain proportion of sporadic CJD cases may result from exposure to prion-contaminated surgical instruments. Because of the rarity of CJD, confirming or refuting such an association in a small proportion of patients is extremely difficult.

A possible risk of occupational transmission of CJD to health professionals has been raised repeatedly. This risk has recently been reviewed in a study published in Eurosurveillance [112]. The authors used various data sources, including published case reports, case-control studies, and surveillance data from 21 countries contributing to the EuroCJD program. A wide variety of health professionals had been reported with CJD, but the study findings did not suggest increased risk of CJD among health professionals [112].

Sporadic CJD is a heterogeneous disorder which can be further subdivided into five different subtypes based on the Western blot characteristics of protease-resistant fragment of PrP^{Sc} and the polymorphism at codon 129 of the host prion protein gene. These different subtypes, first proposed by Parchi et al., correspond with characteristic clinical and neuropathologic phenotypes (Table 47.2) [113–118]. The most common subtype is associated with a 21 kDa PrP^{Sc} fragment, designated type 1, and the presence of methionine at the polymorphic codon 129 of the prion protein gene. The phenotypic expression does not necessarily neatly fit into these various categories in some patients. In fact, both type 1 and type 2 prion fragments have been reported to coexist in up to 25 % of sporadic CJD patients [113, 119].

A cluster of sporadic prion disease cases termed variably protease-sensitive prionopathy (VPSPr) with a phenotype distinct from other known subtypes of sporadic CJD was recently reported in the United States [120]. The major distinguishing characteristics of VPSPr include sensitivity of the agent to proteinase-K digestion and clinicopathologic manifestations different from sporadic CJD patients. On Western blot analysis, the electrophoretic profile of proteinase-K-resistant fragments from VPSPr patients shows a ladder-like pattern with five major bands corresponding to fragments with different molecular weights. This pattern is clearly different from that seen in sporadic CJD patients but shares some similarities with that observed in some GSS patients. However, no prion protein gene mutations have been identified in association with VPSPr [120]. All the three codon 129 polymorphisms have been identified in patients with VPSPr with slightly variable clinical and histopathologic phenotypes, indicating that the polymorphism at codon 129 modifies the phenotype as it has been shown to do in sporadic CJD patients and several genetic prion diseases.

3.2 Iatrogenic CJD

Iatrogenic CJD, which accounts for <1 % of CJD cases, is associated with transmission of the CJD agent via medical interventions such as administration of contaminated human pituitary hormones and the use of contaminated dura mater and corneal grafts and neurosurgical equipment [121]. Iatrogenic transmission of CJD was first identified by Duffy et al. in 1974 in a patient who, 18 months before CJD onset, received a corneal graft obtained from a donor who died of confirmed CJD [122]. Since then, 10 cornea-associated CJD cases have been reported worldwide, but definitive evidence of CJD in the donors was available for only two of the cases [121, 123, 124]. In the remainder, information on the donors was unavailable or they died of conditions other than a prion disease [124]. Sporadic CJD unrelated to transplanted corneas is expected to occur among elderly patients with a history of cornea transplantations because of a large number of such patients alive in the United States (over 30,000 corneal transplantations are performed annually). Using statistical analysis, Maddox et al. have suggested that one coincidental sporadic CJD case among patients with a history of cornea transplantation is expected to occur every 1.5 years in the United States [124].

In 1977, CJD transmission was reported in two unusually young patients who underwent EEG procedures with implantation of depth electrodes 16–20 months before CJD onset; several months earlier, the electrodes were implanted in a patient who subsequently died of confirmed CJD [125]. Experimental implantation of the EEG electrodes into a chimpanzee 18 months after their original use transmitted CJD, demonstrating that the electrodes were indeed contaminated with the CJD agent [126].

Almost a decade after iatrogenic transmission of CJD was first reported in the corneal recipient and via EEG electrodes, other modes of CJD transmissions were identified, including the use of contaminated cadaveric human growth hormone in 1985 and dura mater grafts in 1987 [59, 127–132]. Reports of these initial iatrogenic CJD cases in the United States were followed by identification of similar cases in other countries [121, 128, 133–151]. Human growth hormone-associated CJD cases were primarily reported in France, the United Kingdom, and the United States. In these countries, large cohorts of young individuals were given human growth hormone (hGH) injections as a treatment for stunted growth resulting from growth hormone deficiency. Extraction of hGH by batch processing of pituitaries from multiple cadaver donors may have led to contamination of an entire batch if one of the donors was in the preclinical phase of disease or had died of CJD. In the United States, after identification of the first three hGH-associated CJD cases, a cohort of >6,000 recipients of hGH sponsored by the National Hormone and Pituitary Program were enrolled in a follow-up study. Among this cohort, as of April 2012, a total of 29 hGH recipients have developed CJD [121, 152]. All of these patients began their treatment prior to the introduction of a size exclusion chromatography purifying step in 1977. To date, no patient who began treatment with hGH purified using this method has developed CJD. A recent analysis of the US data indicated that the absence of cases among patients treated after 1977 may represent a real difference in risk between hGH produced before and after that year [152]. Worldwide, a total of 226 hGH-associated CJD patients have been identified, including the 29 US cases, 119 cases from France, and 65 cases from the United Kingdom [121]. The attack rate ranged from 0.4 % in the United States to 6.3 % in France. Incubation periods ranged from 5 to 42 years with a mean of 17 years [121].

Beginning in the mid-1980s, a parallel iatrogenic CJD outbreak was occurring among patients who received dura mater grafts. The initial case was identified by an astute physician in the United States in a patient who received Lyodura, a brand of dura mater graft processed by a German company [131, 133]. Subsequent to this report, several cases of dura mater graft-associated CJD cases were identified, including 142 from Japan, 14 from Spain, 13 from France, 10 from Germany, 9 from Italy, 8 from the United Kingdom, 5 each from Australia and the Netherlands, 4 each from Canada and the United States, and a smattering of cases from other countries [121]. Over 60 % of the worldwide 228 dura mater graft-associated CJD cases were reported from Japan, where Lyodura was used in much higher quantities than elsewhere [121, 153, 154]. In addition, isolated cases of CJD associated with Tutoplast, another brand of dura mater graft produced by a different German company, have been reported, including in the United States and Japan [137, 153, 154]. The higher number of iatrogenic CJD transmissions associated with Lyodura is believed to be due to the sourcing and processing practices prevalent at the company in the 1980s before revisions were made in response to the occurrence of the initial few Lyodura-associated CJD cases. Almost all Lyodura-associated CJD cases received products processed before these revisions were made. The mean incubation period for the cases identified worldwide was 12 years with a range of 1.3–30 years [121].

4 Bovine Spongiform Encephalopathy and Variant Creutzfeldt-Jakob Disease

4.1 Bovine Spongiform Encephalopathy

Prion diseases attracted unprecedented scientific and public attention after a large outbreak of BSE in cattle emerged in the United Kingdom in the mid-1980s and spread to other countries [155–157]. This attention increased dramatically when strong scientific evidence indicated in 1996 that the

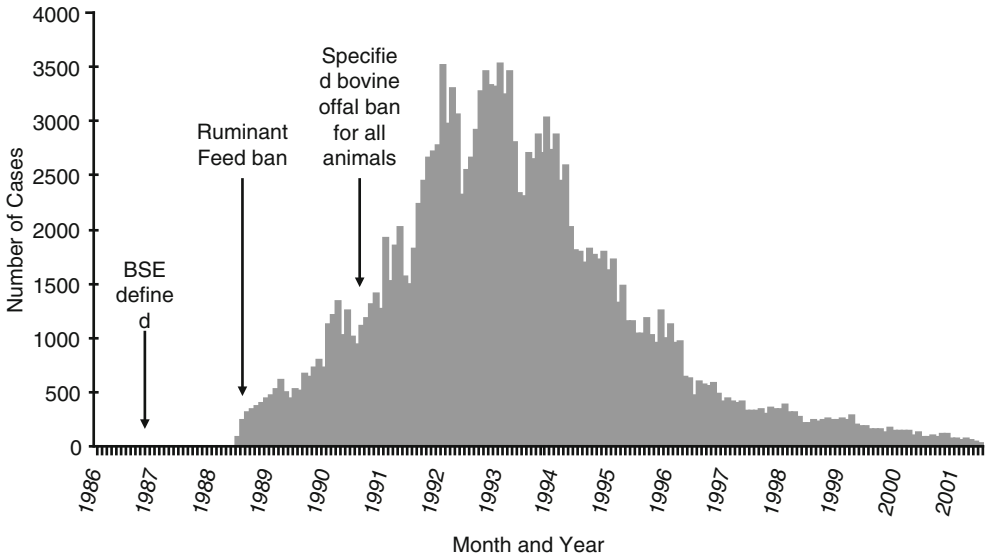

Fig. 47.3 Epidemic curve of bovine spongiform encephalopathy outbreak, the United Kingdom

BSE agent has been transmitted to humans causing a new form of CJD, later termed variant CJD [158]. The implication that prion diseases could be transmitted via contaminated food or bovine-derived products potentially exposing a large number of consumers sent shockwaves through the cattle industry and international beef trade. By far the largest number of BSE cases was reported from the United Kingdom, followed by several other European countries, including Ireland and Portugal [27, 133].

Although BSE was first recognized in the United Kingdom in 1986, undetected cases probably occurred since the early 1980s [159]. The number of UK BSE cases increased rapidly in the second half of the 1980s and early 1990s, peaked in 1992 with 37,280 confirmed cases, and has markedly declined since then (Fig. 47.3). As more stringent control measures were implemented to prevent cattle exposure to meat and bone meal, the number of confirmed UK BSE cases continued its dramatic decline throughout the late 1990s and 2000s consistent with the hypothesis that BSE was orally transmitted via contaminated meat and bone meal [27, 157, 160].

Clinically, the signs of BSE include neurologic dysfunction, including altered behavior, unsteady gait with falling, and abnormal responses to touch and sound [161]. In some animals, the onset of BSE can be insidious and subtle and may be difficult to recognize. During the early phase of the UK BSE outbreak, the public media introduced the popular term "mad cow" disease to describe the strange disease causing fearful and aggressive behavior in some of the cattle infected with BSE.

Although the original source of the BSE outbreak is unknown, the two most accepted hypotheses are cross-species transmission of scrapie from sheep to cattle and the transmission of a spontaneously occurring BSE within the cattle population [157, 160, 162, 163]. The latter hypothesis is predicated on the occurrence of spontaneous BSE, and more convincing data about its occurrence may become available with increased detection and monitoring of rates of atypical as well as classic cases of BSE [164, 165]. Strong epidemiologic evidence indicates that the practice of feeding cattle protein derived from rendered animal carcasses presumably contaminated with the scrapie or spontaneous BSE agent may have triggered the cattle epidemic [157]. In the past, cattle feed rendering in the United Kingdom involved several treatment steps, including exposure of the feed to prolonged heating in the presence of a hydrocarbon solvent. Some researchers have suggested that omission of these steps in the late 1970s and early 1980s in the United Kingdom contributed to the emergence of BSE by allowing scrapie or spontaneous BSE infectivity to survive the rendering process [157, 162, 166, 167]. Regardless of the origin of BSE, the epidemiologic evidence indicates that feeding cattle rendered BSE-infected carcasses greatly amplified the BSE outbreak. Several other factors may have contributed to the emergence of BSE in the United Kingdom, including a relatively high rate of endemic scrapie, a high population ratio of sheep to cattle, and the inclusion of rendered meat and bone meal at high rates in cattle feed.

Since the BSE outbreak was first detected, an estimated >2 million cattle have been infected with BSE in the United Kingdom [133, 167]. Approximately half of these BSE-infected cattle would have been slaughtered for human consumption, potentially exposing millions of UK residents [133, 168, 169]. Beginning in 1988, UK animal and public health authorities implemented several protective measures to prevent further exposure of animals and humans to BSE-infected cattle products. The implementation of these measures, particularly animal feed bans, led to a dramatic decline in the UK BSE outbreak (Fig. 47.3).

Because cattle carcasses were included in the production of animal feed, potential transmission of BSE to other animals was considered during the early phase of the BSE outbreak in the United Kingdom. BSE-like dis-

eases were identified in zoo animals (ungulate spongiform encephalopathy) beginning in the late 1980s and in domestic cats (feline spongiform encephalopathy) beginning in 1990, indicating the potential for the BSE agent to cross the species barrier and infect other animals [28, 170]. This development increased the concern about the possible transmission of BSE to humans and led to the establishment of enhanced CJD surveillance in the United Kingdom.

BSE was reported for the first time outside the United Kingdom in Ireland in 1989 and in Portugal and Switzerland in 1990. By August 2006, the number of countries that reported one or more BSE cases in native cattle increased to 25, including 21 countries in Europe. The four countries outside Europe that reported BSE cases are Canada, Israel, Japan, and the United States. The BSE outbreak appears to be declining in most European countries, although small numbers of cases continue to occur.

In North America, BSE was first detected in 1993 in a cow that had been imported into Canada from the United Kingdom. Rendered remains of imported cohorts of this cow may have been responsible for the BSE cases subsequently identified during 2003–2011 among cattle born in Canada. One of these cases was identified in Washington State but was later traced to a farm in Canada [171]. As of May 2012, a total of 19 BSE cases have been identified in Canada, and at least 13 of these cases were born after the 1997 ruminant feed ban which was implemented to prevent BSE transmission among cattle [172]. Because of the continued occurrence of new BSE infections after the 1997 ruminant feed ban, US and Canadian authorities tightened the specified risk material ban in 2007 by excluding potentially infectious nervous tissues from all animal feed. In 2005 and 2006, respectively, nonclassic forms of BSE termed atypical BSE were confirmed in an approximately 12-year-old cow born and raised in Texas and a 10-year-old cow from Alabama [173, 174]. The source of BSE infection for these two cows remains unknown. In 2012, as part of USDA's ongoing surveillance, a third BSE case was identified in a dairy cow aged over 10 years in California. Similar to the previous 2 BSE cases reported in the United States, the third case was reported to have atypical BSE. The initial two cases were reported with H–type and the third case with L-type BSE. The occurrence of these cases renewed speculations that atypical BSE may in fact constitute a prion disease that arises spontaneously among older potentially predisposed cattle.

4.2 Variant Creutzfeldt-Jakob Disease

In 1996, the identification of a cluster of young patients (median age, 28 years) with a prion disease was reported in the United Kingdom as part of the CJD surveillance system that was established in response to concerns about the potential spread of BSE to humans [158]. Because of the patients' unusually young age and the distinct clinical and neuropathologic findings, which were different from patients with the classic form of CJD, the occurrence of the cluster was believed to signify the transmission of BSE to humans. Since 1996, variant CJD cases increased in number and geographic distribution, and strong scientific evidence supported initial suspicions that variant CJD was indeed BSE in humans [175, 176]. As of June 2014, a total of 229 variant CJD patients were reported worldwide, including 177 patients from the United Kingdom; 27 from France; 5 from Spain; 4 from the United States; 3 from the Netherlands; 2 each from Canada, Italy, and Portugal; and 1 each from Japan, Saudi Arabia, and Taiwan [177]. Seven of the non-UK variant CJD patients (2 each from the United States and Ireland and 1 each from Canada, France, and Japan) were believed to have acquired variant CJD during their past residence or visit in the United Kingdom. The third US and second Canadian vCJD patients were believed to have acquired the disease during their residence in Saudi Arabia.

Variant CJD can be distinguished from the more common classic CJD by the clinical and laboratory findings (Table 47.3) [133, 175, 178]. The median age at death of variant CJD patients is 40 years younger than that of sporadic CJD patients (28 and 68 years, respectively); the median illness duration for vCJD is longer (14 months) than that of sporadic CJD (<6 months). The distinguishing clinical features of variant CJD include a predominantly psychiatric manifestation at onset with delayed appearance of frank neurologic signs and the appearance of sensory abnormalities with dysesthesia and paresthesia [175]. On the MRI, a typical "pulvinar sign" is often demonstrated in a majority of vCJD patients consisting of a hockey-stick-like, symmetrical hyperintensity in the pulvinar region relative to the intensity in other structures [179]. The diagnostic EEG finding that is common in classic CJD patients is very rare in patients with variant CJD. All variant CJD patients tested to date had methionine homozygosity at the polymorphic codon 129 of the prion protein gene [177, 180]. This homozygosity is present in approximately 35–40 % of the general UK population. A definitive diagnosis of variant CJD requires laboratory testing of brain tissues. In addition to the spongiform lesion, neuronal loss, and astrogliosis typical of most prion diseases, the neuropathology in variant CJD is characterized by the presence of numerous "florid plaques," consisting of amyloid deposits surrounded by a halo of spongiform lesions [177, 181] (See Fig. 47.4).

Studies in the United Kingdom have indicated the probable secondary person-to-person transmission of the variant CJD agent in three patients by blood (non-leukodepleted red blood cells) collected 17–40 months before variant CJD onset in the donors [177, 182–187]. The incubation period in

Table 47.3 Clinical and pathologic characteristics distinguishing variant Creutzfeldt-Jakob disease (variant CJD) from classic CJD

Characteristic	Variant CJD	Classic CJD
Median age (range) at death (years)	28 (14–74)	68 (23–97)[a]
Median duration of illness (months)	13–14	4–5
Clinical presentation	Prominent psychiatric/behavioral symptoms, painful sensory symptoms, delayed neurologic signs	Dementia, early neurologic signs
Periodic sharp waves on electroencephalogram	Almost always absent	Often present
"Pulvinar sign" on magnetic resonance imaging[b]	Present in >75 % of cases	Very rare or absent
Presence of "florid plaques" on neuropathologic sample	Present in great numbers	Rare or absent
Immunohistochemical analysis of brain tissue	Marked accumulation of PrP-res[c]	Variable accumulation
Presence of agent in lymphoid tissue	Readily detected	Not readily detected
Increased glycoform ratio on Western blot analysis of PrP-res	Present	Not present
Genotype at codon 129 of prion protein	Methionine/methionine[d]	Polymorphic

[a]U.S. CJD surveillance data 1979–2001
[b]Symmetrical high signal in the posterior thalamus relative to that of other deep and cortical gray matter
[c]Protease-resistant prion protein
[d]A patient with preclinical vCJD related to bloodborne transmission was heterozygous for methionine and valine

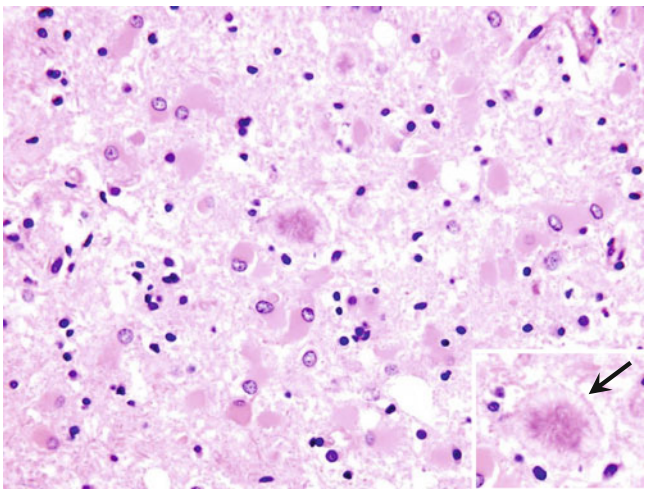

Fig. 47.4 Variant CJD neuropathology. Cerebral cortex shows marked astroglial reaction and the occasional presence of relatively large florid plaques surrounded by vacuoles (*arrow in insert*). Frontal cortex, hematoxylin and eosin stain

these patients ranged from 6.5 to 8.5 years, and all three had methionine homozygosity at the polymorphic codon 129 of the prion protein gene. A fourth patient who was heterozygous at codon 129 had laboratory evidence of vCJD but died of a non-neurologic condition >5 years after receipt of red blood cells from a donor diagnosed with vCJD 18 months after donation [182]. Because a large proportion of the UK population has potentially been exposed to the BSE agent, concerns still exist about additional secondary spread of the agent via blood products and possibly via contaminated surgical instruments. Testing of retrospectively collected appendectomy samples from 12,674 UK residents identified three positive samples, indicating an estimated prevalence of 237 vCJD infections per million populations [188]. Two of the three positive samples with prion protein gene analysis were homozygous for valine at codon 129.

4.3 Atypical Bovine Spongiform Encephalopathy

Since 2003, BSE cases with histopathologic features and Western blot characteristics of the infecting prions distinct from that of the classic form of BSE were increasingly identified from several European countries, Canada, Japan, and the United States [173, 189–193]. Based primarily on the molecular weight of the unglycosylated, proteinase-K-resistant fragment of the PrP^{Sc}, atypical BSE cases were classified into two groups. The fragment with a higher molecular weight than the classic type (C-type) was designated the H-type, and the fragment with a lower molecular weight than the C-type was designated the L-type [194, 195]. It is widely believed that these different properties of the infecting prions may represent different strains of the BSE agent. Initially, the L-type BSE was called bovine amyloid spongiform encephalopathy or BASE because of the presence of amyloid plaques in histopathologic preparations of brain tissues from infected cattle [189]. Because the H- and L-types of BSE seem to be rare and because they tend to occur in older cattle, some researchers have suggested that these atypical BSE cases arise spontaneously as a result of sporadic, random mutations of the prion protein gene [195]. However, the possibility that they may also be strain variations of the large C-type BSE outbreak should not be excluded. Regardless of the origin of atypical BSE, its continued occurrence could still pose a risk of contamination of the animal feed and human food supplies. Spontaneous occurrence of BSE may actually be an ominous sign because feed control measures may not completely eliminate its occurrence and herald the need for continuing surveillance and maintaining effective feed bans even after the C-type BSE outbreak is under control.

Whether or not atypical BSE can be transmitted to humans and the possible phenotype it may represent remain unknown. The possible transmission of L- and

H-type BSE to humans has been assessed using transgenic mouse models expressing the human prion protein homozygous for methionine at codon 129. L-type BSE transmitted to the transgenic mice with no apparent barrier, whereas C-type BSE had a substantial transmission barrier; no transmission of the H-type was reported [196, 197]. Transmission of L-type BSE to lemurs has also been reported via intracerebral and oral challenges and to cynomolgus macaques by intracerebral inoculation [191, 198]. A comparison between the pathogenesis of classic and atypical BSE is currently an area of active research because this could have potential implications for possible exposure to humans and animals [199–201].

5 Chronic Wasting Disease and Interspecies Transmission

CWD was first identified in the late 1960s as a fatal wasting syndrome of captive mule deer in research facilities in Colorado and subsequently in a similar facility in Wyoming. It was not recognized as a spongiform encephalopathy until 1977 [202, 203]. CWD was recognized as a disease of free-ranging animals in the early 1980s, and by the mid-1990s, its endemic occurrence was reported in deer and elk in a contiguous area in northeastern Colorado and southeastern Wyoming. Mule deer, white-tailed deer, and Rocky Mountain elk are the major known natural hosts for CWD [204]. In 2005, a hunter-killed moose was confirmed with CWD in Colorado, suggesting that this member of the deer family is also a natural host. As of December 2013, CWD has been identified in free-ranging cervids in 18 US states (Fig. 47.5) and 2 Canadian provinces and in greater than 100 captive herds in 15 states and provinces in North America.

The occurrence of CWD in free-ranging animals is spreading to wider geographic areas, and prevalence is increasing in many CWD endemic locations. Parts of Wyoming now have CWD prevalence rates approaching 50 % in mule deer, and prevalence in areas of Colorado and Wisconsin is less than 15 % in deer. The prevalence of CWD in elk is generally lower than in deer but can reach 10 % in parts of Wyoming. Known risk factors for CWD include sex and age of the animals, with adult male deer having the highest disease prevalence [203, 205]. Polymorphisms in the prion protein gene correlate with the incidence of CWD in deer and elk but remain less understood than the strong genetic influence described for scrapie in sheep [206–209]. The long-term effects of CWD infection on the dynamics of cervid populations are unclear. Epidemiologic modeling studies have predicted that CWD infection could have negative effects on the density of free-ranging cervid populations. These long-term effects may be influenced by variations in hunting management practices and persistence of the CWD agent in the environment [210, 211]. CWD infection in free-

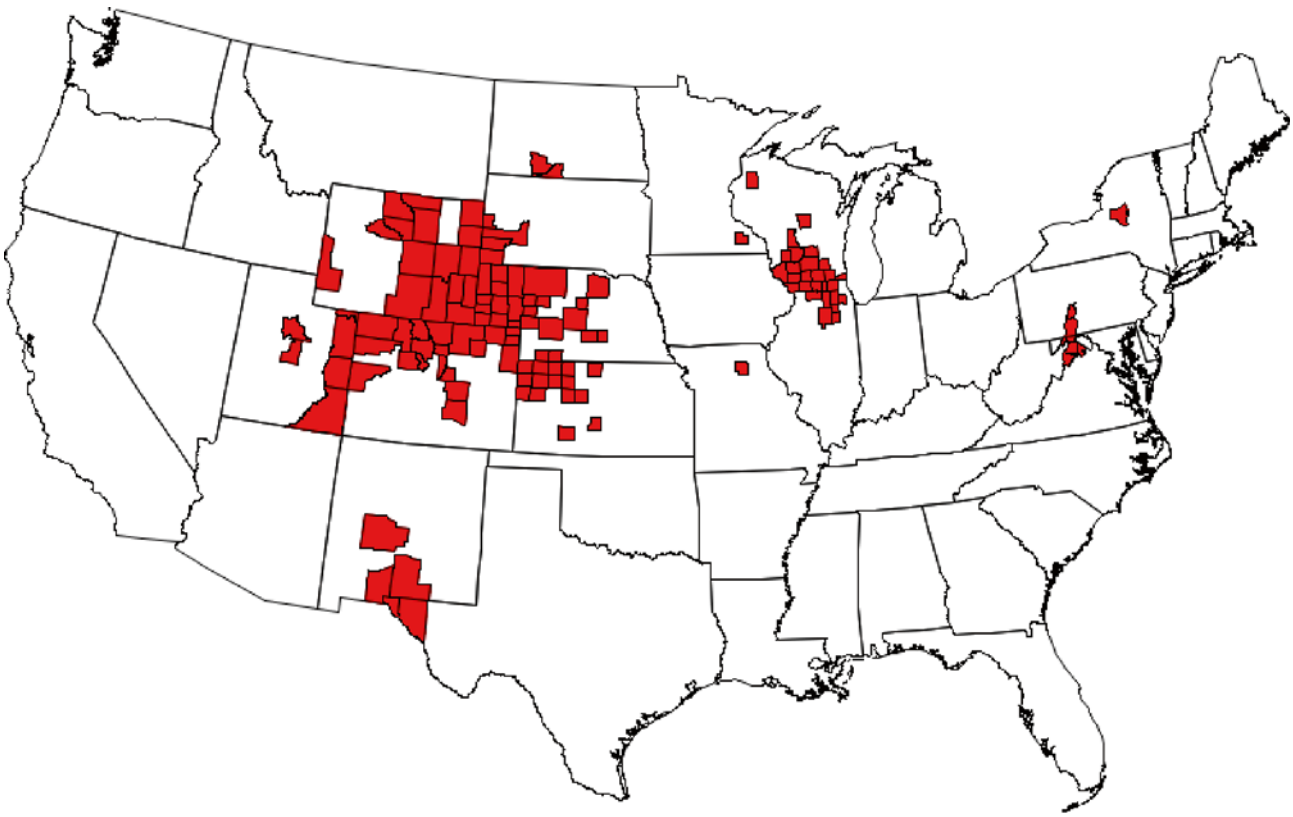

Fig. 47.5 Chronic wasting disease among free-ranging cervids by county, the United States, July 2012

ranging mule deer has been associated with large decreases in cervid populations in Boulder, Colorado, an area with a high rate of CWD infection [205]. Consistent with the clinical manifestations of the disease, deer with CWD are weaker and are preyed upon by mountain lions more readily than healthy deer [205]. They are also more likely to be involved in collisions with vehicles, further contributing to thinning of cervid populations [212].

5.1 CWD Transmission in the Natural Host

Horizontal transmission of the CWD agent is a major mechanism of disease spread in the wild. CWD prions can enter the environment through shedding from diseased animals and from decomposing carcasses. The CWD agent is shed from infected deer in urine, feces, saliva, blood, and antler velvet, and shedding can occur during the preclinical phase of CWD or from clinically affected animals [213, 214]. CWD prions are present in many organs and tissues of an infected cervid, including skeletal muscle, cardiac muscle, fat, lymphoid tissues, and peripheral and central nervous system tissues [209, 215, 216]. Ingestion is an effective route of CWD agent transmission among cervids and lesions in the oral cavity can facilitate entry of the agent enhancing the transmission of CWD [217, 218]. Prions bind to a range of soils and soil minerals and retain the ability to replicate [219–223]. Ingestion and inhalation of soil by cervids has been hypothesized to play an important role in CWD transmission [224, 225]. Nasal inoculation has been shown to be an efficient route of prion transmission [226, 227]. Consistent with the role of the environment in CWD transmission, exposure of CWD-naïve deer to drinking water, feed buckets, and bedding used by CWD-infected deer resulted in the naïve deer developing CWD [228].

5.2 Interspecies CWD Transmission

Human prion disease with evidence of a link with CWD has not been identified despite several epidemiologic investigations of suspected cases [202, 229–232]. Consistent with this observation, in vitro prion conversion assays indicate that the efficiency of human PrP^C conversion by CWD prions is low. In addition, transgenic mice expressing human PrP^C are not susceptible to CWD infection [233–235]. However, the CWD agent has been transmitted to squirrel monkeys by intracerebral and oral routes of inoculation, while cynomolgus macaques, which are genetically closer to humans, are resistant to CWD infection [236]. Transmission of CWD to non-cervid species has not been observed under natural conditions. Raccoons, opossums, and coyotes who may scavenge CWD-infected carcasses have not been shown to be infected with CWD in Wisconsin [237]. Transmission of CWD to cattle has not been observed in experimentally controlled environmental exposure studies [209]. However, CWD has been transmitted to cattle, sheep, goats, mink, ferrets, voles, and mice by intracerebral inoculation [209, 238–240]. Limitations of the aforementioned negative transmission studies include the small number of animals inoculated, which would be unlikely to identify low transmission rates, and the fact that strain variations have not been fully accounted for. Compelling evidence suggests the existence of distinct CWD strains which may influence host range, pathogenicity, and the zoonotic potential of CWD [241–243]. Currently, knowledge about the natural distribution and prevalence of CWD strains in free-ranging cervids is very limited. Interactions of the CWD agent with the environment particularly soil may alter CWD strain properties or exert selective pressure on different strains, further complicating the interpretation of CWD transmission studies [219]. Although the negative transmission studies provide reassurance indicating the existence of a substantial species barrier protecting humans from CWD transmission, the animal model studies should be interpreted with caution. Epidemiologic surveillance is ongoing to monitor the zoonotic transmission of CWD to individuals who hunted in areas where the disease has been endemic for decades. Although these studies have not detected any evidence of CWD transmission to humans, long-term follow up is necessary because of the long incubation period associated with any potential zoonotic transmission.

6 Prion Diseases of Humans Associated with Genetic Mutations

One of the intriguing properties of prion diseases in humans is the fact that they can be both infectious and heritable. The inherited or genetic forms of prion diseases are associated with insertion, deletion, or point mutations of the open reading frame of the prion protein gene [113, 244]. At least 24 different point mutations of the prion protein gene have been described in association with human prion diseases [113, 245]. These genetic prion diseases have widely varying clinical and neuropathologic manifestations and account for 10–15% of prion diseases in humans. Historically, genetic forms of prion diseases, in part based on their phenotypical expression, are classified as familial CJD, GSS, and FFI. They mostly follow an autosomal dominant inheritance pattern and have high penetrance. Beginning in 1989, many types of insertion mutations associated with markedly heterogeneous phenotypes have been reported in familial clusters.

6.1 Familial CJD

Patients with familial CJD generally have clinicopathologic phenotype similar to sporadic CJD. The disease has a

dominant inheritance pattern, and over half of affected family members carrying the mutation eventually die of CJD [2]. Familial CJD has been reported among family clusters in many countries, including from Canada, Europe, Japan, Israel, the United States, and several Latin American countries [2, 113, 244, 246–250]. The largest familial cluster was reported among Jews of Libyan and Tunisian origin, in rural Chile, and in Slovakia. It is most frequently associated with a mutation substituting glutamic acid with lysine at codon 200 of the prion protein gene. Arguably, familial CJD associated with codon 200 mutation is the most common heritable form of prion disease in humans. Perhaps the next relatively common form of familial CJD is associated with a mutation at codon 178 substituting aspartic acid with asparagine. This mutation has been reported in families originating from England, Finland, France, Hungary, and the Netherlands [245, 251]. A pedigree analysis of the original Finish family indicated that codon 178 mutation could have a disease penetration rate of 100 % [252]. Familial CJD with codon 178 mutation occurs when the mutant allele coding for asparagine at codon 178 also codes for valine at codon 129 [2, 253]. Compared with sporadic CJD patients, familial CJD patients with codon 178 mutation tend to have illness onset at an earlier age (mean, 46 years). About 12 other less frequent mutations with phenotypical expressions resembling familial CJD have been reported from many countries.

6.2 Gerstmann-Sträussler-Scheinker Syndrome

GSS bears the names of the three physicians who first described the disease in 1936 [56]. The original Austrian family spanning many generations reported by these physicians was later shown to carry the codon 102 mutation, which we now know is the most common form of GSS. Since its first report, the term GSS is used to describe a heterogeneous group of inherited human prion diseases that are characterized by a long duration of illness (median: ~5 years but sometimes exceeding 20 years) and the presence of numerous PrP-amyloid plaques, primarily in the cerebellum.

At least 13 different types of prion protein gene mutations or a combination of mutations in at least 56 kindred or families have been reported in association with the GSS phenotype. Familial clusters with the GSS phenotype have been reported from Canada, Europe, Japan, Israel, Mexico, and the United States [2, 245]. Many of the GSS mutations are associated with a greater degree of variability in the disease phenotype than other inherited forms of prion diseases. The most frequent GSS mutation results in a substitution of leucine for proline at codon 102 of the prion protein gene and is coupled with methionine at the polymorphic codon 129 of the mutant allele [254]. Patients with this mutation commonly manifest with cerebellar dysfunction, including ataxia and dysarthria, movement disorders, and possibly dementia and akinetic mutism. In some patients with the GSS 102 mutation, the illness can last for up to 6 years.

GSS with codon 105 mutations substituting proline for leucine or serine has been reported in several Japanese families. Patients predominantly have spastic paraparesis associated with cerebellar dysfunction and dementia and a neuropathologic picture of numerous amyloid plaques with neurofibrillary tangles and absence of spongiform changes. A large US family of German descent followed up for decades with an illness resembling GSS has been shown to have a mutation at codon 117 substituting alanine for valine [255]. Patients in this family cluster predominantly presented with dementia and pyramidal and extrapyramidal signs with minimal signs of cerebellar dysfunction. Arguably the largest and perhaps most studied family cluster with GSS was identified in the US state of Indiana with over 70 affected family members identified over 6 generations, and a total of over 1,000 family members involved in the investigation [256–263]. Many patients in this cluster had a mutation at codon 198 substituting phenylalanine for serine. At least six additional mutations of the prion protein gene have been reported in patients manifesting with a phenotype resembling GSS.

6.3 Fatal Familial Insomnia

The name FFI was first used in 1986 to describe a patient who predominantly presented with insomnia and autonomic dysfunction and had a history of other family members in several generations who had died of a similar illness [264–266]. Neuropathologic studies in FFI predominantly show marked involvement of the thalamus, resulting in a clinical phenotype characterized often by intractable insomnia and autonomic nervous system dysfunction, including abnormalities in temperature regulation, increased heart rate, hypertension, and sexual and urinary dysfunction [266–272]. The neuropathologic lesions are more severe in the thalamus than other regions of the brain. FFI is primarily associated with a mutation at codon 178 of the prion protein gene resulting in a substitution of aspartic acid with asparagine in combination with methionine at the polymorphic codon 129 of the mutant allele. The mutation seems to follow an autosomal dominant inheritance pattern. FFI has been identified in Australia, Canada, Japan, the United States, and several European countries. Recently, patients with no prion protein gene mutations but having clinical and pathologic manifestations indistinguishable from that seen in FFI patients have been reported. These seemingly sporadic cases with no family history of a similar disease are now recognized as sporadic fatal insomnia (sFI) and are classified as one of the subtypes of sporadic CJD [273, 274].

6.4 Codon 129 Polymorphism

The prion protein gene in humans exhibits a polymorphism at codon 129 which codes either for methionine or valine. Approximately 40 % of predominantly Caucasian populations are homozygous for methionine, 50% heterozygous with methionine and valine, and 10 % homozygous for valine. Methionine and valine homozygosity seem to be overrepresented among sporadic CJD (84 %) and hGH-associated CJD (67 %) patients, indicating that it may influence disease susceptibility in some individuals [121]. To date, all variant CJD patients who have been tested are homozygous for methionine at codon 129 [180, 181]. Heterogeneity at codon 129 seems to be protective against CJD, but when disease occurs the incubation period is usually prolonged in some iatrogenic CJD patients. The polymorphism also markedly influences the clinicopathologic phenotype of sporadic CJD and several inherited prion diseases. The most striking example of this influence is the phenotype associated with codon 178 mutation that substitutes aspartic acid with asparagine. Patients who have this mutation in combination with methionine on the mutant allele at codon 129 present with the FFI phenotype, whereas patients who have valine at codon 129 of the mutant allele present with the familial CJD phenotype [253]. The codon 129 polymorphism may also influence the age at onset and duration of illness in some prion diseases.

7 Scrapie

Scrapie was first reported in the 1730s in England but has been identified in many countries since then. Although it was recognized as a distinct clinical entity of sheep over 250 years ago, many aspects of the disease including its natural origin in flocks and the precise means by which it usually spreads remain uncertain. Experimentally, the disease was first transmitted by intraocular inoculation of scrapie brain extracts.

Scrapie transmission may occur by different postulated mechanisms. A commonly cited source of transmission, for example, is the placenta and amniotic fluid of scrapie-infected ewes. These tissues are known to harbor the infectious agent and can cause scrapie when fed to other sheep. They may contaminate pastures and barns that, in turn, may remain potentially infectious for years. Another possible source of spread is feces because prion replication occurs in gut lymphoid tissues after oral inoculation in sheep and goats. The importance of oral transmission is supported by experimental studies that detected prions in sheep tonsils examined early during the incubation period. Other poorly defined scrapie transmission mechanisms include: (a) the vertical transfer of the scrapie agent and (b) the possible chance occurrence of scrapie caused by hypothesized rare, spontaneous changes in the animal's cellular prion protein.

Scrapie occurs endemically in many countries, including in Europe and North America. Australia and New Zealand have sizable sheep populations but are generally recognized as free of the disease. To protect their "scrapie-free" status, these countries have established extensive safeguards to prevent the introduction of scrapie into their herds from imported animals.

The breed of sheep and polymorphisms of the prion protein gene can greatly influence susceptibility to scrapie [275–277]. Experimental transmissions with scrapie-infected tissues, for example, have confirmed the differing susceptibility to scrapie of different breeds of sheep. Other studies of Suffolk sheep in the United States indicated that susceptibility to scrapie was highly correlated with a polymorphism in the prion protein gene at codon 171 (glycine or arginine); the presence of arginine confers resistance to the disease [278, 279].

7.1 Atypical Scrapie

A new prion disease of sheep was identified in Norway in 1998 (Nor98) and has since been identified in several countries worldwide [280–287]. This new form, commonly referred to as atypical scrapie, differs from the classic form of scrapie in several important ways. The electrophoretic migration of Nor98/atypical scrapie PrP^{Sc} is characterized by lower molecular weight PrP^{Sc} species than that of the classic form [288, 289]. The distribution of PrP^{Sc} in sheep infected with Nor98/atypical scrapie is largely localized to the CNS in contrast to classic forms of scrapie that have a more widespread distribution. Interestingly, Nor98/atypical scrapie is mainly found in sheep with prion protein genotypes that correspond with relative resistance to the classic strains of scrapie [290–293]. Atypical scrapie occurs in older animals compared to classic forms of scrapie, and the incidence of disease is similar in infected flocks compared to the general population, suggesting that animal-to-animal transmission is not a prominent feature of Nor98/atypical scrapie [281, 294–296].

The etiology of Nor98/atypical scrapie is unclear. The predominance of PrP^{Sc} distribution in the CNS, the occurrence of disease in older animals, and the lack of clear evidence of animal-to-animal transmission have led to the hypothesis that Nor98/atypical scrapie may be a spontaneous prion disease with an etiology similar to sporadic CJD [281]. In addition, transmission of Nor98/atypical scrapie to transgenic mice expressing ovine prion protein produces a disease phenotype that is distinct from BSE-infected mice suggesting that Nor98/atypical scrapie is not due to transmission of BSE to sheep [285, 297, 298]. However, recent data indicate that Nor98/atypical scrapie is experimentally transmissible via oral inoculation and that extraneural tissues that are PrP^{Sc} negative as assessed by immunodetection techniques contain significant amounts of prion infectivity when tested using animal bioassays [299]. The implications of these findings are twofold. First, the conditions exist for transmission of Nor98/atypical

scrapie between individual animals (peripheral distribution of agent and oral susceptibility) suggesting that a spontaneous etiology is not the only mechanism of disease occurrence. Second, disease surveillance is based on immunodetection of PrP^{Sc}; therefore, the incidence of disease may be underreported. Clearly much work is needed to resolve the etiology and transmission history of this newly emerging prion disease of sheep.

8 Diagnostic Tests for Prion Diseases

In general, infecting prions can be detected in high concentrations in central nervous system tissues, particularly the brain. Their presence outside of the brain in peripheral tissues varies by the host species and infecting prion strains. In many prion diseases, confirmatory diagnosis requires testing of brain tissues by one of the widely used diagnostic methods, including histopathology using H&E staining, immunohistochemistry after labeling with antibodies directed to certain epitopes of the prion molecule, Western blot techniques, or rapid diagnostic tests developed for BSE screening such as prionics and biorad. Most of these diagnostic methods require protease digestion of biological samples to degrade native PrP^{C} that could interfere with the test results. They rely on the resistance of PrP^{Sc} to protease digestion to detect its presence. In contrast, confirmation-dependent immunoassay (CDI), a test developed by researchers at University of California, San Francisco, does not rely on the protease-resistant nature of PrP^{Sc} to be used as a diagnostic tool [300, 301]. The high-affinity antibodies used in CDI recognize confirmation-dependent epitopes of PrP^{Sc} to distinguish it from that of PrP^{C}.

8.1 Prion Amplification in In Vitro Systems

Current prion diagnostic tests, such as immunohistochemistry and Western blot, mainly rely on the immunodetection of endogenous PrP^{Sc} that is present in the host. They are very specific in the diagnosis of prion diseases but have limited sensitivity of detection of low amounts of PrP^{Sc}. The ability to detect low amounts of PrP^{Sc} would allow for an earlier diagnosis of prion diseases and potentially from easily accessible tissues (e.g., blood) that may harbor low levels of PrP^{Sc} compared to the brain. Recent promising studies have developed in vitro means of prion conversion that allow for the amplification of low levels of PrP^{Sc} to an abundance that is in the range of detection of current immunodetection technologies. In these in vitro systems, the natural properties of prions to recruit and convert PrP^{C} are exploited by mimicking the in vivo prion replication process to amplify and detect even minute amounts of prions in biological samples [302]. Byron Caughey and colleagues first demonstrated in a cell-free system that PrP^{C} could be converted to a PK-resistant form in the presence of PrP^{Sc} [303, 304]. This cell-free conversion assay was useful for understanding the nature of the prion agent, prion strain properties, and the species barrier effect [303]. Cell-free conversion reactions were not very efficient; therefore, such assays had limited ability to be used as diagnostic tools for prion diseases. Claudio Soto and colleagues subsequently developed a system they termed protein misfolding cyclic amplification (PMCA) to increase the efficiency of in vitro conversion assays [305–308]. PMCA utilizes incubation of uninfected brain homogenate that contains PrP^{C} with a biological sample that contains PrP^{Sc} resulting in the conversion of PrP^{C} to PrP^{Sc}. The PrP^{Sc} acts like a seed and elongates into an oligomer by attracting PrP^{C} molecules and incorporating them into the growing oligomer. The rate of conversion is limited by the availability of seeds actively recruiting PrP^{C}. To accelerate the process, a sonication step was introduced to fragment the growing oligomer into smaller units that would serve as multiple seeds to recruit even more PrP^{C}, thereby amplifying the conversion reactions. Serial rounds of incubation and sonication result in a highly efficient in vitro conversion process [307]. PMCA has been shown to amplify PrP^{Sc} from samples containing a single infectious dose to levels that are easily detectible by Western blot analysis. Real-time quacking-induced conversion (RTQuIC) is a modification of PMCA methodology and utilizes recombinant PrP^{C} that has single PrP^{Sc} molecule sensitivity and detects PrP^{Sc} using thioflavin T fluorescence resulting in real-time detection of PrP^{Sc} formation allowing for a more rapid assay [303, 304, 309, 85]. Other promising in vitro technologies include the amyloid seeding assay and the surround optical fiber immunoassay. As with all other highly sensitive diagnostic tests (e.g., PCR), care must be taken to minimize contamination, and in an aspect that is unique to prion diseases, the spontaneous conversion of PrP^{C} to PrP^{Sc} must be controlled for. With these controls in place, PMCA, RTQuIC, and other tests developed using similar methodologies have the potential to be highly sensitive and accurate prion diagnostic tests.

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