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Above: Three Women (1998) by Ezekiel Madiba, Mabopane, South Africa.
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Cover: "When spider webs unite, they can tie up a lion." Adapted from the Red Cross African American HIV/AIDS Program poster series with permission. Copyright April 1992 (revised March 1997).

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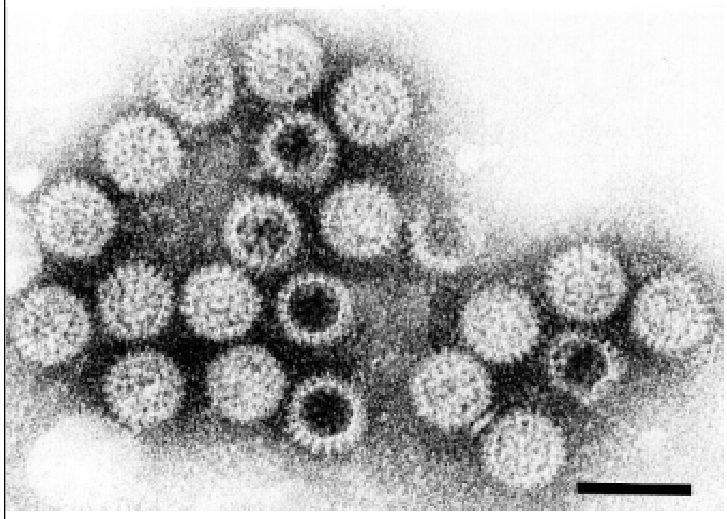

Rotavirus particles visualized by electron microscopy in stool filtrate from child with acute gastroenteritis. Photo courtesy of Dr. Charles D. Humphrey, CDC.

Emerging Infectious Diseases—South Africa

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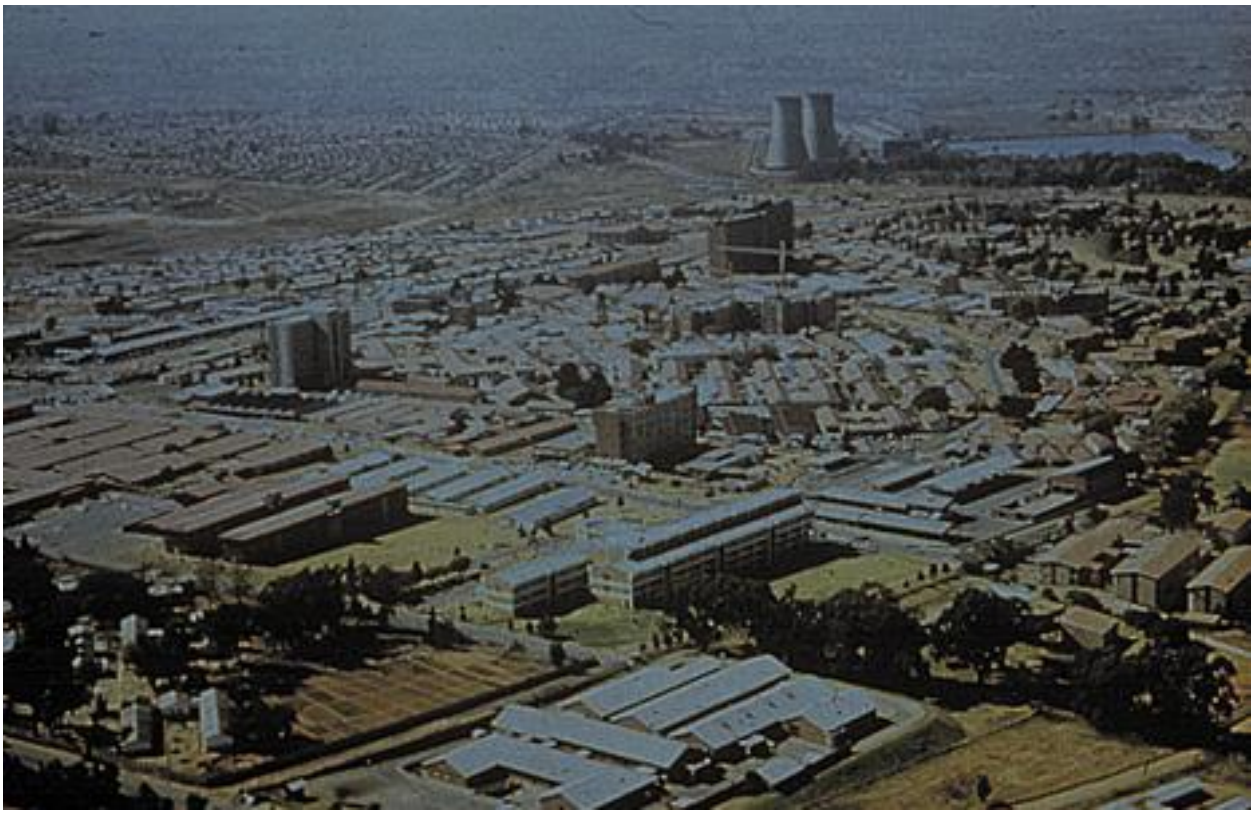
main a major cause of illness and death in South Africa. The medical infrastructure is good in major cities, primary health care is expanding in rural areas, and modern diagnostic facilities are reasonably widely available. Urban areas have a mixture of infectious disease problems encountered in developing as well as industrialized countries, and in the tropical environment of the northeast, malaria and other vector-borne diseases persist.



An aerial view of Chris Hani Baragwanath Hospital, with 3,000 inpatients the largest hospital in the world—Soweto, South Africa. Multiple-drug resistant pneumococci were found here in 1978.

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Emerging Infectious Disease Surveillance

National surveillance of emerging infections in South Africa is in its infancy. A number of infectious diseases are notifiable, but no formal laboratory-based surveillance system is in place to confirm diagnoses or to subtype microorganisms for epidemiology purposes. I have summarized infectious disease trends in South Africa, with special emphasis on the antimicrobial resistance patterns of locally circulating microorganisms.

Bacterial Diseases

Streptococcus pneumoniae

Fully resistant and multiple-drug resistant pneumococci were discovered in South Africa in 1978. The pattern of emergence of pneumococcal resistance in South Africa has been somewhat different from that observed in Western Europe (and more recently in the United States). In those countries, the emergence of resistance was explosive and associated with a limited number of clones, particularly of serotype 23F and 6B, which are associated with multidrug resistance and high-level penicillin resistance. The highly resistant strains initially isolated in South Africa belonged largely to serotype 19A and for some as yet unexplained reason, remain rare. Antimicrobial resistance to penicillin in the pneumococcus in South Africa has reached very high levels (approximately 45% in young children), but most of this resistance is caused by a large number of intermediately penicillin-resistant strains rather than a limited number of clones found internationally. Antibiotic resistance differs in the public versus private health-care sectors of South Africa. The most important difference may be the relative scarcity of macrolide resistance among strains of pneumococci in public hospitals, where these drugs are less often used. Recently, the incidence of pneumococcal disease in both adults and children has doubled as a result of the increased incidence of HIV infection. HIV infection is specifically associated with antibiotic resistance and with childhood serotypes of the pneumococcus (6A, 6B, 9V, 14, 19F, 19A, 23F).

Haemophilus influenzae

In contrast to the United States and Western Europe, South Africa has a low incidence of betalactamase-producing *H. influenzae* (±10%). The reason for the contrast between the low incidence of betalactamase-producing *H. influenzae* and the high incidence of penicillin resistance in the pneumococcus is not known.

Neisseria meningitidis

Over the past few years, the distribution of meningococcal serogroups has been fairly even between groups A, B, and C. An epidemic in Cape Town of group B in the 1980s provided an opportunity to attempt to use an outer membrane protein vaccine, but the epidemic ceased before enough cases could be enrolled in the vaccine trial. An epidemic of group A meningococcal meningitis is ongoing in the countries immediately to the north of South Africa, particularly in Mozambique, and an increasing number of group A strains are being identified, largely in adult refugees from Mozambique. These group A strains have been typed by the World Health Organization Meningococcal Reference Center in Oslo, Norway, and represent a unique South African clone. The epidemic clone associated with the Haj (III-1) in the 1980s was, however, discovered for the first time in South Africa in 1997.

Staphylococcus aureus

The incidence of methicillin-resistant *S. aureus* in South Africa is alarming, with up to 50% of nosocomial isolates being methicillin-resistant. No vancomycin-intermediate *S. aureus* strains as have been reported in the United States and Japan have been identified in South Africa to date. The incidence of methicillin resistance among S. *epidermidis* strains in hospitals is, as expected, even higher.

Enterococcus

The first strains of enterococci resistant to vancomycin (VRE) and teicoplanin, which were due to the presence of the *van* A resistance gene, were described in South Africa only in 1996. These strains were likely imported. The incidence of VRE in South Africa remains low but is expected to increase.

Diarrheal Pathogens

Endogenous transmission of cholera has not been observed in South Africa for the past decade. Epidemics of cholera have occurred in countries to the north, particularly in

Mozambique, and a number of imported cases have been identified each summer in South Africa. The potential for an epidemic in rural areas with poor sanitation and contaminated water exists. *Shigella dysenteriae* type I producing Shiga toxin and associated with hemolytic uremic syndrome was first identified in an epidemic in adults on the South African/Mozambican/Swaziland border in 1993. Subsequently, the same strain has become quite common among children and adults, particularly in KwaZulu-Natal province, and has spread down the east coast to the western Cape. Disease in adults as well as children suggests that this strain has not been endemic in South Africa, at least during the past generation. The strains are resistant to ampicillin and cotrimoxazole but are generally susceptible to nalidixic acid and the fluoroquinolones. Occasional imports of multidrug-resistant typhoid have been identified in KwaZulu-Natal, where there is a large Indian community; these strains likely come from Southeast Asia.

Nosocomial Infections

Extended spectrum betalactamases are endemic particularly among *Klebsiella* species in intensive-care units throughout the country. Gram-negative bacteria producing chromosomally mediated Beta-lactamase are common in all of these settings and *Acinetobacter*-related problems abound in most intensive-care units.

Tuberculosis (TB)

The incidence of TB is increasing in parallel with the HIV epidemic in South Africa. Multiple-drug resistance remains <5% and is commonly associated with noncompliance to drug regimens.

Plague

Low levels of endemic plague are present in rural South Africa, but the last epidemic was in Namibia in the 1980s.

Viral Diseases

HIV infection was not apparent in South Africa until around 1988 when endogenous cases began to emerge. The first reported cases occurred predominantly in homosexual men who had sexual contacts in the United States; in one early case, the patient presumably had heterosexual contact with a sex worker in Central Africa when the disease was first discovered there. The incidence of HIV infection among pregnant African women in South Africa in 1990 was 0.4%. The number of infected persons has doubled at approximately 9-month intervals until now when approximately 20% of adult African men and women are infected in the northern and eastern parts of the country. This number declines to less than 10% in the south, but HIV, although delayed with reference to the rest of Africa, has had and will have a staggering impact on emerging infectious diseases in South Africa. No specific diseases peculiar to South Africa have been found to be associated with HIV infection; major opportunistic infections include TB, cryptococcal disease, cytomegalovirus infections, bacterial diseases such as pneumococcal or salmonella bacteremia, and toxoplasmosis.

Viral Hemorrhagic Fevers

A case of Marburg fever was identified in the 1970s in a tourist visiting several African countries to the north of South Africa; nosocomial transmission to two persons was identified at that time. One case of nosocomial transmission of Ebola has been reported (the patient with the index case was flown down to South Africa from Gabon); however, no endemic transmission of Ebola virus occurs in South Africa. That the nosocomial transmission of Ebola virus was limited to a single person despite the fact that the diagnosis of the index case was not made until a number of weeks after the patient entered the country is a tribute to the standard of medical care in South Africa. Congo Crimean hemorrhagic fever is endemic in southern Africa, but as the vector tick rarely bites humans, not many human cases have been found. Epidemics have been associated with infections in rural communities particularly of farmers and workers who slaughtered infected cattle. An epidemic among ostriches occurred in 1997.

Parasitic Diseases

Malaria remains a serious systemic disease in South Africa. The disease is endemic in the northern and eastern parts of the country where the climate is tropical. After many years of spraying huts with DDT, malaria became rare, but the interruption of the program because of war in the 1970s and 1980s in Mozambique and Zimbabwe (and the development of drug resistance in other insects such as bedbugs,

which reduced the acceptability of the program) has led to a rapid resurgence of malaria. Chloroquine-resistant strains, which were rare in the 1980s, are now common, although quinine-resistant strains have not yet been demonstrated. *Schistosoma haematobium* and *S. mansoni* are endemic in rivers along the eastern part of the country and pose a health risk to nonimmune visitors.

Fungi

Cryptococcus neoformans var gattii and var neoformans represent an increasing fraction of cerebrospinal fluid isolates in South Africa because of the HIV epidemic.

Further Reading

Additional information on emerging infectious diseases in South Africa is available from the South African Virus Laboratories Surveillance Bulletin, which is published by the National Institute for Virology (Private Bag X4, Sandringham, 2131 Johannesburg, South Africa); data can be obtained from the South African Institute for Medical Research Annual Reports (P.O. Box 1038, Johannesburg 2000, South Africa); national antimicrobial susceptibility data are published in the South African Medical Journal by the Antibiotic Study Group of South Africa; and clinically based notifiable diseases are reported to the Department of Health, which produces a free journal called Epidemiological Comments (The Director General, Department of Health, Private Bag X828, Pretoria, 0001, South Africa.) The final source of information on emerging infectious diseases in South Africa, aside from the specific reports that would appear in any international journal is the Proceedings of the Annual Meeting of the Infectious Diseases Society of Southern Africa (membership and meeting information, Dr. Lucille Blumberg, e-mail: lucilleb@mail.saimr.wits.ac.za).

Acknowledgment

I thank Professor H. J. Koornhof for his review and comments on this report.

Quasispecies Structure and Persistence of RNA Viruses

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Viral quasispecies are closely related (but nonidentical) mutant and recombinant viral genomes subjected to continuous genetic variation, competition, and selection. Quasispecies structure and dynamics of replicating RNA enable virus populations to persist in their hosts and cause disease. We review mechanisms of viral persistence in cells, organisms, and populations of organisms and suggest that the critical interplay between host and viral influences (including in some cases the quasispecies organization) is the main driving force for long-term survival of viruses in nature.

The emergence and reemergence of infectious diseases is influenced by the genetics of the infectious agents, the genetics of their hosts and potential new host species, and a considerable number of environmental factors (1-3). The current view proposes a strong stochastic (chance) component regarding the time, place, severity, and epidemiologic impact of infectious disease emergences (2,3). For RNA and possibly some DNA viruses, increasing evidence suggests that genetic variation (mutation, recombination, and genome segment reassortment in the case of multipartite genomes) affects adaptability to environmental changes (4-9). Since some types of adaptation involve changes in host cell specificity (5,6,10), genetic variation of viruses may be involved in the emergence of pathogenic viruses from apathogenic ancestors. Many studies over the last 2 decades have documented the unpredictability of genetic variation in viruses (2,5-7).

In this report, we consider viral persistence in connection with the population structure of RNA viruses, specifically, the extension (in space and time) of the pool of replicating genomes, which are a potential source of variant viruses with altered biologic features. For example, hantaviruses are apathogenic and endogenous to several rodent species. In several geographic areas of the American continent, the unusually mild and wet (El Niño effect) spring seasons of 1992 and 1993 resulted in abundant food and coverage for deer mice, increased numbers of infected deer mice, and increased risk for human infections. These factors led to the newly recognized severe pulmonary syndrome of humans in 1993 (11). Like many pathogenic RNA viruses, hantavirus evolved in humans, and recent epidemiologic evidence suggests human-to-human transmission (12); whether human-to-human transmission is exceedingly rare or is an unusual property associated with Andes virus is not known. The number of carriers, their ability to transport virus (because of their mobility and absence of severe symptoms), and the viral load (number and concentration of infectious particles) in each carrier must be involved in viral emergences and reemergences (2-4,6,13).

Population Complexity of Replicating Genomes: Viral Quasispecies

The viral load in an infected host is both static and dynamic. The static component can be divided into the total number of particles and the numbers and types of mutant viral genomes present in that total. Rather than being homogeneous, RNA virus populations consist of complex distributions of mutant (and sometimes also recombinant) genomes, in a type of population structure known as quasispecies (6,14-16). Assuming a random distribution of

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mutations among genomes, the number of variant genomes in a viral quasispecies increases dramatically with population size (15,17). For example, in a genome distribution with an average of five mutations per genome, the expected number of genomes with 20 mutations is 26 when the population size is $1x10^8$ viral particles but reaches 2,600 when the population size is $2x10^{11}$ particles, as calculated from the Poisson distribution (17). Fitness variations of the individual mutants modify the actual number of genomes in each mutation class (6,14-17). In a horse infected with epizootic Venezuelan equine encephalitis, viral titers in blood reach a high of 10^8 infectious units (i.u.)/ml, or a total of approximately $3x10^{12}$ i.u. These high titers are probably needed for efficient transmission of the virus to insect vectors and for completion of the arbovirus life cycle (18). High titers are also reached in acute infections with HIV-1, hepatitis B virus (HBV, a hepadnavirus replicated by an error-prone polymerase through an RNA intermediate), hepatitis C virus (HCV), influenza virus, the animal foot-and-mouth disease virus (FMDV), and probably many others (7,8).

Increasing evidence indicates that quasispecies evolution may lead to the selection of virulent viruses and to the emergence of new viral pathogens (1-7,10,17,18). For example, a single site at the 5'-untranslated genomic region of coxsackievirus B3 was associated with its cardiovirulent phenotype. Swine vesicular disease, not reported before 1966, may represent a human coxsackie B5 variant adapted to swine. Specific mutations in the RNA of lymphocytic choriomeningitis virus can confer a viral tropism for neurons or for cells of the immune system. Evolution of measles virus can lead to hypermutated forms that have been associated with subacute sclerosing panencephalitis. Major human influenza pandemics have been associated with antigenic shift resulting from genome reassortment between a human influenza virus and an animal influenza virus. Some small DNA viruses possess considerable genetic heterogeneity within infected hosts. Canine parvovirus was probably derived from a feline parvovirus as a result of two amino acid replacements in the viral capsid (1-7,10,17,18).

The viral load during RNA virus replication also has a strong dynamic component. In persons infected with HIV-1, HBV, or HCV, an estimated 10^{10} to 10^{12} new virions are produced each day. For some microorganisms (not only viruses), in what has been termed short-sighted evolution of pathogenic microorganisms (19), virulence may be an inadvertent consequence of mutation and selection in the parasite population. Models of HIV-1 pathogenesis based on the continuous production of antigenic variants have been proposed (8,19,20). As the infection progresses the complexity of antigenically distinct mutants may overwhelm the immune system, leading to AIDS (20). Models of HIV-1 pathogenesis, based on the stimulation of infected T-lymphocytes by secondary antigens (from opportunistic infections), have also been proposed; the progression of HIV infection to AIDS is still poorly understood. In HIV, and many other infections, the evolving viral quasispecies are exploring new mutant variants at astonishingly high rates. The balance between mutation rates and replication rounds is one of the reasons for the great adaptability of RNA viruses (6,7,14-17).

Types of Viral Persistence

"Persistence," which refers to long-term survival of viruses in their hosts, has been described in at least three ways. 1) Long-term survival of virus within a viable cell population occurs when cell disease and destruction are limited and viral genomes replicate in balance with the multiplication of the host cells. 2) Survival of viruses in entire organisms can rarely be reduced to persistence in one cell type since organisms are built as sets of interconnected mosaics of cell types and cell protein effectors; persistence of viruses in organisms often means coping with multitudes of selective forces and defense reactions while allowing the host to survive. 3) A virus can be maintained in nature by the continuous infection of susceptible host organisms, with or without persistence in cells or organisms and with or without long-term stability of viruses as free particles. Genetic, ecologic, and environmental factors exert different influences on these types of persistence. The quasispecies structure of RNA viruses plays an obvious, positive role in some persistence mechanisms. In others the role is more subtle, marginal, or nonexistent. The observations summarized here suggest, however, that persistence is always the result of interactions between viral and host determinants.

Persistence of Viruses in Cells

A variety of mechanisms enable viral genomes to replicate in balance with host cell multiplication. Most of the well-studied mechanisms of persistence of RNA viruses in primary cell cultures or established cell lines involve genetic variation of the virus, the cell, or both. One way to limit cell death is by generating and accumulating defective genomes. Defective genomes depend on a complementing, standard virus for replication; yet they may compete with that standard virus for cellular and viral gene products (which the virus needs to complete its life cycle and kill cells), thus increasing cell survival (21). A classic example is provided by defective interfering (DI) particles of vesicular stomatitis virus (VSV), and related viruses (22). During serial passage of VSV at high multiplicity of infection, DI particles accumulate in a cyclic pattern (23). Standard VSV and DI particles alternate in their dominance in a continuous process of mutant generation, competition, and selection. Excess virus leads to generation and accumulation of DI particles. With excess DI particles, mutant viruses able to escape interference rise to dominance (5). A feedback mechanism is established, which underlines the biologic relevance of the rapid evolutionary potential of RNA genomes (5).

Cell disease can also be limited by infecting cells with limited permissivity for the virus or by selecting noncytolytic variant viruses. In a number of cell-virus systems, cells and viruses coevolve, as documented for reovirus persisting in L cells (24) and for other RNA and DNA viruses (25-28). FMDV illustrates how the quasispecies structure initiates and maintains persistence (26,29). The presence in the FMDV quasispecies of variants with decreased ability to kill BHK-21 cells was not the mechanism (at least the prevailing mechanism) responsible for persistence. Rather, these experiments (29), which measured the proportion of cells that survived an initial cytolytic infection, indicated that a rapid variation of the cells initiated persistence. Indeed, the cells rapidly became more resistant to the infecting FMDV, and the virus became more virulent for the host BHK-21 cells (29). The quasispecies structure and consequent adaptability of RNA genomes do not justify any generalization on the participation of quasispecies, rather than the seemingly more static cellular DNA genomes, in initiation of viral persistence (29). However, the rapid evolution of FMDV toward virulence was very likely facilitated by mutant generation and was essential to sustain persistence (26,29). When the carrier cultures were challenged with FMDVs of distinct degrees of virulence (a population replacement experiment), the endogenous persistent virus was replaced by the externally added virus only when the latter displayed a higher virulence for BHK-21 cells. Thus, virulence can be a positive trait in viral persistence (30), and virulent variants present in the FMDV quasispecies helped maintain persistence when the triggering cellular event had occurred (29,30).

Persistence of Viruses in Organisms

Viral persistence in organisms requires a supply of susceptible cells replicating at the same pace as the virus and the ability to survive the host immune response. With RNA viruses, the continuous production of mutant viruses (inherent to the quasispecies dynamics [14,15]) contributes to virus survival (2,5-7,15-17). Viruses often use alternative receptors and coreceptors, and one or a few amino acid substitutions at exposed surface sites may trigger a shift in receptor specificity (8,10,31). For HIV-1, amino acid substitutions at the surface glycoprotein may effect shifts in receptor use (10,31), and mutations at several genes may promote escape from antibodies or cytotoxic T lymphocytes (CTLs) (8,10); the generation of 10^9 to 10^{10} viral particles per day undoubtedly facilitates escape (8,20). Although evidence of positive (Darwinian) selection of escape mutants is firm, the quasispecies structure and dynamics predict genetic variation in the absence of immune selection (5,32). The two mechanisms are compatible.

Some viruses (HBV, HIV-1, FMDV, measles virus, herpesviruses) may persist after an acute infection, and the dose of infecting virus often determines either clearance or long-term persistence (8,33). Viruses transmitted vertically may induce immune tolerance and persist in adults (33). Viruses may also persist by being sequestered in some privileged sites of an organism, such as the central nervous system, partially hidden from immune attack (33). Ineffective antibody responses may be due to

tolerance, immunosuppression (as a result of some infection, genetic disease, or immunosuppressive treatments), production of nonneutralizing antibodies, or cell-to-cell spread of virus not exposed to immune recognition (33). Viruses that infect lymphocytes or macrophages (HIV, cytomegalovirus, measles virus) may alter immune responses and thus facilitate their own persistence (8,33).

Persistence of Viruses in Nature

All viruses have developed common functional and adaptive strategies; however, the strategies used by DNA and RNA viruses to evade host defenses have distinct features. RNA viruses often exploit mutation to achieve changes in host range and escape antibody and CTL responses (8,10,31,32). Because of their limited genetic complexity (which can be equated with the size of their genomes [of 3 to 30 Kb]), RNA viruses are generally tolerant to high levels of mutagenesis (6,14,16). In contrast, large DNA viruses (of the herpesvirus family, poxviruses, iridoviruses, adenoviruses) have complex genetic information; the need to maintain this information limits their tolerance to mutation. That simple genomes are generally more tolerant to mutagenesis than more complex ones can be argued on the basis of the higher mutation rates observed in simple replicons and the evolution of replication to include proofreading and postreplicative repair functions for the replication of DNA of cells and of at least some of the complex DNA viruses (5,6,14,16). Although antibody and CTL-escape mutants (as well as drug-resistant mutants) have also been described for DNA viruses, the latter have evolved alternative mechanisms to counteract host defenses (34,35). As examples, the adenovirus proteins E3/19K and E1a suppress surface molecules (MHC class I, class II, adhesion molecules) required for T-cell recognition. The Epstein-Barr virus BCRF1 protein is a host interleukin (IL)-10 homologue that activates the IL-10 receptor. Human cytomegalovirus encodes a protein structurally resembling the macrophage inflammatory protein 1α/RANTES receptor. Cytokines regulate immune and inflammatory responses and may trigger antiviral responses in organisms. It is not surprising that DNA viruses causing either persistent or acute infections have evolved to encode homologues of the extracellular binding domains of cytokine receptors (34,35).

Phylogenetic analyses of herpesvirus genomes infecting a broad range of host animal species (the complete genomic nucleotide sequence of 18 herpesvirus genomes is known) suggest a possible cospeciation with their host organisms (36). The capture of cellular genes (and gene assemblies) by DNA viruses to counteract host defense responses agrees with the proposal of a modular origin of viruses (37,38) and has opened a new approach for analyzing new functions related to the immune response in differentiated organisms (35). The selective forces imposed by viral parasites may have contributed to a more rapid diversification of cellular proteins involved in host defense (34). In turn, coevolution may have relaxed the specificity of viral analogues of cellular effectors: the viral chemokines vMIP I and vMIP II of herpes simplex virus-8 bind to a broader range of cellular chemokine receptors (although with lower affinity) than their cellular homologues (39). Genes that have strong sequence identity with cellular counterparts are also encoded by RNA viruses (e.g., sarcoma and leukemia viruses) (34) that tend to exchange genetic material with their hosts. However, evasion strategies based on gene capture and protein mimicry are dominant in DNA viruses, and strategies based on mutant production are widespread among RNA viruses (6-8,16,17,34,35).

Persistence of Viruses at the Population Level

To be maintained in nature and avoid extinction, viruses must have susceptible hosts as well as adaptability to a range of biologic environments (6). Even persistence in an individual host would not help long-term persistence of a viral pathogen in nature, without a number of additional influences (13,19,40), such as the possibility of transmission within the same host species (sexual, perenteral, or respiratory routes) or to a different host species (13,40). Human rhinoviruses do not persist in their hosts and succeed (supported by the high frequency of common colds) in continuous reinfections through aerosols transmission (or other contacts). In the other extreme of life cycle complexity, arboviruses sequentially infect a number of disparate hosts. As an example, insects can transmit Venezuelan

equine encephalitis virus between horses and other mammalian species and can also infect humans, the dead-end point of a complex infectious cycle (18). Evidence indicates that a few point mutations in the viral genome may be sufficient to upset the balance of viral loads in enzootic cycles, render the virus epizootic, and cause severe outbreaks (18). In La Crosse virus, an important cause of pediatric encephalitis, the virus persists in cells of the midgut epithelium of *Aedes triseriatus*. The virus is transovarially transmitted and survives transeasonally in the diapausing mosquito embryo. In quiescent ovaries there is reduced viral replication with limitations in the host-derived 5'-mRNA sequences that prime viral transcription (41). Long-term survival at the population level is associated with persistence and limitation of virulence in the vector mosquito. The complex arboviral life cycle appears to require the fine tuning of a number of factors: the amount of virus in viremia and the duration of viremia, which are likely to contribute to the efficient uptake of virus by the vectors (18), and replication and stability of the virus in the vectors to ensure infection of the mammalian host (18). Perturbations in these, and probably other factors, could lead to viral extinction. What prevents viral extinction? Again, all evidence points to genetic factors of the virus and the hosts together with environmental and ecologic influences (1,3,5-7,17,18). Virus variants unable to fulfill the required processes with the correct timing may be generated, but they would be selected against. We can study only successful examples. Virus variants that do not complete a complex life cycle are yet another example of negative selection (elimination of suboptimal viruses). Negative selection is one of the forces preserving virus variants that are fit in relation to the interactions with their hosts (6,7,14-17,32), a force we believe is responsible for maintaining (at least to some extent) the identity of RNA viruses as disease-causing agents.

The stability of virus particles may also play a relevant role in successful transmission, as documented in aerosol transmission, airborne spread, or mechanical transport of viruses by insects (42). An example is provided by FMDV. In spite of its lability at mildly acidic pH and at moderate temperatures, FMDV is resistant to desiccation and can be transported on dust particles over long distances. Most spectacular is the case of the highly complex and host-specific baculoviruses. They form rod-shaped virions occluded in large capsules made of a viral-coded matrix protein termed polyhedrin. Capsules are uptaken by the insects (of the orders *Lepidoptera* and *Hymenoptera*) and are dissolved by midgut epithelial cells. Thus, capsule formation is responsible for the spread of the virus in the insect population. Again, a great diversity of mechanisms (as varied as those seen at the level of individual organisms or of the cells) operate to ensure viral persistence at the population level.

Basic Issues

The mutant distributions that compose viral quasispecies are the raw material on which selective forces and random sampling events act in the molecular evolution of RNA viruses (5-7,14-18,32,38). In addition to constituting a basic adaptive strategy, the quasispecies genetic organization has a number of biologic implications (5-9,16,32,43), some of which have a direct bearing on viral persistence. It has been wrongly argued that if quasispecies distributions were involved in virus persistence, all RNA viruses would establish persistent infections but that on the contrary, only a minority do. We hope to have shown that persistent infections are unavoidably, necessarily, and evidently the result of an interplay between viruses and their hosts (44). Thus, a quasispecies structure does not imply necessarily that the virus will produce a persistent infection. A potent CTL response may clear a virus infection provided that the viral load has a size amenable to clearing, and this may occur whether the virus is a complex quasispecies or not. In contrast, a similar CTL response confronting a high viral load may frequently fail to clear the infection; in this failure, the presence (in a dynamic quasispecies) of CTL-escape mutants (and many other types of mutants with biologically deviant properties) may be crucial for virus survival, including the establishment of persistence or chronicity. Also, increasing evidence suggests that viruses thought not to persist, such as poliovirus, may actually do so; late postpoliomyelitis syndromes may be one consequence (33,42). Even with the available analytical technology, total clearing of an infecting virus from an organism cannot be guaranteed.

The issue is clear: either we design new antiviral strategies that take into consideration the quasispecies structure of RNA viruses (6,9,43), or viral diseases (classical, new, or reemergent) will remain difficult to control.

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Ecologic Studies of Rodent Reservoirs: Their Relevance for Human Health

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Within the past few years, the number of "new" human diseases associated with small-mammal reservoirs has increased dramatically, stimulating renewed interest in reservoir ecology research. A consistent, integrative approach to such research allows direct comparisons between studies, contributes to the efficient use of resources and data, and increases investigator safety. We outline steps directed toward understanding vertebrate host ecology as it relates to human disease and illustrate the relevance of each step by using examples from studies of hosts associated with rodent-borne hemorrhagic fever viruses.

The practical importance of understanding host and vector ecology has been recognized at least since the early 1900s. Knowledge of the container-breeding habits of *Aedes aegypti* enabled early successes in the control of yellow fever virus transmission and, ultimately, the completion of the Panama Canal in 1914 (1). Diverse applications of vector/reservoir ecology-based measures to prevent zoonotic disease include the prediction of Lyme disease risk by monitoring acorn mast production and its impact on the vertebrate hosts of the tick vectors (2), control of vector populations for *Borrelia burgdorferi* and *Yersinia pestis* through the application of acaricides and insecticides to rodents and deer at feeding stations (3,4), dissemination of bait containing vaccines to control rabies in foxes (5), and use of satellite imagery to predict the activity of Rift Valley fever in East Africa (6,7).

The rodent-borne hemorrhagic fevers, among the most dramatic of recently emerging infectious diseases, are caused by two distinct groups of negative-stranded RNA viruses: the arenaviruses (family *Arenaviridae)* and the hantaviruses (genus *Hantavirus*, family *Bunyaviridae*). With few exceptions, each virus in these two groups is primarily associated with a single species of rodent host of the family *Muridae*. In the specific host, the virus establishes a prolonged infection, which rarely causes disease in the animal. The infected host sheds virus into the environment (in urine, feces, and saliva) for extended periods (8-10). These characteristics are key to the transmission of the viruses to humans (by the inhalation of aerosolized virus) and to other rodents (by horizontal and sometimes vertical mechanisms).

Arenaviruses cause the South American hemorrhagic fevers, which produce hundreds of cases annually, with a case-fatality ratio as high as 33%. The best studied of these agents is Junín virus, which is carried by the corn mouse (*Calomys musculinus*) and causes Argentine hemorrhagic fever (AHF). AHF was first recognized in 1955 on the central pampas of Argentina (11), where before the deployment of a new vaccine in 1992, hundreds of cases occurred each year. Although arenaviral diseases of humans (other than lymphocytic choriomeningitis associated with the introduced Old World rodent *Mus musculus*) have not been recognized in North America, Tamiami virus has been recognized in association with cotton rats (*Sigmodon hispidus*) since 1969 (12), and Whitewater Arroyo virus was identified from wood rats (*Neotoma* species) in the southwestern United States in 1995 (13). The potential of Whitewater Arroyo virus for causing human disease is under investigation.

Hantaviruses cause hundreds of thousands of cases of hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia each year. Hantaviral disease was thought to be rare or

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absent in the United States, although three cases of mild HFRS associated with rat-borne Seoul virus had been described (14). In 1993, the discovery of hantavirus pulmonary syndrome (HPS), which rapidly kills approximately half of those infected, surprised public health officials and virologists in the United States. The causative agent, Sin Nombre virus (SNV), is hosted by the deer mouse, *Peromyscus maniculatus*, and the disease syndrome markedly differs from HFRS. Since 1993, at least 20 additional hantaviruses have been isolated from rodents throughout the Americas; about half are known human pathogens (Figure 1).

Recognition of new rodent-borne diseases has renewed interest in reservoir host ecology in the United States, and recognition of HPS in South America has prompted field studies in Paraguay, Argentina, Chile, and Brazil. In 1997, representatives from 13 American countries, meeting in Lima, identified surveillance in humans and rodents as a priority for combating emerging hantaviral disease. Reservoir studies are being designed and conducted in many parts of the world by mammalogists, ecologists, and vector-control personnel. However, sanitarians and others with no specific training in such areas are being asked to conduct reservoir studies in the course of investigating disease outbreaks. Rarely are these professionals trained in the safe handling of animals potentially infected with viruses capable of causing fatal disease.

Figure 1. Distribution of recognized, autochthonous, New World hantaviruses.
* = known human pathogens.

To promote discussion of study design, data collection, and appropriate and safe methods for reservoir studies, we review prior efforts and lessons learned. Although specific examples are restricted to the rodent-borne hemorrhagic fever viruses, many of the generalizations can be applied to other pathogens maintained by small-mammal reservoirs.

An Outline for Reservoir Studies

Assuming that the primary reservoir host has already been implicated, the following consecutive but overlapping steps can promote an understanding of rodent reservoir ecology as it relates to human disease: 1) determination of the geographic distribution of the host; 2) determination of the geographic range of the pathogen within the host range; 3) determination of the regional distribution of the host and pathogen among the distinct biomes or habitat types; 4) determination of the relative prevalence of infection among demographic subpopulations of the host (e.g., males versus females and adults versus juveniles); 5) elucidation of the temporal and fine-scale spatial patterns of host-pathogen dynamics through prospective, longitudinal studies; and 6) development of an integrative time- and place-specific predictive model.

Examples from studies of hantaviruses and arenaviruses illustrate how each step is relevant to human health.

Geographic Distribution of the Host

The geographic distribution of the host(s) defines the maximum area in which the disease can be endemic. For most small-mammal species in North America, these distributions are known relatively precisely (15). For example, the deer mouse is one of the best-studied small-mammal species in the world, and its distribution throughout North America is well defined (Figure 2). Fewer data are available on the distribution of Central and South American species. The range of the cotton rat, *S. hispidus*, which is the reservoir of both Black Creek Canal virus (a hantavirus that causes HPS) and Tamiami virus (an arenavirus not associated with human disease) is well defined in the United States. However, the southern limits of the species' distribution are unclear (16).

Problems in defining species' distributions are exacerbated by imprecise taxonomy. The multimammate rat, *Mastomys natalensis,* was

originally described as the reservoir for Lassa fever in West Africa. Subsequent taxonomic studies have shown that several morphologically similar animals in sub-Saharan Africa actually form a species complex (17). The phylogeny of this complex is being studied by cytologic and molecular techniques (18), but it will be years before the geographic distributions of each species can be mapped and used to interpret the restricted distribution of Lassa fever.

Andes virus, associated with *Oligoryzomys longicaudatus,* causes HPS in the northern Patagonian regions of Argentina and Chile (19). Another hantavirus, Oran virus, also causes HPS and is apparently associated with the same rodent species in extreme northern Argentina (19). However, the population of *O. longicaudatus* in northern Argentina appears to be disjunct from the population that follows the Andean cordillera and includes northwestern Patagonia (20). Careful taxonomic and distributional studies are required to determine if these disjunct rodent populations represent distinct species and to define the potential HPS-endemic areas associated with each virus.

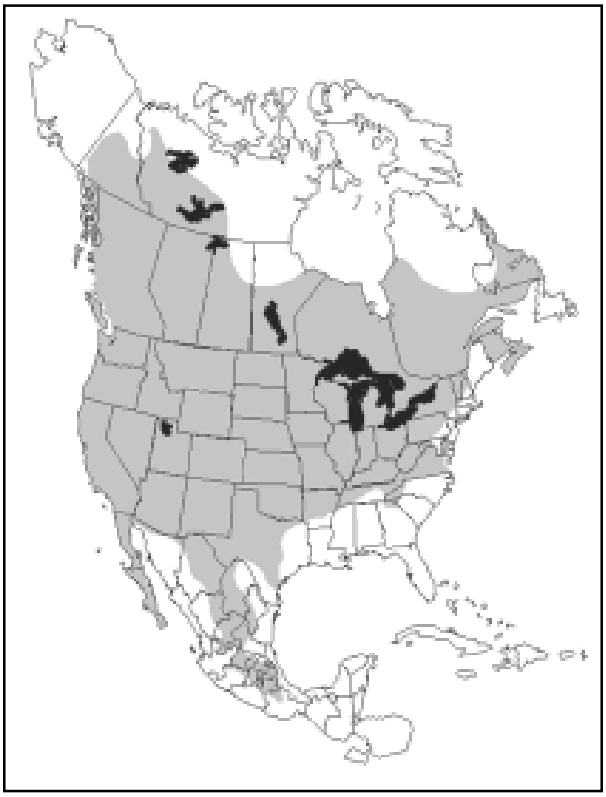

Figure 2. Geographic distribution of the deer mouse, *Peromyscus maniculatus* (shaded) (48).

Geographic Distribution of the Pathogen within the Host Range

The extent of the geographic area in which the infected host can be found indicates the area in which human disease can be endemic. In some cases, host and pathogen distributions are largely coincident. SNV infection has been found in deer mice throughout North America north of Mexico, with the exception of Alaska (Ksiazek et al., unpub. obs.; 21,22). *Rattus norvegicus* is found throughout most of the world, and infection with Seoul virus has been reported on every continent except Antarctica (23).

However, for many arenaviruses, including Junín, Guanarito, and Tamiami, and for some hantaviruses, the area in which the disease is endemic apparently includes only a small portion of the range of individual host species (Figure 3). The reservoir for Laguna Negra virus, which causes HPS in Paraguay, is *Calomys laucha*, and approximately 12% of the *C. laucha* tested from the Chaco region of western Paraguay are infected with Laguna Negra virus (24). *C. laucha* is also one of the most common rodents in central Argentina, yet testing of thousands of samples has provided no evidence of Laguna Negra virus in central Argentina (25; Levis et al., unpub. obs.). However,

Figure 3. Distribution of the corn mouse, *Calomys musculinus* (dots; 20), and disease-endemic area of Argentine hemorrhagic fever (AHF) (shaded).

the distributional data of *C. laucha* indicate that populations in Paraguay are disjunct from those in Argentina and Uruguay. Additional distributional, taxonomic, and laboratory studies are required to determine whether genetic differences in host populations are influencing susceptibility to infection or other factors are influencing virus distribution in a uniformly susceptible population.

The geographic range of infection by the pathogen within reservoir populations may be dynamic, not static. In 1955, the AHF-endemic area included approximately 10,000 km^2 centered around the town of Junín in Buenos Aires province. By 1985, the AHF-endemic area encompassed more than 150,000 km^2 in four provinces (26). Prevalence of infection in reservoir populations may be very high in some localities and very low, or absent, at nearby sites, for both arenaviruses (27) and hantaviruses (28,29). It is likely that pathogens periodically become extinct in local reservoir populations, only to be reintroduced later from neighboring populations. The dynamics and factors controlling these processes are unknown but indicate that, although a single cross-sectional survey approximates the range of infection, repeated surveys or longitudinal studies are necessary to elucidate temporal and spatial patterns.

Finally, in many instances, reservoir surveillance provides a clearer definition of potential disease-endemic areas than human disease surveillance, even when the incidence of human disease is extremely low (e.g., in HPS-endemic areas), and cases where the use of a vaccine may mask human exposure (e.g., in the AHF-endemic area).

Distribution of the Pathogen among Habitats

The distribution of the host and the pathogen among different biotic communities and habitat types indicates the risk for human infection in various habitats within a geographic region. The deer mouse, a habitat generalist, was found in all eight biomes studied in the four-state region of the initial HPS outbreak in the United States (28). Prevalence of SNV infection, however, varied among habitat types, being lowest at the climatic and altitudinal extremes (desert and alpine tundra) and highest at middle-altitude habitats such as piñon-juniper woodland, where most HPS cases occurred during the 1993 outbreak.

The corn mouse, reservoir for Junín virus, was described as a crop field specialist (30), and virus transmission was said to occur when farmers working in their fields inhaled aerosols of virus shed by infected rodents (31). However, during detailed studies of habitat preferences in the AHF-endemic area (Figure 4), the corn mouse was rarely found in crop fields. Instead, it inhabited the more stable, weedy fence lines and roadsides bordering crop fields. These results suggest that humans might become infected with Junín virus in border habitats and that cutting or burning vegetation in these habitats might help decrease the incidence of AHF.

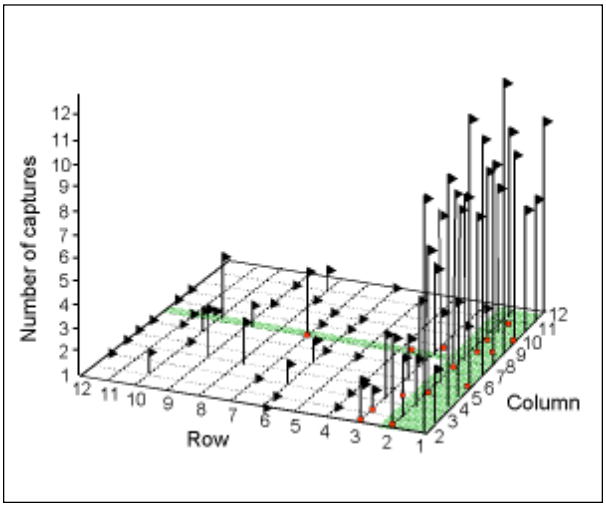

Figure 4. Schematic of mark-release-recapture grid in central Argentina. The green shaded areas are roadside (rows 1 and 2) and fence-line (column 7) habitats. Unshaded areas are crop fields. Intersections of dotted lines represent the 144 trap stations of the 12 x 12 trapping grid. The height of the flagpoles shows the cumulative numbers of corn mouse captures at each trap station of the 30-month sampling period. Red boxes are the locations of antigen-positive captures. Reprinted with permission from (32).

Relative Frequency of Infection in Subpopulations of the Host

The relative frequency of infection in different demographic subgroups within the host population suggests potential mechanisms of pathogen transmission within host populations. An association of antibody prevalence with age, suggesting horizontal (rather than vertical) transmission, has been documented for Norway rats infected with Seoul virus (33); deer mice, western harvest mice, and brush mice infected

with SNV or SNV-like viruses (28); cotton rats infected with Black Creek Canal virus (29); and corn mice infected with Junín virus (33). For Norway rats, deer mice, and cotton rats, a J-shaped curve of antibody prevalence with age was apparent; very young animals had prevalence rates similar to those of some adults. This pattern may be explained by the presence of maternal antibody in the offspring of infected females; as this apparently protective antibody wanes, animals become infected.

Fighting and biting among adult male rodents may be a common mechanism of virus transmission. Scars or wounds on rodents can indicate intraspecific aggressive encounters. A significant association between the acquisition of scars and the development of antibody to Seoul virus was demonstrated for rats in Baltimore (34), implying that virus was transmitted by biting during fights. In Argentina, male *C. musculinus* had more scars than females, and adult males with scars were more often infected with Junín virus than adult males without scars. In North America, the prevalence of antibody to SNV among male deer mice, brush mice, and western harvest mice is at least twice that among females (28), and scarring appears associated with antibody in some of these species. However, infection patterns vary. Male-to-female infection ratios are approximately 2:1 for deer mice and SNV, but two studies of harvest mice found infection of 40% to 50% in males (21,28), whereas no females were infected. Although scars and antibody to Seoul virus were highly correlated for Norway rats in Baltimore, antibody prevalence did not differ between male and female rats (34). These species-specific differences are presumably related to differences in behavior and, consequently, to differences in specific mechanisms of viral transmission. Further study of these mechanisms may lead to an understanding of the timing and conditions under which increased transmission in reservoir populations takes place, which, in turn, would lead to improved prediction of how and when humans become infected.

Temporal Dynamics of Host and Pathogen Populations

Longitudinal studies of reservoir populations are needed to identify 1) the effects of infection on individuals and populations of host species, 2) the seasonal and year-to-year fluctuations in incidence and prevalence as well as the sequence and duration of infection in the host, and 3) the environmental variables associated with changes in host density or rates of transmission. The mark-release-recapture method, important for longitudinal studies of small-mammal populations, establishes permanent trapping plots operated for several nights at standard intervals (usually monthly for disease studies) and maintained for several years. Captured animals are anesthetized, identified, weighed, measured, sampled (e.g., blood and oral swab), marked with a unique number, and released at the exact site of capture. Animals may be recaptured in subsequent months so that growth rates, movement, reproductive condition, and infection status can be monitored over time. Environmental variables, such as temperature, rainfall, and vegetation, relate to reservoir population dynamics. Mark-release-recapture studies should continue long enough to monitor the effects of unusual events, such as the El Niño southern oscillation (ENSO).

The mark-release-recapture method was used in a 3-year study of corn mouse populations on the Argentine pampas (33). AHF epidemics are highly seasonal, with the peak number of cases and peak rodent populations occurring in May when autumn crops are harvested. During the first 2 years, corn mouse population densities were relatively low, and the magnitude of the

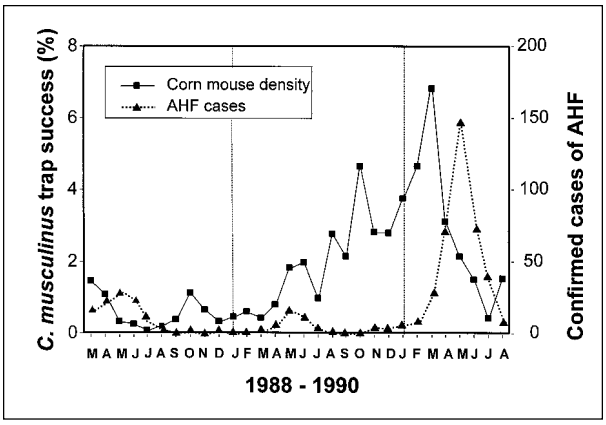

Figure 5. Mean monthly trap success for *Calomys musculinus* (number of captures per 100 trap nights) and numbers of confirmed cases of Argentine hemorrhagic fever (AHF) in central Argentina, March 1988 to August 1990. Reprinted with permission from (33).

annual AHF epidemics was also low (Figure 5). During the third year, unusually mild weather resulted in reproduction throughout the winter, high overwinter survival (35), and a very high population base at the beginning of the spring reproductive season. The following summer had abundant rainfall, vegetation remained green, and the usual midsummer decline in rodent populations did not occur. Consequently, corn mouse populations and numbers of infected corn mice reached extremely high levels by early autumn; an unusually severe epidemic of AHF cases followed (Figure 5; 33).

Longitudinal field studies also show the effects of infection on host populations. Contrary to indications from laboratory studies (10), Junín virus infection had no effect on the growth, movement, or longevity of corn mice (33), which implies that laboratory infection may not mimic natural transmission and illustrates the importance of investigating transmission mechanisms under field conditions.

Because the animals are released, mark-release-recapture studies do not provide data on diet, reproduction, precise population age structure (using molar wear patterns or eye lens weights), and variation in genetics of host and pathogen populations. Complementary investigations in which animals are collected for species identification and harvesting of tissues are therefore essential.

Predictive Models

The goal of reservoir ecology is to integrate and apply the data toward the development or refinement of predictive models (Figure 6) that help public health authorities identify specific times and locations that may pose a threat to public health. In addition to early warning capability, these models may suggest when, where, and how to intervene (e.g., in the form of reservoir control or habitat modification) to break the transmission cycle or otherwise decrease the incidence of human infection.

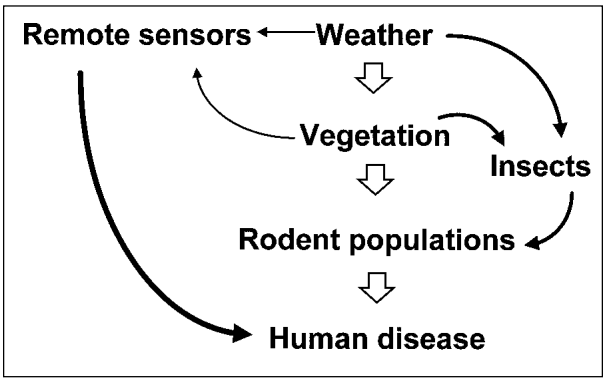

Figure 6. Simplified hypothetical model of interactions among ecosystem components within disease-endemic areas for rodent-borne zoonotic disease. Left-hand side of model demonstrates potential use of remote sensors (satellites) for predicting relative risk for human disease.

Figure 6 is a simplified schematic for such a model. We assume that the risk for human disease is directly related to reservoir population density because increased density increases the probability of human contact with infected rodents. Data on the incidence of HFRS due to Puumala virus in Sweden and studies of rodent population densities at HPS case and control households in the United States support this assumption (36,37). We also assume that reservoir population density is influenced by characteristics of the biotic environment (e.g., habitat quality and food supply), which in turn are influenced by characteristics of the physical environment (e.g., climate, weather, and edaphic conditions). Accurate predictive models are not yet available, but recent developments are promising. The clear correlation between reservoir population density and the risk for AHF indicates that monitoring of rodent populations might give public health professionals a 2- to 3-month predictive capability—enough time to make advisory warnings but not enough to intervene with a vaccination program or integrated pest management. The Argentine model also provided clues about factors contributing to the rodent irruption. The proper model might have allowed prediction of the rodent irruption and increased risk to humans a year before the epidemic.

Continuous measurement of environmental variables wherever rodent-borne zoonoses are endemic may not be necessary. Remote sensing and geographic information systems have measured vegetation indexes that help predict changing risk for zoonotic diseases (6,38). Integration of remote sensing with long-term studies measuring environmental conditions on the ground may provide the key to predicting future risk for virus transmission. A more complete understanding of generalized climatic patterns, such as ENSO, and their effects on local environmental variables may lead to even earlier predictive capabilities.

The Future

An otherwise complete and carefully executed study can be rendered useless if the species from which each sample was taken cannot be confirmed. Except in the case of mark-release-recapture studies, voucher specimens for all captured small mammals should be archived, and museum accession numbers should be cited in reports of these studies. Even if field identifications are accurate according to existing nomenclature, taxonomic revisions may leave the source of a pathogen in question unless the voucher is available for reexamination. For example, the recent search for the reservoir of Ebola virus near Kikwit, Republic of the Congo, resulted in the identification of at least seven taxa of rodents and insectivores that are preliminarily considered new species (39).

Where resources are available, a field study is enhanced by the maintenance of specimens in a museum frozen tissue collection. These specimens can be used by future investigators for genetic, taxonomic, and microbiologic studies. A listing of museums (in the Western Hemisphere) maintaining mammal collections, including frozen tissue collections, is available (40), as are instructions for preparation and maintenance of voucher and tissue specimens (41-43).

Safety

Although the precise circumstances of their transmission to humans are often unclear, zoonotic pathogens are associated with direct or indirect exposure to infected hosts. Modes of human infection include inhalation of infectious aerosols, direct contact of contaminated fomites with broken skin or mucous membranes, animal bites, or arthropod vectors. Mammalogists and others who work with reservoir populations are likely at increased risk. Safe methods and proper protective equipment must be used in handling potentially infected rodents. In handling reservoir species for known human pathogens such as hantaviruses and arenaviruses, protective clothing, latex gloves, and respirators with high-efficiency particulate air filters should be used. Animals should be anesthetized to minimize the risk for injuries, and the use of sharp instruments and needles for collection should be minimized. Safety guidelines are available in English (41,44) and Spanish (45).

Investigators studying relatively benign or less easily transmitted agents (e.g., *Borrelia*) should be alert to the potential presence of more virulent agents associated with any wild animals they may be handling. Certainly not all arenavirus- and hantavirus-host associations have been described, and some species, such as the white-footed mouse and the cotton rat, are known hosts for multiple agents.

Conclusions

Reservoir studies are an essential component of any integrated public health response to established or emerging zoonotic diseases. The proposed algorithm facilitates study of reservoir population ecology as it relates to human disease. Each step improves understanding of the ecology and epizootiology of hantaviruses and arenaviruses. The approach is offered as one model to stimulate discussion of appropriate methods and as a conceptual framework for other investigators to critique and improve. Other techniques, such as radio-tracking (46) and the use of injectable passive integrative transponders (PIT tags) (47), may better identify and track movements of individual animals. Methods and approaches will evolve as our knowledge of the subject matter increases. All the steps may not be appropriate in all cases, and many are beyond the capability of any single investigator or institution. For example, the delineation of the geographic range of any mammal species requires intensive taxonomic, genetic, and ecologic studies and could require the collaboration of investigators from several countries. These studies will be greatly facilitated by properly archived specimens from previous reservoir studies.

The phylogenetic relationship and distribution of pathogens may help elucidate the taxonomic relationships between closely related reservoir species. Although we have specifically addressed field studies, parallel laboratory studies of hosts and pathogens are needed for maximum benefit from these field studies. For example, studies of host and virus genetics, host specificity, and pathogenesis and viral shedding may elucidate 1) the roles of cospeciation and coevolution in the associations of hantaviruses and arenaviruses with their hosts, 2) mechanisms of viral persistence, and 3) the fundamental question of defining viral species.

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Diphtheria in the Former Soviet Union: Reemergence of a Pandemic Disease

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The massive reemergence of diphtheria in the Newly Independent States of the former Soviet Union marked the first large-scale diphtheria epidemic in industrialized countries in 3 decades. Factors contributing to the epidemic included a large population of susceptible adults; decreased childhood immunization, which compromised what had been a well-established childhood vaccination program; suboptimal socioeconomic conditions; and high population movement. The role of a change in the predominant circulating strains of *Corynebacterium diphtheriae* in this epidemic remains uncertain. Massive, well-coordinated international assistance and unprecedented efforts to vaccinate adults were needed to control the epidemic.

In the 1990s, a massive epidemic throughout the Newly Independent States of the former Soviet Union marked the reemergence of epidemic diphtheria in industrialized countries. Diphtheria had been well controlled in the Soviet Union for more than 2 decades after universal childhood immunization was initiated in the late 1950s (Figure 1). Although all of the Newly Independent States were affected, three quarters of the more than 140,000 cases (Table 1) and two thirds of the more than 4,000 deaths reported since 1990 (1-3) were reported by the Russian Federation.

Massive efforts to vaccinate both children and adults are bringing the epidemic under control; in 1996, 20,215 cases were reported, a 60% decrease from the 50,425 cases reported in 1995 (4), and, in 1997, 6,932 cases were reported as of February 1998 (World Health Organization [WHO], unpub. data). The European Regional Office of WHO considers the outbreak nearly under control in Armenia, Azerbaijan, Belarus, Estonia, Latvia, Lithuania, Moldova, the Russian Federation, Turkmenistan, and Uzbekistan; in the five remaining republics, control is improving, but continuing efforts are needed to stabilize the situation.

This epidemic, primarily affecting adults in most Newly Independent States of the former Soviet Union, demonstrates that in a modern society diphtheria can still spread explosively and cause extensive illness and death. Intense international efforts have focused on aiding the affected countries and understanding the reasons for the epidemic. The study of this resurgence, especially as it relates to diphtheria resurgence in other industrialized countries, may elucidate the potential for the reemergence of other vaccine-preventable diseases.

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Epidemiology of Diphtheria

Prevaccine Era

In the prevaccine era, diphtheria was a dreaded, highly endemic childhood disease found in temperate climates. Despite a gradual decline in deaths in most industrialized countries in the early 20th century, which was associated with improving living standards, diphtheria remained

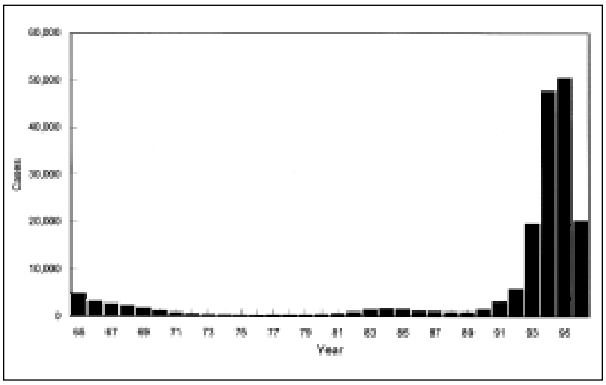

Figure 1. Reported diphtheria cases in the Soviet Union and the Newly Independent States, 1965–96.

Table 1. Diphtheria incidence in the Newly Independent States (NIS) of the former Soviet Union, 1991–1996

Country	Population (millions)	Cases (per 100,000) 1991	1992	1993	1994	1995	1996
Russia	149.90	1,869 (1.25)	3,897 (2.60)	15,211 (10.15)	39,582 (26.41)	35,652 (23.78)	13,604 (9.08)
Kazakstan	17.46	30 (0.17)	45 (0.26)	82 (0.47)	489 (2.80)	1,105 (6.33)	455 (2.61)
Kyrgyzstan	4.7	10 (0.21)	4 (0.09)	6 (0.13)	299 (6.36)	693 (14.74)	412 (8.77)
Tajikistan	6.02	5 (0.08)	14 (0.23)	680 (11.30)	1,912 (31.76)	4,455 (74.00)	1,464 (24.32)
Turkmenistan	4.16	4 (0.10)	22 (0.53)	3 (0.07)	60 (1.44)	87 (2.09)	80 (1.92)
Uzbekistan	22.83	9 (0.04)	29 (0.13)	137 (0.60)	224 (0.98)	638 (2.79)	160 (0.70)
Central Asia	55.17	58 (0.11)	114 (0.21)	908 (1.65)	2,984 (5.41)	6,978 (12.65)	2,571 (4.66)
Armenia	3.74	0 (0.00)	0 (0.00)	1 (0.03)	36 (0.96)	29 (0.78)	11 (0.29)
Azerbaijan	7.46	36 (0.48)	55 (0.74)	141 (1.89)	841 (11.27)	883 (11.84)	114 (1.53)
Georgia	5.49	7 (0.13)	3 (0.05)	28 (0.51)	294 (5.36)	419 (7.63)	346 (6.30)
Caucasus	16.69	43 (0.26)	58 (0.35)	170 (1.02)	1171 (7.02)	1,331 (7.97)	471 (2.82)
Ukraine	52.47	1,103 (2.10)	1,567 (2.99)	2,987 (5.69)	2,990 (5.70)	5,280 (10.06)	3,156 (6.01)
Moldova	4.36	14 (0.32)	22 (0.50)	35 (0.80)	376 (8.62)	418 (9.59)	97 (2.22)
Belarus	10.33	26 (0.25)	66 (0.64)	120 (1.16)	230 (2.23)	322 (3.12)	179 (1.73)
Western NIS	67.16	1,143 (1.70)	1,655 (2.46)	3,142 (4.68)	3,596 (5.35)	6,020 (8.96)	3,432 (5.11)
Estonia	1.57	7 (0.45)	3 (0.19)	11 (0.70)	7 (0.45)	19 (1.21)	14 (0.89)
Latvia	2.65	5 (0.19)	8 (0.30)	12 (0.45)	250 (9.43)	369 (13.92)	112 (4.23)
Lithuania	3.77	1(0.03)	9 (0.24)	8 (0.21)	38 (1.01)	43 (1.14)	11 (0.29)
Baltics	7.99	13 (0.16)	20 (0.25)	31 (0.39)	295 (3.69)	431 (5.39)	137 (1.71)
All NIS	296.91	3,126 (1.05)	5,744 (1.93)	19,462 (6.55)	47,628 (16.04)	50,412 (16.98)	20,215 (6.81)

one of the leading causes of childhood death until widespread vaccination was implemented. In England and Wales, as recently as 1937 to 1938, diphtheria was second only to pneumonia among all causes of childhood death (5), with an annual death rate of 32 per 100,000 in children less than 15 years of age.

Most urban residents acquired immunity to diphtheria by the age of 15 years (6); only a minor portion of diphtheria cases were in adults. Only approximately 15% of children with immunity to diphtheria had had typical clinical diphtheria; the other 85% had milder symptoms or asymptomatic infections (6). Children of preschool and elementary school age had the highest attack rates. The age of school entry was associated with increased risk for disease (7). Peaks of endemic diphtheria in the fall (8) were often attributed to the opening of schools. Crowding and low socioeconomic conditions were other risk factors (9).

Superimposed on the high endemic disease rates was a rough periodicity of incidence with peaks every several years (5). Epidemic waves characterized by extremely high incidence and deaths were sporadic: Spain in the early 1600s (10), New England in the 1730s (10,11), and Western Europe from 1850 to 1890 (12,13). The factors governing diphtheria periodicity are not understood.

Vaccine Era in Western Europe and the United States

In the United States, Canada, and many countries in Western Europe, the widespread use of diphtheria toxoid for childhood vaccination beginning in the 1930s and 1940s led to a rapid reduction in diphtheria incidence (14). However, in the 1930s, gradual rises in diphtheria incidence to 200 cases per 100,000 in the prewar period occurred in Germany and several other central European countries with partially implemented vaccination programs. The onset of World War II in 1939 and the occupation by German troops of many Western European countries led to the last diphtheria pandemic in Western industrialized countries. Although diphtheria incidence had been very low before the war, Holland, Denmark, and Norway had severe epidemics following occupation by German soldiers. Newly developed biotyping methods confirmed that endemic disease in prewar Germany was associated with strains of gravis biotype and that epidemics in occupied countries were associated with the introduction of gravis strains (15-17).

Data from a World War II epidemic in Halifax, Nova Scotia, suggest the high epidemic potential of these strains (18). Diphtheria was endemic in Halifax (30 to 80 cases per year),

primarily among children in poor neighborhoods. Although not all isolates were biotyped, mitis strains seemed to be predominant. Gravis organisms, presumably related to the strains introduced into Norway by German troops, were introduced into Nova Scotia in September 1940 by Norwegian sailors with diphtheria, leading to an outbreak of 649 cases by July 1941. All biotyped strains were gravis. At first, secondary cases occurred predominantly among school-aged children; following cases were among adults, especially women. A study of more than 1,000 school children in February 1941 found gravis carriage rates of up to 30% in schools in poor neighborhoods. An apparently limited introduction of new strains resulted in an epidemic while circulation of the previously endemic strains had not.

Diphtheria incidence continued to decline steadily throughout the vaccine era in the United States (Figure 2) and (after the immediate postwar period) in Western Europe. Cases of clinical diphtheria have become extremely uncommon; several European countries have not reported a case of diphtheria in more than 20 years (12,19). Residual indigenous cases have been concentrated among incompletely vaccinated or unvaccinated persons of low socioeconomic status. In the United States, the decline was interrupted in the late 1960s by a small resurgence of diphtheria lasting until 1975 (20). Until this time, the predominant biotype in the United States, especially in the Southeast, was mitis, although gravis outbreaks were common in the West. Intermedius cases were uncommon, and outbreaks were rare (21). Between 1969 and 1975, outbreaks caused by intermedius strains were reported in economically depressed populations in Chicago (22), San Antonio (23), Phoenix (24), the Navajo reservation in Arizona and New Mexico (25), Seattle (26), and communities in eastern Washington State (Centers for Disease Control and Prevention [CDC], unpub. data). Since 1975, cases of respiratory diphtheria and isolation of toxigenic strains of *Corynebacterium diphtheriae* have been extremely rare in the United States (19,27).

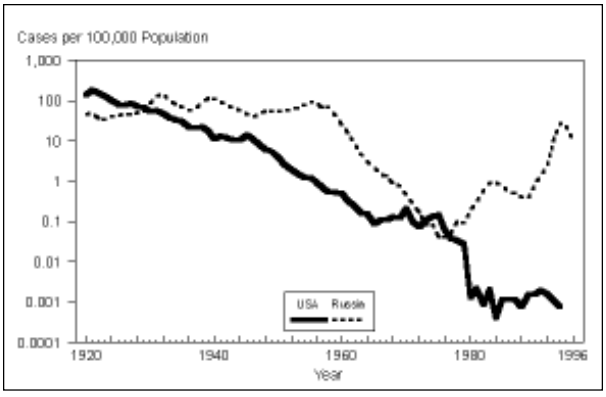

Figure 2. Diphtheria Incidence—United States and Russian Federation, 1920–1996.

Prevaccine and Early Vaccine Era in the Soviet Union

Diphtheria incidence in Russia was high throughout the first half of the 20th century (Figure 2); more than 750,000 cases were reported in Russia alone in the 1950s. Although immunization against diphtheria began in some areas of the Soviet Union as early as the 1920s, it was only in 1958 that universal childhood immunization began throughout the Soviet Union (28). By 1963, the incidence in the Soviet Union had decreased 15-fold compared with 1958 and elimination was thought attainable. From 1965 until 1980, the Soviet childhood vaccination schedule mandated five doses of high antigenic content diphtheria vaccine by school entry and a school booster (Table 2). Diphtheria incidence levels continued to drop and in the mid-1970s approximated levels in the United States; only 199 cases (0.08 cases per 100,000) were reported in the Soviet Union in 1975 and only 198 cases in 1976. As in the prevaccine era, most cases were in children; in 1975 and 1976, 62% of the 109 cases in the Russian Federation were in children under 15 years of age (N.M. Maksimova, pers. comm.). During this period, diphtheria incidence was higher in the Central Asian republics (14).

Resurgence and Stabilization of Diphtheria, 1977-1989

In 1977, diphtheria started to gradually make a comeback in the Soviet Union; incidence peaked in 1984 (1,609 cases, 0.9 per 100,000 population). The cases were concentrated in Russia, especially Central European Russia and the Russian Far East (29,30). In the Russian Federation, the resurgence was associated with a change in the predominant circulating biotype—from gravis (75% of cases 1975 to 1976) to mitis (60% of cases 1977 to 1981, 80% of cases 1982 to 1986). The circulating gravis strains belonged to several phage types and serotypes (31).

Table 2. Diphtheria vaccination schedule (Soviet Union 1965–1991, Russian Federation 1991–97, Ministry of Health, Russian Federation)

	Year[a]					
		1980		1987		
Age	1965	Alternative A	Alternative B	Alternative A	Alternative B	1994
3-18 mo (infancy)	DTP (3 doses)	DTP (3 doses)	DT or Td (2 doses)	DTP (3 doses)	DT or Td (2 doses)	DTP (3 doses)
18-36 mo[b]	DTP	DTP	DT or Td	DTP	DT or Td	DTP
6 yr	DTP	Td		Td		
9 yr				Td		
11-12 yr		Td		Td		Td
16 yr		Td (after 1983)		Td		Td

[a]Antigenic content of Russian-manufactured vaccine: DTP = 15 lf units diphtheria toxoid per dose; DT = 30 lf units diphtheria toxoid per dose; Td = 5 lf units diphtheria toxoid per dose; d = 5 lf units diphtheria toxoid per dose.
[b]To be given 12-18 months after last infancy dose.

In 1978, for the first time most cases occurred in adults and, although incidence rates rose in all age groups, the rates in adults rose more rapidly and to higher levels than in children. The proportion of cases associated with mitis strains was also lower in children, suggesting that children were initially relatively protected or not exposed during this epidemic (29,30). In response to the resurgence, Soviet public health authorities intensified diagnostic and case investigation efforts, called for widespread vaccination of adult occupational groups at high risk, and in some localities with multiple cases, called for widespread community adult immunization (30).

However, changes in the immunization schedule during this period encouraged less intensive vaccination of children. Use of an alternative schedule of fewer doses of lower antigenic content (adult formulation) vaccine was allowed beginning in 1980; in 1986, the school entry booster dose was dropped, lengthening to 7 years the interval between recommended childhood booster doses after a primary series (Table 2). In addition, an increasing number of conditions were considered temporary or permanent contraindications to childhood vaccination. At the same time, public support for childhood vaccination programs fell, at least in some areas, for several reasons. Many childhood vaccine-preventable diseases had low incidence rates. In addition, a vocal antiimmunization movement received favorable press coverage in an atmosphere of increased distrust of government during perestroika (1985 to 1991). Participants in focus groups conducted in 1996 on diphtheria vaccination vividly recalled these reports and said that the reports had made them more fearful of vaccinating themselves or their children (N. Keith, pers. comm.). Childhood vaccination rates declined during the 1980s, with coverage of infants with a primary diphtheria toxoid series dropping to 70% or below for most of the decade (14). Many children vaccinated in the Russian Federation received low antigen content vaccine as their primary series, especially in some areas such as Moscow and St. Petersburg. In 1989 in Russia, more than 25% (a higher proportion than in any other republic [32]) of vaccinated children received primarily adult formulation vaccine preparations lacking the pertussis component.

After 1984, diphtheria incidence gradually declined to 839 cases (0.3 per 100,000) in 1989, although remaining above the nadir reached in 1976 to 1977. Most cases were reported by the Russian Federation; although most were in adults, the proportion of cases in children rose gradually throughout the 1980s (Figure 3).

The military may have played an important role in the persistence and spread of diphtheria

Figure 3. Reported cases and proportion of cases among children <15 years, Russian Federation. Data provided by the Russian Ministry of Health.

during the 1980s. Military service remained universal, and recruits were not routinely vaccinated against diphtheria until 1990. The source of one prolonged Russian outbreak (Kovrov District, Vladimir Oblast, 1982 to 1987) was diphtheria cases among a unit of military recruits from Central Asia that spread into the civilian population through social functions (V.A. Grigorevna, pers. comm.). Investigations in military units in various parts of Russia between 1983 and 1987 found carrier rates of toxigenic *C. diphtheriae* of up to 5.0% (33). Although cases in the military were (and still are) not reported to the Ministry of Health or included in the reported incidence data, some published data exist. An outbreak in Kzyl-Orda, Kazakstan, in 1988 began among the military but spread to the civilian population, causing 58 cases (34). From 1987 to 1990, most adult diphtheria patients seen at the Botkin Infectious Disease Hospital, one of two treatment units for diphtheria in Moscow, were members of the military (35). On the basis of published data, between 1990 and 1992 the minimum incidence of diphtheria in the military was 21 cases per 100,000 service members (36), six times higher than that of the civilian population (37). This incidence ratio was even more disproportionate in the late 1980s (32,36).

Beginning in 1986, gravis strains accounted for an increasing proportion of isolates, and in 1989, accounted for 52% of reported cases. One hundred and fifty-six diphtheria strains from various sites in Russia from 1985 to 1994 were analyzed by multilocus enzyme electrophoresis (MEE) and ribotyping. Although the mitis strains predominant in the 1980s were of multiple electrophoretic and ribotypes, most of the gravis strains from 1986 to 1989 belonged to a group of strains closely related by both MEE and ribotyping; this epidemic clone became predominant overall in the 1990s epidemic in Russia (38).

Epidemic Diphtheria, 1990 to 1996

Spread from the Center: 1990 to 1992

In 1990, 1,431 cases were reported in the Soviet Union, a 70% increase over 1989 (32). Cases were heavily concentrated in the Russian Federation (1,211 cases), especially Moscow City and Oblast (541 cases combined), and the three Pacific Coast oblasts of Khabarovsk, Primorye Krai, and Sakhalin Island (109 cases). In 1991, 3,126 cases were reported from the now Newly Independent States; epidemic diphtheria had reached St. Petersburg City and Oblast (246 cases) and in the Ukraine, Kiev (372 cases), Kharkov (129 cases), and Lvov (190 cases). Further spread within Russia, the Ukraine, and Belarus accounted for most of the 5,744 cases reported in 1992 (14).

Explosion and Spread: 1993 to 1994

In 1993, the number of reported diphtheria cases surged to 19,462; epidemic diphtheria became established throughout urban Russia, the Ukraine, and Belarus. Russia alone reported 15,211 cases, an increase of 290% from 1992, with more cases reported in each succeeding month. For the first time in the epidemic, a pronounced seasonal incidence was seen (Figure 4), and the incidence rate in children exceeded that in adults by 60%. Elsewhere, Azerbaijan reported 141 cases and in the aftermath of a civil war, an epidemic sprang up in Tajikistan (680 cases) that spilled over into neighboring areas of densely populated Uzbekistan (137 cases). Cases had also increased in other Newly Independent States, although the incidence was still less than 1 per 100,000.

In 1994, epidemic diphtheria was reported from all states except Estonia, where most of the adult population had been vaccinated in 1985 to 1987. Russia had 39,582 (83%) of the 50,412 cases reported by the Newly Independent States. In Central Asia, areas of high incidence included regions adjacent to Russia in Kazakstan and regions adjacent to Tajikistan in both Uzbekistan and Kyrgyzstan, where the outbreak reportedly

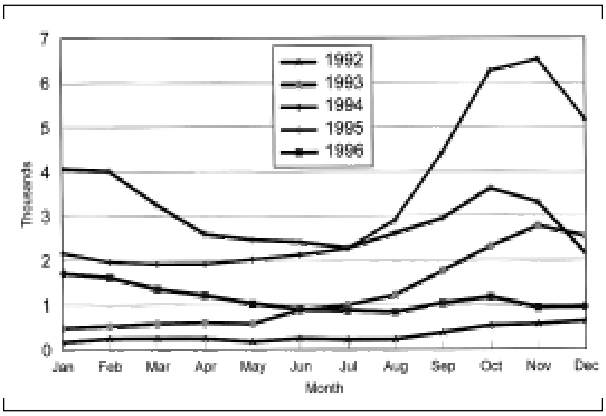

Figure 4. Diphtheria cases in the Russian Federation, 1992–96. 1994 = 39,582; 1995 = 35,652 (-10%); 1996 = 13,604 (-62%).

began with eight cases in a refugee family from Tajikistan (39).

In Russia, the Ukraine, Belarus, and the Baltics, most cases occurred in adults (3). In Russia in 1993, two thirds of cases were in persons older than 14 years. The highest incidence rates were among school-aged children and adolescents (12.4 to 18.2 per 100,000) and in adults ages 40 to 49 years (16.7 per 100,000); 45% of all deaths and the highest death rate (1.3 per 100,000 population) were reported among persons 40 to 49 years of age. Incidence rates dropped sharply in persons above age 50 (2.8 per 100,000) (40). Similarly in the Ukraine, adolescents 15 to 19 years of age and adults 40 to 49 years of age had the highest incidence rates (41). In several areas, most reported adult diphtheria cases were in women; women accounted for 60% of adult cases in St. Petersburg in 1991 to 1992 (42) and for 64% in three Russian regions in 1994 to 1995 (43). In the three regions, incidence rates among women 20 to 49 years of age were 68% higher than the rates among men of that age.

Biotype gravis strains have been predominant in Russia in the 1990s. Molecular studies using ribotyping and MEE demonstrated emergence of an epidemic clone of closely related strains (38,44). In the Ukraine, Belarus, the Baltics, and northern Kazakstan, the predominant strains were gravis biotype; in Tajikistan, Uzbekistan, Kyrgyzstan, and southern Kazakstan, the mitis biotype strains predominated.

Public Health Response and Control of the Epidemic: 1995 to 1996

Initial diphtheria control efforts were hindered by shortfalls in strategy and vaccine supply. In the early years of the epidemic, public health officials concentrated on improving childhood coverage rates and on vaccinating adults in occupational groups perceived at high risk; vaccination of all adults was not directed by Russian public health authorities until 1993. The resulting unprecedented demand for adult formulation vaccine was met with stepped-up Russian vaccine production during 1994 to 1995 when nearly 80 million doses of adult formulation vaccine was produced (compared with less than six million in 1992 [Russian Federation Ministry of Health, unpub. data]).

Implementation efforts focused on vaccinating adults at work sites, followed by intensified efforts including house-to-house visits, to reach and vaccinate nonworking adults. Similarly, to further raise childhood coverage, a shortened list of contraindications was adopted and the use of full-strength vaccine preparations in the primary series was increased. In October 1994, the school-entry booster dose was reinstituted.

By the end of 1995, considerably improved coverage in children in Russia was reported (93% coverage with primary series at 1 year reported compared with 68.7% in 1991). Adult coverage with one or more doses in the previous 10 years was estimated at 75%; between January 1993 and December 1995, 70 million adults were vaccinated in the Russian Federation (45). In 1995, the incidence (24 cases per 100,000 population) decreased 10% from 1994. In 1996, 13,604 cases (9 per 100,000) were reported, a further 62% decline (2,4). In 1997, a provisional total of 4,057 cases were reported to WHO (WHO, unpub. data).

Unlike Russia, other Newly Independent States of the former Soviet Union states were not producers of diphtheria vaccine. The disruption of vaccine supply and economic difficulties associated with the dissolution of the Soviet Union were reflected in sharp drops in childhood vaccination rates in the early 1990s in the Central Asian and Caucasian republics (3).3 In 1994 to 1995, WHO, United Nations Children's Fund (UNICEF), other agencies, and Newly Independent States' governments developed and adopted an epidemic control strategy that aimed at rapidly raising adult and childhood coverage through nationwide immunization campaigns. Subsequently, a massive international effort, involving governmental and nongovernmental organizations and United Nations agencies and coordinated by an oversight committee, the Interagency Immunization Coordinating Committee, proved successful in mobilizing resources, purchasing and delivering vaccine to the Newly Independent States, and providing technical assistance to implement the strategy (2,3).

In 1995 and 1996, the Newly Independent States raised adult and childhood coverage and began to control the epidemic. All states made widespread use of workplace vaccinations for adults and other intensified vaccination tactics; these efforts resulted in increased adult coverage. The strategy was implemented with highly successful national mass campaigns in Moldova, Tajikistan, Latvia, Lithuania, and Azerbaijan, which achieved high adult coverage and rapid steep declines in incidence. All

countries also made efforts to increase childhood coverage and to limit contraindications and the use of lower antigen content vaccine; routine childhood coverage exceeded 90% in most countries (2). Most countries reinstated a school-entry revaccination, and many of the national mass vaccination campaigns focused on children and adolescents. In 1996, 6,611 diphtheria cases were reported in the Newly Independent States excluding Russia, a 55% decrease compared to 1995; in the first quarter of 1997, 885 cases were reported, a 57% decrease compared with the first quarter of 1996 (4). WHO has received provisional reports of 2,875 cases for the full year of 1997, although data are not complete for a few countries (WHO, unpub. data).

Factors Contributing to the Diphtheria Epidemic in the Newly Independent States

The control of epidemic diphtheria by childhood vaccination has been one of the outstanding successes of medicine in this century. Most Western industrialized countries have nearly eliminated this disease; many developing countries have progressively increased vaccination coverage by introducing diphtheria toxoid into vaccination programs. Global diphtheria incidence declined approximately 70% between the mid-1970s and the early 1990s (14). The diphtheria epidemic in the Newly Independent States raised numerous concerns about the efficacy of diphtheria control programs and of the diphtheria vaccine itself. However, case-control studies in the Ukraine and in Moscow demonstrated that three or more doses of Russian-manufactured diphtheria toxoid was highly effective in preventing disease in children (46). The rapid decline in disease incidence with increased vaccination coverage among both adults and children provides strong evidence of the continued effectiveness of diphtheria vaccine.

Numerous factors appear to have contributed to the epidemic: 1) increased adult susceptibility, which is reflected in the age distribution of cases and deaths; 2) increased susceptibility of children; 3) a clone of closely related strains of *C. diphtheriae*, gravis biotype, associated with most of the cases in Russia, even though its role remains uncertain; 4) highly crowded urban populations and service in the military; 5) the breakup of the former Soviet Union, perhaps by disrupting vaccine supply to all countries other than Russia and initiating large-scale population movements throughout the Newly Independent States.

Increased Adult Susceptibility

Arguably the most important factor for the diphtheria epidemic was the development of large populations of adults susceptible to the disease as a consequence of successful childhood vaccination programs. The decreased opportunity for naturally acquired immunity, along with the waning of vaccine-induced immunity in the absence of routine adult revaccination, has resulted in a high proportion of adults susceptible to diphtheria as documented by serologic studies in many countries (12). In the United States, a trend to increased diphtheria incidence in older age groups was noted before the near-total disappearance of diphtheria (47). In several developing countries that have conducted immunization programs for more than 10 years, recent small epidemics have shown a similar increase in the proportion of adult cases (12).

Adults born between the early 1940s and the late 1950s in the Russian Federation and some other Newly Independent States were at the highest risk for never having acquired immunity to diphtheria; during their childhood, diphtheria incidence was decreasing, but not all children were reached by newly implemented vaccination programs. The gap in immunity in this age group, observed in serologic studies (48,49), was reflected in the very high numbers of deaths and illnesses among persons 35 to 50 years of age.
Adults with similar high susceptibility to diphtheria in the United States and Western Europe are likely to be older than susceptible adults in the former Soviet Union because of the earlier implementation of vaccination programs in the West. The only sizeable diphtheria outbreaks in the United States that involved predominantly adults occurred in the early 1970s in Arizona and Washington. At this time, the analogous cohort of U.S. adults born just before widespread immunization would have been approximately 30 to 50 years old. Points of maximal population susceptibility to epidemic adult diphtheria therefore may depend on the age distribution of adults susceptible to the disease and the frequency of high contact rates for these susceptible adults (e.g., military service for young adults, care of school-aged children for young and middle-aged adults, homelessness among young and middle-aged men).

A high rate of adult susceptibility to diphtheria is a necessary but not sufficient precondition for the development of epidemic diphtheria in adults. The United States and most other European countries have high rates of adult susceptibility but have not had sustained chains of transmission, despite documented importations. In Poland and Finland, which border on the Newly Independent States that had diphtheria epidemics, multiple documented importations since 1990 have led to only very limited secondary transmission; these countries have maintained very high levels of childhood coverage against diphtheria (50).

In the epidemic of the Newly Independent States, sustained transmission in adults may have been limited to certain focal groups. In addition to military units, high-level transmission between adults (clusters of multiple cases and high rates of carriage) was demonstrated in other adult groups characterized by crowding, low levels of hygiene, and high contact rates, such as the homeless and patients in neuropsychiatric hospitals (40,42); in routine work settings, clusters of cases were rare, and the carrier rates among adult contacts of cases were very low.

Increased Childhood Susceptibility

By 1990, on-time coverage of infants and young children had fallen because of changes in childhood vaccination recommendations and practices and increased population skepticism regarding vaccination, which was greatly exacerbated by an active antivaccination movement. Vaccination was frequently delayed and temporary contraindications to vaccination were extremely common. In addition, many children were considered to have permanent contraindications to vaccination, large numbers of children received lower antigen content vaccines in the primary series, and the interval between childhood booster doses was lengthened. The lowered coverage with pertussis-containing vaccine has been linked to a resurgence of pertussis in the Russian Federation (51). Some data exist on the direct impact of these changes on diphtheria incidence; in Russia and the Ukraine, incidence was higher among unvaccinated than vaccinated children, and lack of vaccination was a strong risk factor for severe disease (40). A case-control study after the reinstitution of the school entry booster dose found an interval of greater than 5 years since the last dose to be a strong risk factor for disease (52). No data regarding the effect of the increased use of lower antigen content vaccine are available.

Although since the mid-1970s most of the diphtheria cases in the Newly Independent States have been in adults, throughout the 1980s and early 1990s the proportion of diphtheria cases in children increased during the period of worsening overall control of diphtheria in all age groups (Figure 3). The increase in diphtheria cases among children suggests an important role for the susceptibility of children in the dissemination of diphtheria. In addition, most reported carriers in the Russian Federation were children in both the 1980s (31) and 1990s (Russian Federation Ministry of Health, unpub. data). Clusters of multiple cases in schools and within families were prominent features in this epidemic, as in previous ones (40). Some data suggest that a proportion of the disease in adults may represent 'sentinel' events and may have been transmitted from ill or asymptomatic children; these data include the much higher disease incidence among women compared with men (despite no evidence of lower serologic immunity [50,53]) and the large proportion of adult cases linked with multiple asymptomatic child carriers (CDC and Russian Federation Ministry of Health, unpub. data). In this respect, epidemic diphtheria in the era of adult susceptibility may resemble epidemic influenza, in which studies suggest that schoolchildren are a very important population in transmission and spread, although the bulk of severe disease occurs in their adult contacts (54).

Change in Biotype or Epidemic Clone

Changes in the circulating strains of *C. diphtheriae* could be responsible for the cyclicity and episodic epidemic waves associated with diphtheria incidence in the prevaccine era. The outbreaks in Western Europe during World War II, in the United States in the 1970s, and in the Soviet Union/Newly Independent States in the mid-1970s and the late 1980s were associated with the emergence of a new biotype. Host factors (such as antimicrobial immunity) could contribute to the epidemic potential of a newly introduced strain, but microbial factors cannot be excluded. However, the role of antibacterial immunity in preventing infection with *C. diphtheriae* has not been studied since the 1930s, and no microbial factors have been identified that distinguish

epidemic from nonepidemic strains. The molecular characterization of an epidemic clone of gravis strains associated with the current epidemic in most parts of Russia supports a role for a change in the agent in the development of the epidemic in the Newly Independent States of the former Soviet Union; however, other countries of the Newly Independent States have had epidemic diphtheria linked to strains of both mitis and gravis biotypes.

Although the source of the epidemic strains in Russia is unclear, persistent foci of diphtheria in Russia are a possible source. Russia was never totally free of reported cases of diphtheria, and recent reports of persistent disease-endemic foci in the United States (55) and Canada (56) suggest that circulation of toxigenic strains of *C. diphtheriae* can occur for prolonged periods even in the absence of recognized clinical cases, at least in certain communities. Other suggested sources include persistent diphtheria foci in the Central Asian countries or importation by returning military units from the war in Afghanistan between 1979 and 1990.

Soviet Economic Development and the Post-Soviet Economic Crisis

Soviet populations were highly urbanized, but because of economic growth lagging behind that in Western Europe and the United States, most city dwellers lived in crowded apartments. Many of the amenities conducive to decreasing transmission of bacteria were deficient or lacking, especially in public areas, including routine access to functioning faucets for hand washing. A case-control study of diphtheria cases in Georgia found an increased risk for diphtheria associated with sharing utensils, cups, glasses, or a bedroom and with decreased bathing (57).

The economic crisis of the post-Soviet period in all Newly Independent States may have worsened these living conditions and contributed to the epidemic. The crisis indisputably led to lowered childhood coverage in the Central Asian and Caucasian republics in the early 1990s and may have contributed to the high proportion of childhood diphtheria cases reported from many of these republics.

Militarization

The Soviet Union was the most extremely militarized large country in the world, with 1.4% of its population armed (58). The high level of militarization and the lack of routine immunization of recruits resulted in bringing large numbers of susceptible adults together from all parts of the immense country under conditions of crowding and suboptimal hygiene. These adverse conditions may have played a role in the development of the epidemic as suggested by the high diphtheria incidence in the military early in the 1990s.

Increased Travel and Mass Population Movement

Other major changes after the breakup of the Soviet Union in 1991 included loosening of controls on movement within countries and increasing movements of populations between the Newly Independent States. During the Soviet era, movement was restricted by regulations and housing shortages. The success of control measures in the epidemic of the early 1980s may have been enhanced by slower dissemination of toxigenic strains due to movement restrictions. Similarly, while the epidemic clone was already established in the Newly Independent States by 1991, epidemic spread may have been facilitated by the mass movements of populations, primarily the repatriation of ethnic Russians from Central Asian and Caucasian countries and the flight of refugees from fighting in Georgia, Armenia, Azerbaijan, and Tajikistan.

Factors in Successful Epidemic Control

The epidemic was controlled by vaccination efforts that achieved very high childhood coverage and unprecedented coverage in adults. The control strategy was developed and refined on the basis of epidemiologic analyses of disease incidence and population immunity. Implementation of the strategy for all of the Newly Independent States, except Russia, required massive international assistance; the instrumental role of the Interagency Immunization Coordinating Committee in successfully coordinating the multiple partners in this effort should serve as a model for future international public health emergencies. Finally, an effective, although underfunded, system of primary health care and public health centers had functioned throughout the Soviet Union for decades. The health workers of this system played a critical role in rapidly implementing the control measures once adequate strategies and material resources were identified.

Lessons for Other Potential Reemerging Diseases

The epidemic in the Newly Independent States was unexpected; however, many of the factors apparently contributing to the epidemic coincide with factors important in the emergence of infectious diseases (Table 3) (59) and are connected with other diseases and other countries. An increased susceptibility in adults to childhood diseases is a predictable consequence of successful childhood vaccination programs with vaccines that produce less than lifelong immunity; this type of susceptibility has been suggested as one factor contributing to the current increase in pertussis in the United States. Although extreme loss of confidence in immunization may not occur often in other countries, an increasing population resistance to childhood vaccination as a result of adverse publicity and a diminished perception of risk occurs commonly (60) and has contributed to outbreaks of pertussis in England and Sweden (61,62). The role of changes in etiologic agents contributing to emergence is under study for many diseases. A recent outbreak of pertussis in the Netherlands may be due to a change in the predominant circulating strains resulting in decreased vaccine efficacy in children (63). Rapid urbanization with large segments of the population living under suboptimal hygienic conditions is characteristic of rapidly industrializing nations, and mass population movements are regular accompaniments of sociopolitical instability. The reemergence of diphtheria in the Newly Independent States of the former Soviet Union demonstrates the continued threat of this disease and of other infectious agents that may exploit similar social and political vulnerabilities.

Table 3. Factors influencing the emergence of diphtheria, Newly Independent States (NIS), 1990–1996.

Technology and industry
Population of susceptible adults
Human demographics and behavior
Population resistance to vaccinating children
Changes in childhood vaccination schedule
High levels of militarization
Decreased social controls, increased travel
Microbial adaptation and change
Change in biotype or emergence of epidemic clones
Economic development and land use
Highly crowded and intense urbanization, substandard housing
Breakdown of public health measures
Decreased immunization in Central Asia and Caucasus due to breakup of Soviet Union
International travel and land use
Repatriation of Russian population from republics
Refugees from Tajikistan, refugees in Georgia

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Cell-to-Cell Signaling and *Pseudomonas aeruginosa* Infections

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Pseudomonas aeruginosa is a bacterium responsible for severe nosocomial infections, life-threatening infections in immunocompromised persons, and chronic infections in cystic fibrosis patients. The bacterium's virulence depends on a large number of cell-associated and extracellular factors. Cell-to-cell signaling systems control the expression and allow a coordinated, cell-density–dependent production of many extracellular virulence factors. We discuss the possible role of cell-to-cell signaling in the pathogenesis of *P. aeruginosa* infections and present a rationale for targeting cell-to-cell signaling systems in the development of new therapeutic approaches.

Pseudomonas aeruginosa as a Human Pathogen

Pseudomonas aeruginosa, an increasingly prevalent opportunistic human pathogen, is the most common gram-negative bacterium found in nosocomial infections. *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases (1), 12% of hospital-acquired urinary tract infections (2), 8% of surgical wound infections (3), and 10% of bloodstream infections (4). Immunocompromised patients, such as neutropenic cancer and bone marrow transplant patients, are particularly susceptible to opportunistic infections. In this group of patients, *P. aeruginosa* is responsible for pneumonia and septicemia with attributable deaths reaching 30% (5,6). *P. aeruginosa* is also one of the most common and lethal pathogens responsible for ventilator-associated pneumonia in intubated patients (7), with directly attributable death rates reaching 38% (8). In burn patients, *P. aeruginosa* bacteremia has declined as a result of better wound treatment and dietary changes (removal of raw vegetables, which can be contaminated with *P. aeruginosa*, from the diet) (3). However, *P. aeruginosa* outbreaks in burn units are still associated with high (60%) death rates (9). In the expanding AIDS population, *P. aeruginosa* bacteremia is associated with 50% of deaths (10). Cystic fibrosis (CF) patients are characteristically susceptible to chronic infection by *P. aeruginosa*, which is responsible for high rates of illness and death in this population (11). The capacity of *P. aeruginosa* to produce such diverse, often overwhelming infections is due to an arsenal of virulence factors (Figure 1). Many extracellular virulence factors secreted by *P. aeruginosa* have been shown to be controlled by a complex regulatory

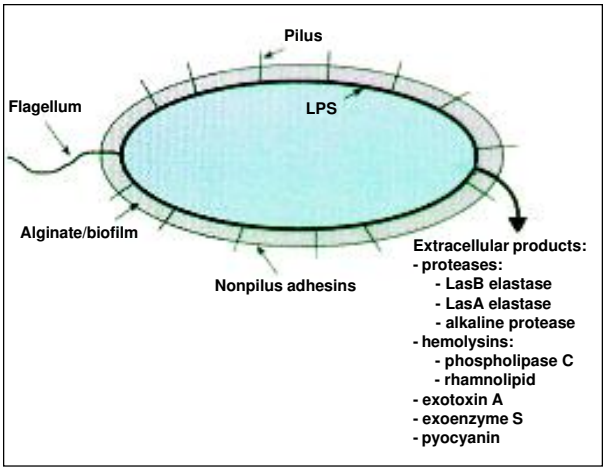

Figure 1. Virulence factors of *Pseudomonas aeruginosa. P. aeruginosa* has both cell-associated (flagellum, pilus, nonpilus adhesins, alginate/biofilm, lipopolysaccharide [LPS]) and extracellular virulence factors (proteases, hemolysins, exotoxin A, exoenzyme S, pyocyanin).

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circuit involving cell-to-cell signaling systems that allow the bacteria to produce these factors in a coordinated, cell-density–dependent manner (12). In this article we describe major virulence factors of *P. aeruginosa* and the possible involvement of cell-to-cell signaling in the pathogenesis of acute *P. aeruginosa* infection. We also summarize data suggesting that these regulatory systems could be exploited for the design of therapeutic interventions.

Pathogenesis of *P. aeruginosa* Infections

P. aeruginosa (family *Pseudomonadaceae),* an aerobic, motile, gram-negative rod able to grow and survive in almost any environment, lives primarily in water, soil, and vegetation. However, despite abundant opportunities for spread, *P. aeruginosa* rarely causes community-acquired infections in immunocompetent patients. As a result, the pathogen is viewed as opportunistic. The different phases of *P. aeruginosa* infection are shown in Figure 2.

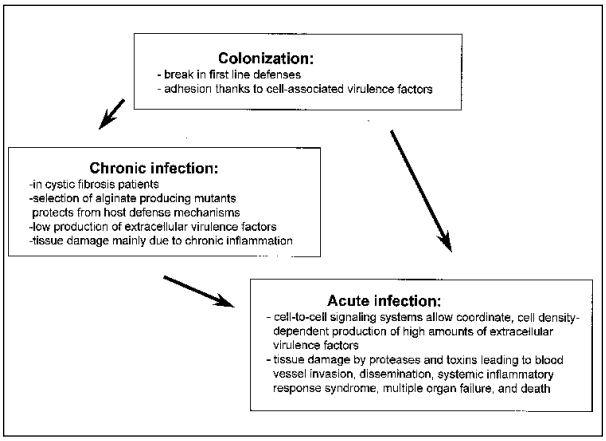

Figure 2. Model of the different phases of *Pseudomonas aeruginosa* infection. After an initial colonization phase, mostly dependent on cell-associated virulence factors, the infectious process evolves either to a chronic infection characterized by low production of extracellular virulence factors or to an acute infection characterized by high production of cell-to-cell signaling dependent virulence factors. During acute exacerbation of chronic infection, the production of cell-to-cell signaling dependent virulence factors is highly increased.

Colonization: The Predominant Role of Cell-Associated Virulence Factors

To initiate infection, *P. aeruginosa* usually requires a substantial break in first-line defenses. Such a break can result from breach or bypass of normal cutaneous or mucosal barriers (e.g., trauma, surgery, serious burns, or indwelling devices), disruption of the protective balance of normal mucosal flora by broad-spectrum antibiotics, or alteration of the immunologic defense mechanisms (e.g., in chemotherapy-induced neutropenia, mucosal clearance defects from cystic fibrosis, AIDS, and diabetes mellitus).

The first step in *P. aeruginosa* infections is colonization of altered epithelium. The pathogen colonizes the oropharynx of up to 6% and is recovered from the feces of 3% to 24% of healthy persons (2). In contrast, up to 50% of hospitalized patients are at high risk for *P. aeruginosa* colonization (2). Adherence of *P. aeruginosa* to epithelium is probably mediated by type 4 pili similar to those of *Neisseria gonorrhoeae* (13). Several other nonpilus adhesins responsible for the binding to mucin have been described, but their role in the infection process remains unclear (14). Flagella, which are primarily responsible for motility, may also act as adhesins to epithelial cells (15).

From Colonization to Acute Infection: The Role of Extracellular Virulence Factors

P. aeruginosa produces several extracellular products that after colonization can cause extensive tissue damage, bloodstream invasion, and dissemination (Figure 1). In vivo studies have shown that mutants defective in the production of exotoxin A, exoenzyme S, elastase, or alkaline protease are essential for maximum virulence of *P. aeruginosa;* however, the relative contribution of a given factor may vary with the type of infection (16). Many of these factors are controlled by regulatory systems involving cell-to-cell signaling. We will summarize the known biologic effects of the most-studied extracellular virulence factors associated with acute *P. aeruginosa* infection.

Exotoxin A is produced by most *P. aeruginosa* strains that cause clinical infections. Like

diphtheria toxin, *P. aeruginosa* exotoxin A catalyzes ADP-ribosylation and inactivation of elongation factor 2, leading to inhibition of protein biosynthesis and cell death (17). Exotoxin A is responsible for local tissue damage, bacterial invasion (18), and (possibly) immunosuppression (19). Purified exotoxin A is highly lethal for mice which supports its role as a major systemic virulence factor of *P. aeruginosa* (18).

Exoenzyme S is also an ADP-ribosyl transferase, but unlike exotoxin A, it preferentially ribosylates GTP-binding proteins such as Ras (20). This exoproduct is responsible for direct tissue destruction in lung infection (21) and may be important for bacterial dissemination (22).

Two hemolysins, phospholipase C and rhamnolipid, produced by *P. aeruginosa,* may act synergistically to break down lipids and lecithin. Both may contribute to tissue invasion by their cytotoxic effects. Rhamnolipid, a rhamnose-containing glycolipid biosurfactant, has a detergentlike structure and is believed to solubilize the phospholipids of lung surfactant, making them more accessible to cleavage by phospholipase C (23). The resulting loss of lung surfactant may be responsible for the atelectasis associated with chronic and acute *P. aeruginosa* lung infection (24). Rhamnolipid also inhibits the mucociliary transport and ciliary function of human respiratory epithelium (25). However, the relative role of rhamnolipid in acute or chronic infection is not known.

Proteases are assumed to play a major role during acute *P. aeruginosa* infection. *P. aeruginosa* produces several proteases including LasB elastase, LasA elastase, and alkaline protease (12). The role of alkaline protease in tissue invasion and systemic infections is unclear; however, its role in corneal infections may be substantial (26,27). The ability of *P. aeruginosa* to destroy the protein elastin is a major virulence determinant during acute infection. Elastin is a major part of human lung tissue and is responsible for lung expansion and contraction. Moreover, elastin is an important component of blood vessels, which rely on it for their resilience. The concerted activity of two enzymes, LasB elastase and LasA elastase, is responsible for elastolytic activity (28). Elastolytic activity is believed to destroy elastin-containing human lung tissue and cause the pulmonary hemorrhages of invasive *P. aeruginosa* infections. LasB elastase is a zinc metalloprotease that acts on a number of proteins including elastin. LasB elastase is highly efficient, with a proteolytic activity approximately 10 times that of *P. aeruginosa* alkaline protease and an activity toward casein approximately four times that of trypsin (28). The LasA elastase is a serine protease that acts synergistically with LasB elastase to degrade elastin. LasA elastase nicks elastin, rendering it sensitive to degradation by other proteases such as LasB elastase, alkaline protease, and neutrophil elastase (28). Both LasB elastase and LasA elastase have been found in the sputum of CF patients during pulmonary exacerbation (29). However, the role of LasB elastase in tissue destruction during the chronic phase of CF is less clear. It has been postulated that during this phase, antibodies present in high titers neutralize LasB elastase, and elastin damaged by minute amounts of LasA is degraded mostly by neutrophil elastase (28). LasB elastase degrades not only elastin but also fibrin and collagen (30). It can inactivate substances such as human immunoglobulins G and A (31), airway lysozyme (32), complement components (33), and substances involved in protecting the respiratory tract against proteases such as α-1-proteinase inhibitor (34) and bronchial mucus proteinase inhibitor (35). Therefore, LasB elastase not only destroys tissue components but also interferes with host defense mechanisms. Studies in animal models show that mutants defective in LasB elastase production are less virulent than their parent strains (16,36,37), which supports the role of LasB elastase as a virulence factor.

Cell-to-Cell Signaling: A Global Regulation System of *P. aeruginosa* Extracellular Virulence Factors

Cell-to-Cell Signaling Systems

P. aeruginosa appears to control the production of many of its extracellular virulence factors by a mechanism that monitors bacterial cell density and allows communication between bacteria by cell-to-cell signaling. Bacteria are able to sense their environment, process information, and react appropriately; however, their ability to sense their own cell density, to communicate

with each other, and to behave as a population instead of individual cells has only recently been understood (38,39). This phenomenon, called quorum-sensing or cell-to-cell signaling, is a generic phenomenon described in many gram-negative (40) and gram-positive bacteria (41).

The first cell-to-cell signaling system described is the *lux* system, responsible for the cell-density–dependent control of bioluminescence genes by the marine bacteria *Vibrio fischeri* (38). Cell-to-cell signaling systems of gram-positive bacteria involve peptide pheromones as signals (41). In contrast, cell-to-cell signaling systems of gram-negative bacteria, with the exception of *Ralstonia solanacearum,* in which an endogenous fatty acid derivative has been suggested as a cell-to-cell signal (42,43), are composed of a small molecule called an autoinducer, which is synthesized by a LuxI-type autoinducer synthase and a LuxR-type transcriptional activator protein (R-protein) (Figure 3) (38). The various autoinducers described in gram-negative bacteria are homoserine lactone-based molecules that differ between one another in length and substitutions on their acyl side chains. At low cell density, autoinducer is synthesized at basal levels and is thought to diffuse into the surrounding media, where it becomes diluted. With increasing cell density, the intracellular concentration of autoinducer increases until it reaches a threshold concentration. At this critical concentration, the autoinducer has been proposed to bind to a specific R-protein (40). The R-protein itself is not active without the corresponding autoinducer, and it is the R-protein/autoinducer complex that is proposed to bind to specific DNA sequences upstream of target genes enhancing their transcription (44,45). The resulting increase in expression of these genes can reach 1,000-fold. The autoinducer, therefore, allows the bacteria to communicate with each other (cell-to-cell signaling), to sense their own density (quorum-sensing), and together with a transcriptional activator to express specific genes as a population instead of individual cells.

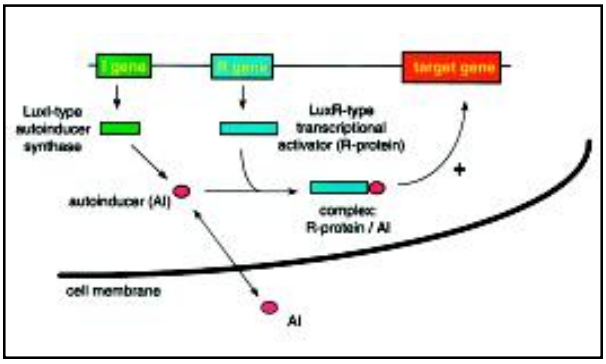

Figure 3. Cell-to-cell signaling systems. Cell-to-cell signaling systems are composed of two genes. The *I* gene encodes an autoinducer synthase and the *R* gene encodes a transcriptional activator protein (R-protein). The autoinducer synthase is responsible for the synthesis of an autoinducer molecule (AI), which crosses the cell membrane. With increasing cell-density the intracellular concentration of AI reaches a threshold level, and the AI then binds to the transcriptional activator. The complex R-protein/AI activates the expression of specific target genes.

The *las* Cell-to-Cell Signaling System of *P. aeruginosa*

The first cell-to-cell signaling system described in *P. aeruginosa* was shown to regulate expression of LasB elastase and was therefore named the *las* system (46). The *las* cell-to-cell signaling system is composed of *lasI,* the autoinducer synthase gene responsible for the synthesis of 3-oxo-C12-HSL (*N*-[3-oxododecanoyl]-L-homoserine lactone, previously named PAI-1 or OdDHL), and the *lasR* gene that codes for a transcriptional activator protein (Figure 4) (47,48). The *las* cell-to-cell signaling system regulates *lasB* expression and is required for optimal production of other extracellular virulence factors such as LasA protease and exotoxin A (49). *LasI* is the most sensitive gene to activation by LasR/3-oxo-C12-HSL (50). The preference for the *lasI* promoter allows an initial rapid rise in autoinducer synthesis, which increases the amount of 3-oxo-C12-HSL available to bind to LasR. This autoinduction hierarchy is responsible for a dramatic increase of expression of virulence genes (such as *lasB*) once a critical cell density has been reached. Recently, the *las* system has also been shown to activate the *xcpP* and *xcpR* genes that encode proteins of the *P. aeruginosa* secretory pathway (51). 3-oxo-C12-HSL alone has been suggested to contribute to the virulence of *P. aeruginosa* because it has some immunomodulatory activity (52). The *las* cell-to-cell signaling system is positively controlled by GacA (53), as well as by

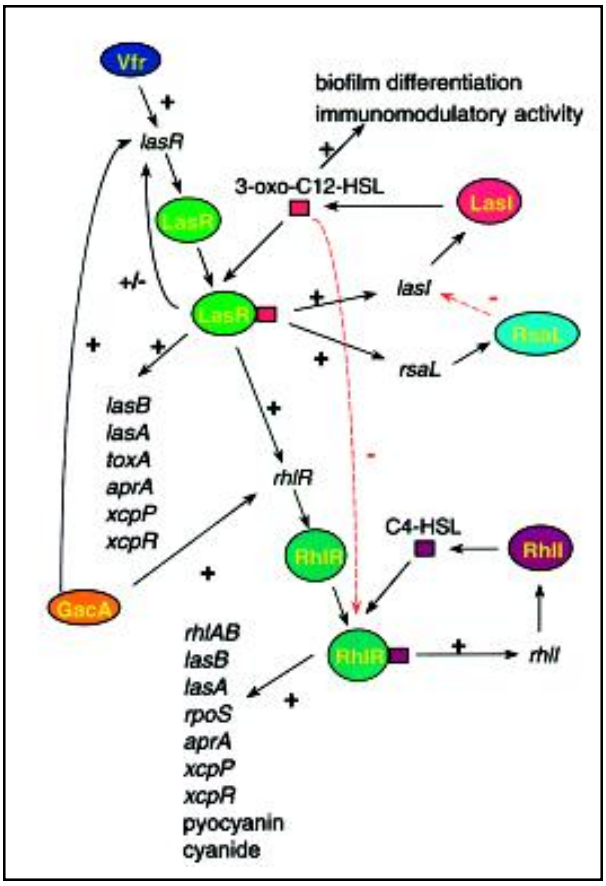

Figure 4. The cell-to-cell signaling circuitry of *P. aeruginosa*. The *las* cell-to-cell signaling system controls the *rhl* cell-to-cell signaling system in a hierarchy cascade. The LasR/3-oxo-C12-HSL complex activates the transcription of *rhlR*, and 3-oxo-C12-HSL blocks the activation of RhlR by C4-HSL. The *las* system itself is controlled positively by Vfr and GacA, and negatively by RsaL. 3-oxo-C12-HSL is required for biofilm differentiation and has immunomodulatory activity. Both cell-to-cell signaling systems regulate the expression of numerous genes (*lasB*: LasB elastase, *lasA*: LasA elastase, *toxA*: exotoxin A, *aprA*: alkaline protease, *xcpP* and *xcpR*: genes of the *xcp* secretory pathway, *rhlAB*: rhamnosyltransferase required for rhamnolipid production, *rpoS*: stationary phase sigma factor).

Vfr, which is required for the transcription of *lasR* (54). An inhibitor, RsaL, that represses the transcription of *lasI,* has also been described (de Kievit et al., unpub. data). The multiple regulatory levels of the *las* cell-to-cell signaling system and the various genes under its control highlight the importance of this system for *P. aeruginosa*.

The *rhl* Cell-to-Cell Signaling System of *P. aeruginosa*

P. aeruginosa has a second cell-to-cell signaling system, named the *rhl* system because of its ability to control the production of rhamnolipid. This system is composed of *rhlI*, the C4-HSL (*N*-butyrylhomoserine lactone, previously named PAI-2 or BHL) autoinducer synthase gene, and the *rhlR* gene encoding a transcriptional activator protein (Figure 4) (55-59). This system regulates the expression of the *rhlAB* operon that encodes a rhamnosyltransferase required for rhamnolipid production (60). The *rhl* system is also necessary for optimal production of LasB elastase, LasA protease, pyocyanin, cyanide, and alkaline protease (53,57,61,62). Therefore, like the *las* cell-to-cell signaling system, the *rhl* system, sometimes referred to as vsm (virulence secondary metabolites), regulates the expression of various extracellular virulence factors of *P. aeruginosa*. Interestingly, the *rhl* system also regulates the expression of *rpoS,* which encodes a stationary sigma factor (δ^S) involved in the regulation of various stress-response genes (63,64).

The Cell-to-Cell Signaling Hierarchy in *P. aeruginosa*

Recent data have shown that the *las* and *rhl* cell-to-cell signaling systems of *P. aeruginosa* interact. Both systems are highly specific in that their respective autoinducers are unable to activate the transcriptional activator protein of the other system (i.e., 3-oxo-C12-HSL cannot activate RhlR, and C4-HSL cannot activate LasR) (57,62). It has also been shown that the R-protein/autoinducer complexes prefer certain promoters that they will activate; LasR/3-oxo-C12-HSL preferentially activates *lasB* over *rhlA*, and RhlR/C4-HSL preferentially activates *rhlA* over *lasB* (57,62). However, neither system is completely independent of the other. The LasR/3-oxo-C12-HSL complex activates the expression of *rhlR* placing the *las* system in a cell-to-cell signaling hierarchy above the *rhl* system (Figure 4) (63,65). Moreover, 3-oxo-C12-HSL can bind to RhlR, blocking the binding of C4-HSL to its transcriptional activator *rhlR* (65). The *las*

system therefore controls the *rhl* system at both a transcriptional and posttranslational level. Another yet unidentified regulatory mechanism directly influencing the expression of *rhlRI* has been suggested (63). *Rhl* system regulation of such important genes as *rpoS* could explain why multiple levels of controls are required for its tight regulation.

Cell-to-Cell Signaling: A Powerful System to Overcome Host Defenses during Acute Infection

Cell-to-cell signaling systems might enable *P. aeruginosa* to overcome host defense mechanisms. Isolated production of extracellular virulence factors by a small number of bacteria would probably lead to an efficient host response neutralizing these compounds. However, the coordinated expression of virulence genes by an entire bacterial population once a certain density has been reached might allow *P. aeruginosa* to secrete extracellular factors only when they can be produced at high enough levels to overcome host defenses. These factors could alter the precarious balance between host defenses and production of bacterial toxins, leading to invasion of blood vessels, dissemination, systemic inflammatory-response syndrome, and finally death. Even appropriate antibiotic therapies are often unable to stop this course; therefore, the process must be blocked early, before virulence gene expression can be coordinated.

Biofilms and Cell-to-Cell Signaling

The most striking feature of persistent *P. aeruginosa* infections in CF patients is the selection of mucoid mutants producing the exopolysaccharide alginate (a polymer of mannuronic and guluronic acid) (11). These mutant bacteria grow inside a biofilm (defined as microcolonies surrounded by an exopolysaccharide) as a survival strategy because the surrounding matrix protects bacteria from phagocytes and complement activity (11). *P. aeruginosa* growing in an alginate "slime matrix" are resistant to antibiotics (e.g., aminoglycosides, ß-lactam antibiotics, fluoroquinolones) and disinfectants (11). The exact nature of the increased resistance is unclear but has been attributed to slow growth, penetration barriers, ß-lactamase production, and other factors. *P. aeruginosa* also produces other less well-defined biofilms essential in the colonization of indwelling devices such as catheters. Recently, the *las* cell-to-cell signaling system has been shown to be involved in the differentiation of *P. aeruginosa* biofilms (66). A mutant defective in the production of 3-oxo-C12-HSL formed an abnormal biofilm that, in contrast to the wild type biofilm, was sensitive to low concentrations of the detergent sodium dodecyl sulfate (SDS). Furthermore, the addition of 3-oxo-C12-HSL to the culture media restored production of a differentiated, SDS-resistant biofilm by the mutant (66). Whether the formation of an undifferentiated biofilm renders this mutant more sensitive to antibiotics is still unknown. Also unclear is whether 3-oxo-C12-HSL is required for the differentiation of alginate biofilms. The link between 3-oxo-C12-HSL and biofilm differentiation highlights the broad range of systems controlled by cell-to-cell signaling in *P. aeruginosa*.

Interference with Cell-to-Cell Signaling: A Potential Therapeutic Approach against *P. aeruginosa*

The increasing emergence of bacterial strains resistant to antibacterial drugs is a major challenge. *P. aeruginosa* is one of the most problematic human pathogens, showing intrinsic resistance to many structurally unrelated antibiotics. Resistance mechanisms include low outer membrane permeability or multidrug efflux pumps (tetracycline, imipenem, fluoroquinolones, aminoglycosides) or production of antibiotic modifying enzymes (aminoglycosides, ß-lactams) (67,68). Previous exposure to antibiotics often leads to multidrug-resistant *P. aeruginosa* strains. Moreover, the eradication of colonization with *P. aeruginosa* is almost impossible in CF patients because of the emergence of multidrug-resistant strains and the protective effects of alginate (69). Therefore, major efforts have been made to find new therapeutic interventions against *P. aeruginosa*. A tempting approach is to reduce the production of extracellular virulence factors. In this regard, the interference with cell-to-cell signaling systems is very attractive. Experimental evidence for effective interference with cell-to-cell signaling systems has been recently

provided by the use of autoinducer analogs (70-72) or furanone compounds (73). The LasR/3-oxo-C12-HSL complex, which plays a central role in controlling virulence gene expression in *P. aeruginosa,* is essential not only for the production of elastase and other proteases required for tissue invasion but also for the control of the *rhl* cell-to-cell signaling system in a hierarchical cascade and for the differentiation of biofilm. Mutants with a nonfunctional *las* cell-to-cell signaling system are unable to produce several virulence factors (including elastase) and have significantly reduced virulence in a neonatal mouse pneumonia model (37). Therefore, the LasR/3-oxo-C12-HSL complex has become a potential target for new therapeutic interventions. Analogs of 3-oxo-C12-HSL (74) are under investigation for their ability to act as 3-oxo-C12-HSL antagonists, which block activation of LasR and thereby interfere with virulence gene expression. The fact that 3-oxo-C12-HSL can block the bioactivity of C4-HSL (65) suggests that it might be possible to interfere efficiently with cell-to-cell signaling in *P. aeruginosa*. As elements essential for the synthesis of autoinducers have recently been described, drugs inhibiting the biosynthesis process could also be designed (75-80). These approaches could reduce the production of virulence factors, and by interfering with biofilm differentiation they could also render colonizing *P. aeruginosa* more susceptible to antibiotics and biocides. Combination therapies, including antibiotics and drugs reducing virulence factor production, might have an important role in the treatment of CF and intensive-care patients.

As with any therapeutic approach against *P. aeruginosa,* the possible emergence of mutations that would prevent the desired effect is a concern. We have recently described spontaneous mutants that were restored in the production of extracellular virulence factors (elastase and rhamnolipid) in the absence of a functional *las* cell-to-cell signaling system (81). We were unable to isolate mutants restored in virulence factor production when both the *las* and the *rhl* cell-to-cell signaling systems were deficient. These results suggest that therapeutic interventions directed only against the *las* cell-to-cell signaling system are likely to fail because of the emergence of resistant strains able to produce virulence factors despite an inactive *las* system (81). However, if both the *las* and the *rhl* cell-to-cell signaling systems are blocked, *P. aeruginosa* might be unable to restore the production of cell-to-cell signaling dependent virulence factors; this approach may efficiently reduce virulence factor production and high death rates associated with *P. aeruginosa.*

Cell-to-Cell Signaling Systems in Other Human Gram-Negative Pathogens

Other gram-negative human pathogens including *Serratia marcescens*, *S. liquefaciens, Aeromonas hydrophila, Vibrio cholerae, V. parahaemoliticus*, *Yersinia enterocolitica*, *Enterobacter agglomerans, Hafnia alvei*, and *Rahnella aquatis* have been shown to produce homoserine lactone-based autoinducer molecules. These organisms are thought to contain cell-to-cell signaling systems analogous to the *las* and *rhl* systems of *P. aeruginosa* (39,40). *Burkholderia cepacia* (formerly known as *Pseudomonas cepacia*) is an opportunistic pathogen responsible for fatal pulmonary infections in CF patients (11). Virulence factor production of this pathogen can be enhanced by *P. aeruginosa* exoproducts, and this interspecies communication may be due to homoserine-lactone signals (82). Whether *B. cepacia* contains cell-to-cell signaling systems is still unknown. As the number of bacteria known to use cell-to-cell signaling systems increases, therapeutic interference with cell-to-cell signaling might expand to other human pathogens.

Conclusions

The pathogenesis of *P. aeruginosa* is clearly multifactorial as underlined by the large number of virulence factors and the broad spectrum of diseases the bacterium causes. Many of the extracellular virulence factors required for tissue invasion and dissemination are controlled by cell-to-cell signaling systems involving homoserine lactone-based signal molecules and specific transcriptional activator proteins. These regulatory systems enable *P. aeruginosa* to produce virulence factors in a coordinated, cell-density–dependent manner that could allow the bacteria to overwhelm the host defense mechanisms. Interference with cell-to-cell signaling dependent virulence factor production is a promising therapeutic approach for reducing illness and death associated with *P. aeruginosa*

colonization and infection. The growing number of human pathogens found to contain cell-to-cell signaling systems highlights the importance of exploring interference with bacterial cell-to-cell signaling for new therapeutic interventions.

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Rotavirus

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Rotavirus, the most common diarrheal pathogen in children worldwide, causes approximately one third of diarrhea-associated hospitalizations and 800,000 deaths per year. Because natural infection reduces the incidence and severity of subsequent episodes, rotavirus diarrhea might be controlled through vaccination. Serotype-specific immunity may play a role in protection from disease. Tetravalent rhesus-human reassortant rotavirus vaccine (RRV-TV) (which contains a rhesus rotavirus with serotype G3 specificity and reassortant rhesus-human rotaviruses with G1, G2, and G4 specificity) provides coverage against the four common serotypes of human rotavirus. In clinical trials in industrialized countries, RRV-TV conferred 49% to 68% protection against any rotavirus diarrhea and 61% to 100% protection against severe disease. This vaccine was licensed by the U.S. Food and Drug Administration on August 31, 1998, and should be cost-effective in reducing diarrheal diseases in industrialized countries. The vaccine's efficacy and cost-effectiveness in developing countries should be evaluated.

In 1973, Bishop and colleagues observed by electron microscopy, in the duodenal epithelium of children with diarrhea, a 70-nm virus, subsequently designated rotavirus (Latin, *rota* = wheel) because of its appearance (Figure 1) (1). Before this discovery, a bacterial, viral, or parasitic etiologic agent could be detected in only 10% to 30% of children with diarrhea. Within 5 years, rotavirus was recognized as the most common cause of diarrhea in infants and young children worldwide, accounting for approximately one third of cases of severe diarrhea requiring hospitalization (2-4). In the United States, approximately 2.7 million children <5 years of age are affected by rotavirus diarrhea each year, resulting in 500,000 physician visits and 50,000 hospitalizations at an estimated $274 million in medical care and more than $1 billion in societal costs (5,6). The cost of rotavirus diarrhea is even higher in developing countries, where 20% to 70% of hospitalizations and 800,000 of the three million deaths per year from diarrhea are caused by this pathogen (Figure 2) (7,8). Recognition of rotavirus as a major cause of diarrhea in children led to extensive research for interventions to reduce the incidence of this disease.

Because rotavirus infects virtually all children ≤ 3 to 5 years of age in both industrialized and developing countries, improving water, food, and sanitation appeared unlikely to reduce disease incidence. Instead, early studies identified epidemiologic features indicating that rotaviral disease might best be

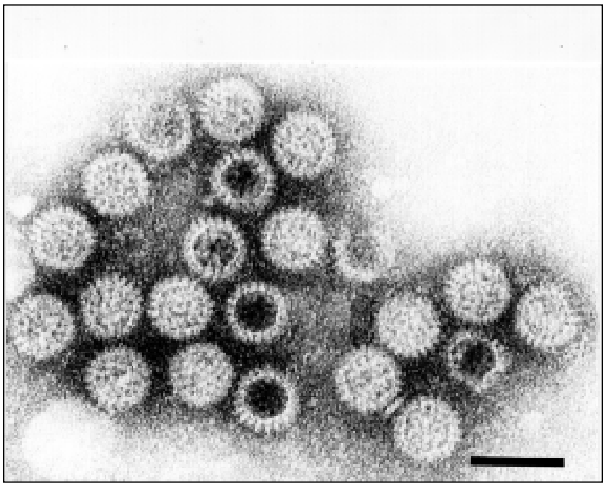

Figure 1. Rotavirus particles visualized by immune electron microscopy in stool filtrate from child with acute gastroenteritis. 70-nm particles possess distinctive double-shelled outer capsid. Bar = 100 nm.

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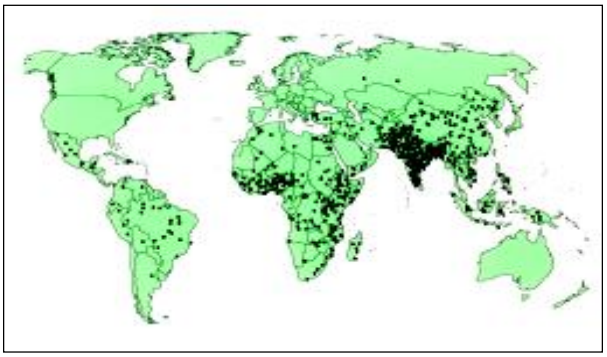

Figure 2. Estimated global distribution of the 800,000 annual deaths caused by rotavirus diarrhea. Reprinted with permission from (8).

controlled through vaccination. Natural immunity was suggested by the infrequent occurrence of more than one episode of rotavirus diarrhea in a child and decreased incidence of disease with increasing age (9). Furthermore, protection increased with each new infection and was greatest against moderate to severe disease, less against mild illness, and least against asymptomatic infection (10). These findings implied that an attenuated rotavirus vaccine that simulates natural infection could induce protective immunity and that more than one dose of vaccine may be required.

Several rotavirus vaccine candidates developed in the past 3 decades have proven safe and effective in clinical trials. On August 31, 1998, only 25 years after the discovery of rotavirus, a live, oral, tetravalent rhesus-human reassortant rotavirus vaccine (RRV-TV) was licensed by the U.S. Food and Drug Administration, and this vaccine may soon be available for immunization of children. This paper reviews the biologic and epidemiologic characteristics of rotavirus, discusses the development of rotavirus vaccines, and identifies research needs for expediting the introduction of rotavirus vaccines into childhood immunization programs.

Rotavirus Characteristics

Morphology and Classification

Rotavirus, an icosahedral virus in the family *Reoviridae*, has a distinct morphologic appearance by negative-stain electron microscopy (3). The viral capsid is triple-layered; the inner layer (core) contains the virus genome, which comprises 11 segments of double-stranded RNA, each coding for products that are either structural viral proteins (VP) or nonstructural proteins (NSP) (Figure 3). The segmented genome of rotavirus readily reassorts during coinfection, a property that has been used in

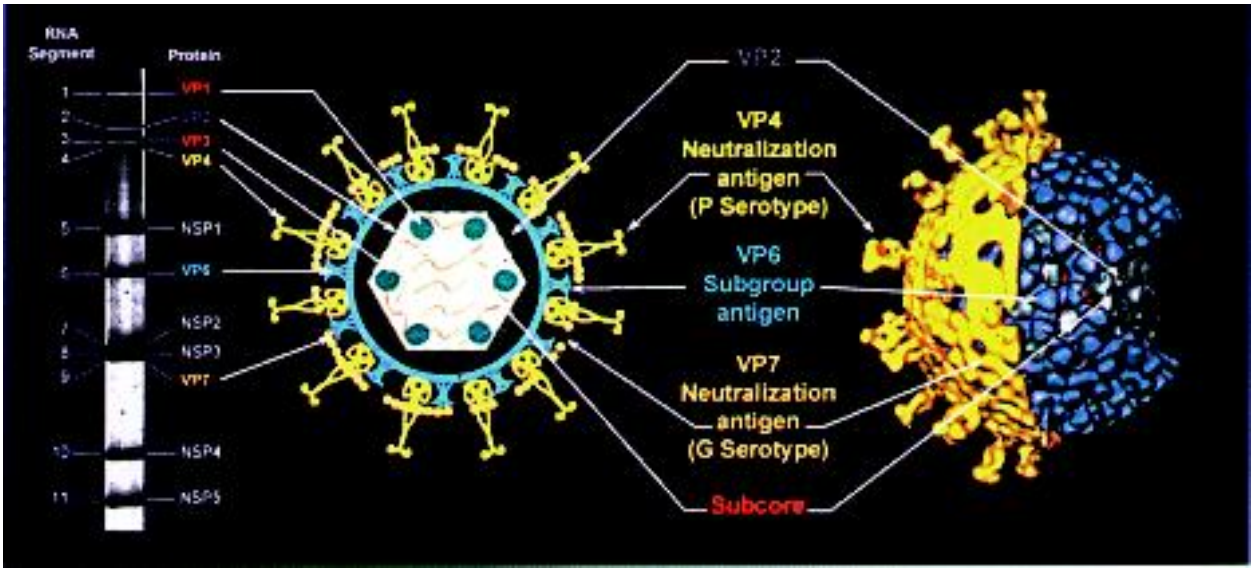

Figure 3. Gene coding assignments and three-dimensional structure of rotavirus particles. Double-stranded RNA segments separated on polyacrylamide gel (left) code for individual proteins, which are localized in the schematic of virus particle (center) or in different protein shells of virus (right). Outer capsid proteins VP4 and VP7 are neutralization antigens, which induce neutralizing antibody; protein that makes up intermediate protein shell, VP6, is the subgroup antigen. Reprinted with permission from (4).

developing vaccines and undoubtedly plays a role in virus evolution.

The major antigenic properties of rotaviruses—group, subgroup, and serotype—are determined by the viral capsid proteins. Rotavirus has seven major groups (A-G); most human strains belong to group A, although groups B and C have occasionally been associated with human illness. The product of the 6th gene of group A rotaviruses encodes VP6, the most abundant viral protein, which is the major determinant of group reactivity, the target of common diagnostic assays, and contains the antigen used to further classify rotaviruses into subgroups I and II. The outer capsid proteins, VP7, the glycoprotein or G-protein (encoded by gene 7, 8, or 9, depending on the strain), and VP4, the protease-cleaved or P-protein (encoded by gene segment 4), determine the serotype specificity and form the basis of the binary classification (G and P type) of rotaviruses. Both G and P proteins induce neutralizing antibodies and may be involved in protective immunity.

Global Distribution of Rotavirus Strains

Fourteen G serotypes of rotavirus, 10 of which occur in humans, have been defined by cross-neutralization studies with polyclonal animal serum samples; these serotypes correlate with antigenic specificities of the VP7 glycoprotein. The characterization of P serotypes has been difficult because adequate reagents are not available. Eight P serotypes of human rotaviruses have been characterized. Additional VP4 gene variants have been identified, so ultimately the number of P serotypes may exceed 20. Theoretically, 80 different strains of rotavirus could result from various combinations of the known 10 G and 8 P serotypes of human rotaviruses. For vaccine development purposes, it is fortunate that only four common strains (P[8]G1; P[8]G3; P[8]G4; and P[4]G2) of rotavirus predominate globally (Figure 4) (11). However, the prevalence of rotavirus strains varies considerably from one geographic area to another, and unusual strains are common in several developing countries (e.g., unusual P[6] strains, including those with serotype G9 specificity, accounted for 9.5% of all rotaviruses from a multicenter collection in India) (12).

Epidemiology

Rotaviruses are ubiquitous; 95% of children worldwide are infected by 3 to 5 years of age. The

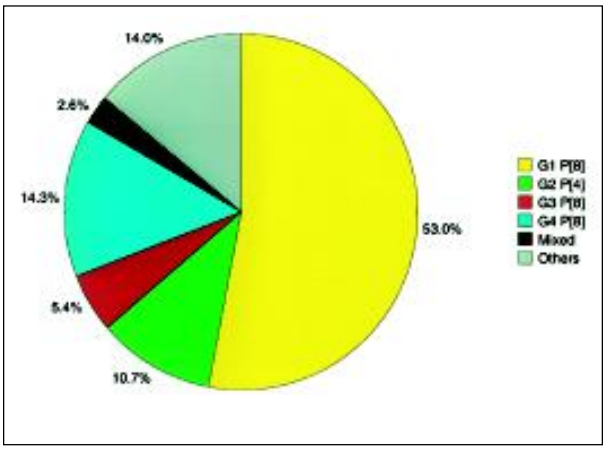

Figure 4. Distribution of rotavirus strains from a global collection of 2,748 strains. "Others" includes strains that were not typable. Adapted from (11).

prevalence of rotavirus infection in neonates has not been systematically examined, but high infection rates were documented in newborns in six hospitals in India (13). These infections, frequently asymptomatic, were caused by unusual strains of rotavirus. The incidence of clinical illness peaks among children ages 4 to 36 months, who are also at greatest risk for severe disease requiring hospitalization. Rotavirus infections of adults are usually subclinical but occasionally cause illness in parents of children with rotavirus diarrhea, immunocompromised patients (including those with HIV), the elderly, and travelers to developing countries. In temperate climates, rotavirus diarrhea occurs predominantly during the fall and winter; in tropical settings and in developing countries, seasonality is less marked (14). Peak rotavirus activity in the United States begins in the Southwest in autumn (October through December) and ends in the Northeast during spring (March through May) (Figure 5) (15); the reason for this pattern is unknown.

Rotaviruses are shed in large numbers during episodes of diarrhea, and usually are detectable by antigen enzyme immunoassays (EIA) up to 1 week after infection or for more than 30 days in immunocompromised patients. A recent study showed that as many as 30% of immunocompetent infants with severe rotavirus diarrhea may have virus detectable by polymerase chain reaction (PCR) for more than 25 days after hospital admission. The predominant

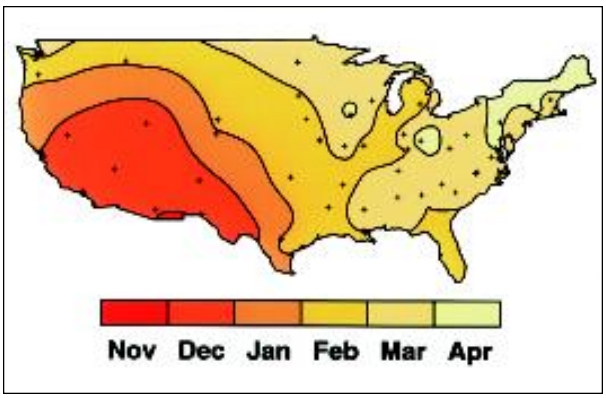

Figure 5. Average time of peak rotavirus activity in the contiguous 48 states, United States, July 1991 to June 1997. This contour plot was derived using the median value for time of peak activity for each laboratory. The surveillance system and analytic methods used to create this map are described in greater detail in (15). + indicates location of laboratories participating in the surveillance system.

mode of rotavirus transmission is fecal-oral. Spread through respiratory secretions, person-to-person contact, or contaminated environmental surfaces has also been speculated because of the high rates of infection in the first 3 years of life regardless of sanitary conditions, the failure to document fecal-oral transmission in several outbreaks of rotavirus diarrhea, and the dramatic spread of rotavirus over large geographic areas in the winter. Animal-to-human transmission does not appear to be common, although human rotavirus strains that possess a high degree of genetic homology with animal strains have been identified (16). Animal strains of rotavirus differ from those that infect humans.

Pathogenesis and Immunity

Rotaviruses infect the mature absorptive villous epithelium of the upper two thirds of the small intestine. After replication in the upper small intestine, infectious particles are released into the intestinal lumen and undergo further replication in the distal areas of the small intestine. Infection is generally confined to the intestinal mucosa. Although rotaviruses can be found in the lamina propria and regional lymphatics, replication at these sites and systemic spread usually do not occur in immunocompetent persons (3).

Despite the superficial nature of mucosal infection, rotaviruses induce both local intestinal and systemic immune responses (17,18). Early animal studies suggested that the presence of rotavirus antibodies in the intestinal lumen (but not in the serum) was correlated with protection against disease. Oral administration of preparations containing rotavirus antibodies has successfully treated chronic rotavirus infection and diarrhea in immunocompromised children (19,20). In a randomized clinical trial, a single oral dose of gamma globulin reduced the duration of illness and the shedding of virus in infants hospitalized with rotavirus diarrhea. These observations indicate that intestinal immunity protects against rotavirus diarrhea and that the success of a rotavirus vaccine will depend, in part, upon its ability to induce mucosal immune responses.

In infants and young children, neutralizing antibodies directed primarily against the G serotype of the infecting strain (homotypic response) develop after primary infection with rotavirus (18). Repeat rotavirus infections elicit both a homotypic and heterotypic (against strains with different G serotypes) antibody response. Protection against rotavirus diarrhea correlates with serum antibody titers following natural infection of young children, and infected children are more protected against reinfection with similar rather than different G serotypes. A protective role of placentally transferred maternal antibody among infants <3 months of age has also been speculated since rotavirus disease is uncommon in this age group. However, serum neutralizing antibody responses among vaccine recipients have sometimes correlated poorly with protection from disease; therefore, the exact role of serum antibody in protection against disease remains unclear.

Rotavirus Vaccines

Monovalent "Jennerian" Vaccines

Initial development of rotavirus vaccines was based on the Jennerian approach, which involved the use of a live, attenuated, antigenically related virus derived from a nonhuman host (21). This approach was prompted by studies indicating that animal and human rotaviruses shared a common group antigen and that experimental animals immunized with animal strains of rotavirus had a significantly lower risk for illness and viral shedding when subsequently challenged with human rotaviruses. Furthermore, neutralizing antibodies to human

rotavirus serotypes in the animal models indicated the potential for cross-protection.

Bovine Vaccines

The first two Jennerian vaccines were developed with bovine rotavirus strains RIT4237 and WC3. The WC3 strain was passaged in cell culture less than RIT4237 and was developed because of concern that excessive passaging of the RIT vaccine might cause overattenuation and diminished efficacy. RIT4237 and WC3 were nonreactogenic and immunogenic when administered to infants 2 to 18 months of age. However, the protection conferred by both vaccines varied greatly in efficacy studies, 0% to 76% against any rotavirus diarrhea and 0% to 100% against severe disease (22-32). A well-defined correlate of protection was not identified, and reasons for the variable efficacy were unknown, although late age at vaccination, timing of vaccination with respect to the onset of the rotavirus season, and variations in the strength and number of doses of the vaccine were proposed as contributing factors. Both vaccines performed less well in developing than in industrialized countries, possibly because of interference by other enteropathogens or inadequate surveillance during follow-up.

Rhesus Vaccine

The third Jennerian vaccine was developed with rhesus rotavirus strain MMU18006, which shares neutralization specificity with human rotavirus G3 strains. Besides sharing antigenic specificity with an epidemiologically important human rotavirus serotype, MMU18006 was suitable for vaccine development because it grew efficiently in cell culture. As in the bovine rotavirus-based vaccines, MMU18006 was safe and immunogenic, although in some trials, one third of infants became febrile 3 to 4 days after vaccination. The reactogenicity of MMU18006 was particularly high in two studies in Finland and Sweden in which 64% and 79% of infants, respectively, became febrile. Most children with febrile responses were >5 months of age; lack of passively transferred maternal antibody might have contributed to the high reactogenicity of the vaccine. As in the RIT4237 and WC3 vaccines, the protective efficacy of MMU18006 in field trials was quite variable, 0% to 60% against any rotavirus diarrhea and 0% to 85% against severe rotavirus diarrhea (33-39).

Reassortant "Modified Jennerian" Vaccines

The greatest efficacy of MMU18006 was observed in a Venezuelan trial in which the rotavirus strain circulating in the community (G3) was the same serotype as the vaccine strain, which suggested that serotype-specific immunity against each of the epidemiologically important strains of human rotaviruses may be required for maximum protection. Similar observations in vaccine challenge cross-protection studies in animals initiated the development of vaccines that used a modified Jennerian approach in which animal-human reassortants expressing VP7 proteins of serotypes 1 through 4 were used as the immunogens.

Rhesus-Human Reassortant Vaccines

Rhesus-human reassortants were generated by coinfecting cell cultures with rhesus rotavirus (RRV) strain MMU18006 (G serotype 3) and human rotavirus strains D (G serotype 1), DS-1 (G serotype 2), and ST3 (G serotype 4). Selection pressure (induced by the addition of neutralizing antibody to VP7 of RRV) produced reassortant strains D x RRV, DS-1 x RRV, and ST3 x RRV, each of which possessed the VP7 gene from HRV serotype 1, 2, or 4 and the other 10 genes from RRV (Figure 6) (40). Because vaccines made from the individual reassortants were safe and immunogenic, RRV-TV was developed incorporating each of the three reassortants and MMU18006 to provide coverage against the four common VP7 serotypes of rotavirus.

RRV-TV testing was initiated at an inoculum of 10^4 PFU of each of the four viruses (i.e., at 4 x 10^4 PFU) and completed at 10^5 PFU, the dose submitted for licensure (i.e., at 4 x 10^5 PFU). In most trials, vaccine was administered orally in three doses separated by at least 3 weeks to optimize the immune response to the component antigens; immunization was completed by the age of 6 to 7 months. Because the vaccine virus strains are acid labile, they are administered with 2.5 ml of citrate-bicarbonate buffer.

Safety. In clinical trials involving approximately 10,000 infants, the tetravalent vaccine has not been associated with any major adverse reactions. In a large multicenter trial in the United States, RRV-TV recipients had a small increase in febrile reactions (axillary temperature >38°C) on day 4 after the first dose (2.2% vs. 0.2%, $p = 0.02$) compared with placebo recipients;

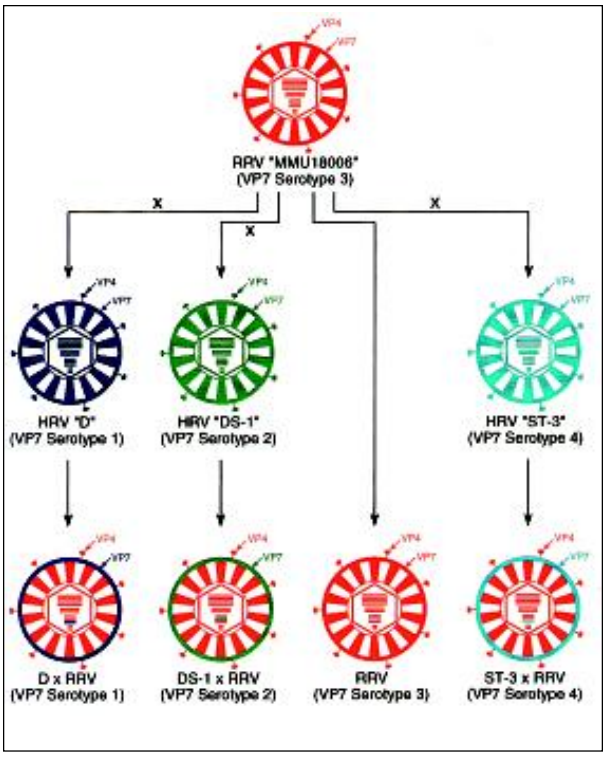

Figure 6. Schematic demonstration of production of rhesus rotavirus (RRV), human rotavirus (HRV) x rhesus rotavirus (RRV) reassortant quadrivalent vaccine with VP7 serotype 1, 2, 3, and 4 specificity. Reprinted with permission from (40).

no significant differences were observed in rates of fever after doses 2 and 3 and of vomiting and diarrhea after any dose (Figure 7). However, the fevers were mild compared with those associated with other childhood vaccines (e.g., diphtheria-tetanus-pertussis vaccine). The results of clinical trials of the monovalent parent RRV strain indicate that febrile reactions may be more frequent or severe among infants who receive the first dose of RRV-TV at >5 months of age.

In the Venezuelan trial of RRV-TV (41), PCR detected vaccine strains of rotavirus (in concentrations too low to be detected by routine methods such as EIA or polyacrylamide gel electrophoresis) in fecal samples from 15% of vaccinated and 13% of unvaccinated children. Although these data suggest the spread of vaccine virus to unvaccinated children, it is not known whether such spread would result in "herd protection" through induction of protective antibody responses in unvaccinated persons. Despite the theoretical risk for transmission of vaccine virus from RRV-TV recipients to

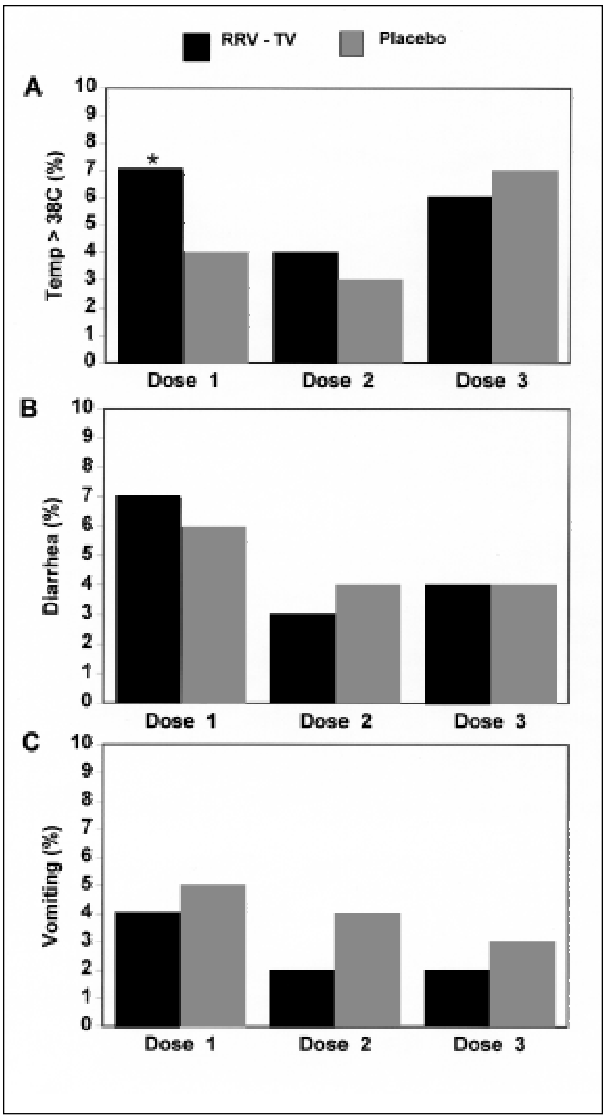

Figure 7. Percentage of children with adverse reactions during 5 days of surveillance following each of three doses of tetravalent rhesus-rotavirus vaccine (RRV-TV) or placebo. A) percentage with fever (as measured by axillary temperatures).*On day 4, 2.2% of RRV-TV recipients vs. 0.2% of placebo recipients became febrile (p = 0.02). B) percentage with diarrhea. C) percentage with vomiting. Adapted from (43).

household members and close contacts with known contraindications for receipt of RRV-TV (such as immunocompromised persons), this risk must be weighed against the benefit of protecting these immunocompromised persons by immunizing young children at risk for wild rotavirus infection.

Efficacy. Seven large efficacy trials have been completed using three doses of RRV-TV: four with the 4 X 10^5 PFU dose submitted for licensure and three with a lower dose (4 X 10^4 PFU) (Table) (41-47). The four trials at the 4 X 10^5 PFU dose yielded similar results: the vaccine demonstrated significant protection against any rotavirus diarrhea (49% to 68%), greater protection against severe rotavirus diarrhea (61% to 91%), and 50% to 100% efficacy in preventing doctor visits for diarrhea. The efficacy of the vaccine in developing countries has been variable, but in a recent trial involving more than 2,200 underprivileged urban children in Venezuela, vaccine efficacy approached levels seen in industrialized countries (48% against all episodes of diarrhea, 70% against episodes requiring hospitalization, 75% against dehydrating illness, and 88% against severe episodes of rotavirus diarrhea) (41). This large trial is the first to clearly show the potential usefulness of rotavirus vaccines in developing countries, where they are most needed.

Bovine-Human Reassortant Vaccines

Bovine-human reassortant rotavirus vaccines include a tetravalent WC3 rotavirus reassortant vaccine with genes coding for the VP7 of three major serotypes of rotavirus (G1, G2, and G3) and W179-4, a human VP4 reassortant with P[8] specificity. Theoretically, this vaccine should induce antibodies broadly reactive to the three common serotypes of rotavirus sharing P[8] specificity, thereby increasing the protective efficacy of this vaccine. In an efficacy trial of a three-dose regimen of the WC3 reassortant vaccine, protection was 67% against all rotavirus diarrhea and 69% against severe rotavirus diarrhea (48).

Other Candidate Vaccines

In clinical trials, no Jennerian vaccine has provided complete protection against rotavirus diarrhea; as a result, several non-Jennerian candidate vaccines are being developed. Vaccines based on neonatal, cold-adapted, and attenuated human strains of rotavirus are under evaluation (49). Other approaches, such as the use of baculovirus-expressed viruslike particles or naked DNA vaccines, are also being used to develop candidate rotavirus vaccines (50,51).

Implementing Rotavirus Vaccines

Introduction of rotavirus vaccines as routine childhood immunizations will be predicated on their safety, efficacy, expected impact, and cost. These aspects need to be addressed separately for industrialized and developing countries because of the difference in the epidemiology of rotavirus, the performance of rotavirus vaccines in clinical trials, and the logistics of implementing new vaccines.

In industrialized countries, the death rate from diarrheal illness has declined to a level that it no longer poses a serious threat to child survival. In the United States, diarrheal illnesses cause approximately 300 deaths per year, accounting for less than 2% of all childhood deaths. However, diarrhea still causes 170,000 hospitalizations each year, resulting in high medical and societal costs. Rotavirus is the most common pathogen identified in children hospitalized with diarrhea and is estimated to account for one third of all diarrheal illnesses. Large

Table. Efficacy of the tetravalent rhesus-human reassortant rotavirus vaccine (RRV-TV) in clinical trials

Country (ref)	Age of vaccinees	No. enrolled vaccine/placebo	Vaccine dose (PFU)	No. doses	Circulating strains	Vaccine efficacy (%) All disease	Vaccine efficacy (%) Severe disease
Industrialized							
Finland (42)	3-5 mo	1,191/1,207	4 x 10^5	3	G1,G2	68	61-100
United States (43)	5-25 wk	403/400	4 x 10^5	3	G1,G3	49	80-100
United States (44)	2-6 mo	347/348	4 x 10^5	3	G3	50	64
United States (45)	4-26 wk	332/330	4 x 10^4	3	G1,G3	57	82
Developing							
Venezuela (41)	8-18 wk	1,112/1,095	4 x 10^5	3	G1	48	88
Peru (46)	2-4 mo	219/209	4 x 10^4	3	G1,G2	24	0-40
Brazil (47)	1-5 mo	233/233	4 x 10^4	3	G1,G2	35	46

multicenter trials in the United States have convincingly demonstrated that RRV-TV can safely prevent half of all mild rotavirus illnesses, 80% of severe episodes, and nearly all hospitalizations associated with rotavirus diarrhea. A routine, universal immunization program against rotavirus would prevent an estimated 227,000 physician visits, 95,000 emergency room visits, and 34,000 hospitalizations from diarrhea among children in the first 5 years of life (6). Therefore, even at a relatively high operational cost, an immunization program against rotavirus is likely to be cost-effective and can be recommended for the United States and probably for other industrialized countries on the basis of similar considerations.

In developing countries, recommending rotavirus vaccines for universal childhood immunization is more complex. With approximately 2,000 children dying each day from rotavirus diarrhea, the potential benefit of rotavirus vaccines is much greater in developing than in industrialized countries. Yet several questions remain unanswered.

Will rotavirus vaccines be as efficacious in developing countries as in industrialized countries? Several factors—younger age at infection, potentially larger inoculum of infection, presence of unusual strains of rotavirus, interference by other enteropathogens, and poorer nutritional status of children—could adversely affect the efficacy of rotavirus vaccines in developing countries. While the high efficacy observed in Venezuela is encouraging, further efficacy studies are needed in other parts of the developing world.

How many doses of vaccine are required for maximum protection against rotavirus diarrhea? In all clinical trials of the tetravalent rotavirus vaccine, three doses were administered to children 4 weeks to 6 months of age. For this reason and because of a lack of good correlation between neutralizing antibody response and protection from disease, it is difficult to determine whether fewer vaccine doses would confer the same level of protection. While fewer vaccine doses are appealing for programmatic and economic reasons, multiple vaccine doses may be required to overcome the factors that could reduce the efficacy in developing countries.

Finally, can the developing world afford another childhood vaccine? A vaccine priced in the $10- to $30-per-dose range may be cost-effective for industrialized countries but unaffordable for developing countries where the total per capita health-care expenditure may be $10 to $20 per year or less. Nonetheless, because the vaccine is prepared from virus grown in tissue culture, the price may be much lower once the developmental costs are recovered and the vaccine is produced and marketed locally. In addition, by recovering a major portion of manufacturing costs from the middle- and upper-class population, vaccine manufacturers may be able to provide vaccine to the underprivileged at a nominal price. Ultimately, the rate at which rotavirus vaccines are incorporated into immunization programs will depend not only on economic considerations but also on their perceived value by national governments and international and bilateral agencies (52).

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Chlamydia pneumoniae and Cardiovascular Disease

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Chlamydia pneumoniae is a ubiquitous pathogen that causes acute respiratory disease. The spectrum of *C. pneumoniae* infection has been extended to atherosclerosis and its clinical manifestations. Seroepidemiologic studies have associated *C. pneumoniae* antibody with coronary artery disease, myocardial infarction, carotid artery disease, and cerebrovascular disease. The association of *C. pneumoniae* with atherosclerosis is corroborated by the presence of the organism in atherosclerotic lesions throughout the arterial tree and the near absence of the organism in healthy arterial tissue. *C. pneumoniae* has also been isolated from coronary and carotid atheromatous plaques. To determine whether chronic infection plays a role in initiation or progression of disease, intervention studies in humans have been initiated, and animal models of *C. pneumoniae* infection have been developed. This review summarizes the evidence for the association and potential role of *C. pneumoniae* in cardiovascular disease.

Chlamydia pneumoniae, a common cause of human respiratory disease, was first isolated from the conjunctiva of a child in Taiwan in 1965 and was established as a major respiratory pathogen in 1983 when it was isolated from the throat of a college student at the University of Washington. *C. pneumoniae* causes approximately 10% of community-acquired pneumonia and 5% of pharyngitis, bronchitis, and sinusitis (1). The clinical symptoms of *C. pneumoniae* pulmonary infections are similar to those caused by other respiratory pathogens, except for a few distinguishing features (1). Subacute onset and pharyngitis are common. Often a biphasic pattern is observed, with pharyngitis resolving before bronchitis or pneumonia develops. Cough is very common and prolonged. Although pneumonia is often relatively mild, recovery is slow, even with antibiotic therapy; and cough and malaise may persist for many weeks.

Considerable knowledge of the epidemiology of *C. pneumoniae* infection has been derived from serologic studies using the *C. pneumoniae*–specific microimmunofluorescence test. *C. pneumoniae* infection is ubiquitous. Virtually everyone is infected at some point in life, and reinfection occurs commonly. Antibodies against *C. pneumoniae* are rare in children under the age of 5, except in developing and tropical countries. Antibody prevalence increases rapidly at ages 5 to 14, reaches 50% at the age of 20, and continues to increase slowly to 70% to 80% at ages 60 to 70 (1).

C. pneumoniae has been associated with other acute and chronic respiratory diseases (e.g., otitis media, chronic obstructive pulmonary disease, pulmonary exacerbation of cystic fibrosis, and asthma) as well as other clinical syndromes (e.g., erythema nodosum, Reiter syndrome, and sarcoidosis [1]). These associations are determined by seroepidemiologic observations, case reports, isolation or direct detection of the organism in specimens, successful response to antichlamydial antibiotics, or a combination of these methods.

The expanding spectrum of *C. pneumoniae* infection has been extended to atherosclerosis and related clinical manifestations such as coronary heart disease, carotid artery stenosis, aortic aneurysm, claudication (occlusion of the arteries of the lower extremities), and stroke. This overview summarizes the studies associating *C. pneumoniae* infection with atherosclerosis and discusses preliminary in vitro and in vivo studies suggesting the plausibility of a causative role.

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Is *C. Pneumoniae* Present in Atherosclerotic Tissues?

Patients with coronary artery disease are significantly more likely than healthy persons to have serologic evidence of past infection with *C. pneumoniae* (2,3). Immune complexes containing *C. pneumoniae* lipopolysaccharide have also been associated with 42- and 98-kDa *C. pneumoniae* species-specific antigens (4). These associations have been confirmed and extended to carotid artery disease and cerebrovascular disease (5,6). Although the high prevalence of *C. pneumoniae* antibodies in the population leaves a very narrow window for demonstrating statistically significant differences between cases and controls, seroepidemiologic studies have shown a consistent association. *C. pneumoniae* antibodies have been associated with coronary heart disease (16 studies) and cerebrovascular disease (2 studies) in 17 of 18 published studies; most had an odds ratio of 2.0 or greater (6). Most were statistically significant, and the risk is independent of other atherosclerosis risk factors (i.e., hypercholesterolemia, cigarette smoking, hypertension, diabetes, and family history). Studies of 2,700 patients and 5,000 controls have demonstrated a serologic association of *C. pneumoniae* antibody and cardiovascular disease (6).

Compelling evidence of the association between *C. pneumoniae* and atherosclerosis has been obtained by polymerase chain reaction (PCR), immunocytochemical (ICC) staining, and electron microscopy, which have detected *C. pneumoniae* in atherosclerotic lesions (Table). Structures found within coronary atheromas were remarkably similar to the pear-shaped elementary body morphologic characteristics described for *C. pneumoniae* (Figure 1) (25). By ICC staining using a *C. pneumoniae*–specific monoclonal antibody (Mab), *C. pneumoniae* was demonstrated within the atherosclerotic lesion in 5 of 7 tissues. By PCR or ICC stain, the organism was detected in 20 of 36 coronary artery tissues from autopsy (7). At the University of Washington, the organism has been detected in coronary, carotid, aortic, femoral, and popliteal atheromas in both early lesions and fibrolipid plaques (7-10,15,18,22,26,27). In all studies using ICC stain, control tissues were stained with control antibody to rule out background staining (Figure 2). The organism was found in tissues of male and female study participants of different ages and ethnic groups. Other investigators have confirmed these findings and have also found the organism in atherosclerotic lesions in iliac arteries and tissues from abdominal aortic aneurysms and

Table. Studies of *Chlamydia pneumoniae* in atherosclerotic tissue

Source of specimens (reference)	Artery	Type of specimen	Atherosclerotic tissue[a] (% positive)
South Africa (7)	Coronary	Autopsy	20/36 (56)
PDAY[b] study (8)	Coronary	Autopsy	8/18 (44)
Univ. Washington (9)	Coronary	Atherectomy	20/38 (53)
Alaskan Natives (10)	Coronary	Autopsy	23/59 (39)
Louisville, Kentucky (11)	Coronary	Vascular surgery	7/12 (58)
Japan (12)	Coronary	Atherectomy	20/29 (69)
Salt Lake City, Utah (13)	Coronary	Atherectomy	71/90 (79)
India (14)	Coronary	Coronary artery bypass	4/40 (10)
California & Univ. Washington (15)	Carotid	Endarterectomy	37/61 (61)
Germany (16)	Carotid	Endarterectomy	7/50 (14)
Canada (17)	Carotid	Endarerectomy	54/76 (71)
Univ. Washington (18)	Aorta	Autopsy	7/21 (33)
Finland (19)	Aorta	Vascular surgery	12/12 (100)
Italy (20)	Aorta	Vascular surgery	26/51 (51)
United Kingdom (21)	Aorta, femoral, iliac	Vascular surgery	15/33 (45)
California (22)	Popliteal femoral	Vascular bypass	10/23 (43)
Finland (23)	Aortic valve	Autopsy	25/46 (54)
Sweden (24)	Aortic Valve	Surgery	19/39 (49)

[a]Number positive by immunocytochemical staining and/or polymerase chain reaction over number tested.
[b]PDAY, pathobiological determinants of atherosclerosis in youth.

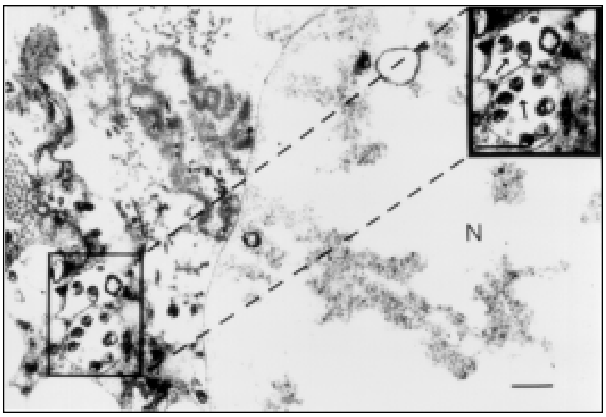

Figure 1. Ultrastructural evidence of *Chlamydia pneumoniae* in coronary atheroma. This transmission electron micrograph demonstrates the presence of endosomes containing *C. pneumoniae* pear-shaped elementary bodies within a foam cell in tissue from coronary artery atheroma. Arrows in inset point to the elementary bodies. Bar=0.5 μm. (Reprinted from Journal of Infectious Diseases (8) with permission from the publisher, University of Chicago Press).

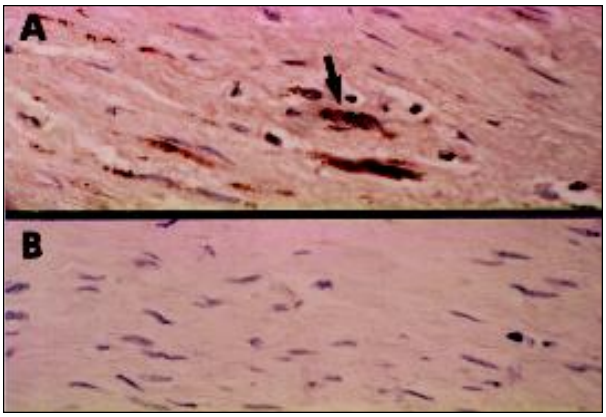

Figure 2. Immunocytochemical staining demonstrating *Chlamydia pneumoniae* in fibrolipid plaque from coronary artery atheroma. Panel A illustrates positive staining of foam cells in the plaque with the *C. pneumoniae*–specific monoclonal antibody TT-401. Panel B shows negative staining of the adjacent section using normal ascites fluid as the control.

aortic valve stenosis (11-14,16,17,19-21,23,24,28). The organism has been found in atherosclerotic lesions in 257 (52%) of 497 tissue specimens (6). In contrast, the organism has been found in only 5% of tissues that appear normal. Remarkably, the detection in diseased arterial tissue compared to normal arterial tissue represents an odds ratio of 10 [95% Confidence Interval (CI) 5-22]. In our studies the organism was not found in cardiovascular tissue that appeared histologically normal (5).

C. pneumoniae has been difficult to isolate from atheromatous tissues, not surprising as isolation from infected tissues in chronic chlamydial infection or after repeated experimental inoculation of animal models is rare. However, the organism is often demonstrated by DNA and antigen detection methods, and a chronic inflammatory response persists. The immunologic characteristics of atherosclerosis are similar to the inflammatory response resulting from chronic infection. In two recent cases, *C. pneumoniae* has been isolated from atheromatous plaques (11,15). The first isolate was obtained during a multicenter study that examined coronary arteries of patients (with or without coronary artery disease) undergoing heart transplants. The organism was found in the coronary artery of 7 of 10 patients with atherosclerosis; it was not found in two patients without evidence of coronary atherosclerosis. *C. pneumoniae* was isolated from the atherosclerotic lesion of one patient with severe coronary artery disease (11).

The second isolate was from carotid atheroma obtained in a study of patients undergoing carotid endarterectomy in Seattle. *C. pneumoniae* was detected in 11 of 16 specimens and was cultured from a patient undergoing elective carotid endarterectomy (15).

In addition to *C. pneumoniae*, other infectious agents including herpes simplex virus (HSV), cytomegalovirus (CMV), and *Helicobacter pylori* have been associated with cardiovascular disease. Only a few studies have concurrently investigated the presence of *C. pneumoniae* and these infectious agents within lesions. In a study of risk factors for atherosclerosis in young persons, 6 of 7 samples with atheromas and 2 of 11 with intimal thickening were positive for *C. pneumoniae*; all 31 unaffected tissues were negative (8). Two specimens from atheromas and two from normal tissues were positive for CMV. Only one coronary atheroma was positive for both CMV and *C. pneumoniae*. In a study investigating the presence of *C. pneumoniae*, CMV, and HSV in atherosclerosis of the carotid artery, *C. pneumoniae* was also detected more

frequently (71%) than CMV and HSV (35% and 10%, respectively). Infection with two or three of these agents occurred in 23.7 and 7.9%, respectively, and *C. pneumoniae* and CMV were independently associated with an increased risk for thrombosis (17). In a study addressing the presence of *C. pneumoniae* or *H. pylori* or both in aortic aneurysm, *C. pneumoniae* was found in 26 of 51 patients with plaques (20). Despite the fact that 47 of 51 patients were seropositive for *H. pylori*, this agent was not detected in any atherosclerotic plaques (20). Thus, although *C. pneumoniae* and HSV or CMV may be found in the same lesion, *C. pneumoniae* has been more frequently found as the only infectious agent.

Does *C. pneumoniae* Play a Role in Atherogenesis?

Despite solid evidence that *C. pneumoniae* exists in atherosclerotic lesions, evidence that the presence of the organism is related to disease pathogenesis is circumstantial. Three possibilities can be examined. The organism 1) persists in vascular cells but does not contribute to pathologic abnormality, 2) causes the initial injury and induces the atherosclerotic process, or 3) accelerates the severity or progression of the disease. If the organism is involved, its role must fit within the context of events in atherogenesis. The early events in lesion development include endothelial injury or activation resulting in monocyte/macrophage adherence to the endothelium, migration to the subendothelium, uptake of oxidized low-density lipoproteins transforming them into foam cells, and release of cytokines. These cytokines upregulate endothelial cell adhesion molecules leading to increased leukocyte adhesion. Platelet aggregation at the site of endothelial damage results in the release of platelet-derived growth factor, which stimulates smooth muscle cell proliferation. Dedifferentiated smooth muscle cells secrete collagen, elastin, and proteoglycans leading to the formation of fibrous tissue. The mature fibrolipid plaque consists of a lipid/cholesterol-rich core surrounded by a fibrous cap composed of matrix elements (29).

Human Tissue Studies

To investigate the hypothesis that *C. pneumoniae* is an "innocent bystander," one study explored how frequently the organism was found in tissues from different anatomic sites. Multiple tissues, including coronary artery, lung, liver, spleen, and bone marrow, were obtained at autopsy from 38 patients, half of whom had cardiovascular disease recognized before death. Twenty-one of the patients had *C. pneumoniae* in one or more tissues. Eighteen had the organism in cardiovascular tissue (11 in cardiovascular tissue only) and seven in both cardiovascular and noncardiovascular tissue. Of the remaining three *C. pneumoniae*–positive patients, two had the organism only in lung tissue and one only in the spleen. While this study shows that *C. pneumoniae* could be detected in noncardiovascular tissue, it also shows the organism is much more frequently found in cardiovascular tissue (27).

Another possible explanation for the frequent presence of *C. pneumoniae* in atheroma is that infected lung macrophages disseminate to any granulomatous tissue. Of 33 surgical or autopsy specimens of granuloma from patients with tuberculosis, leprosy, coccidioidomycosis, Crohn disease, Wegner disease, rheumatoid nodule, giant cell tumor, or sarcoidosis, three contained *C. pneumoniae*, all sarcoid skin granulomas (27). Sarcoidosis is a disease of unknown etiology for which a serologic association with *C. pneumoniae* has been reported (30). These studies suggest that *C. pneumoniae*–infected cells are found preferentially within atheromas.

In Vitro Studies

Consistent with observations in animal models and humans, in vitro studies have demonstrated that vascular cells are susceptible to *C. pneumoniae* infection and that *C. pneumoniae* produces productive infection in human macrophages, endothelial cells, and artery smooth muscle cells (31,32), key cellular components in atherosclerosis. In vitro studies have also measured whether infection leads to the production of immunomodulators. The primary host cells are the epithelial cells that line the trachea and nasopharynx. The first in vitro study addressing a potential role of *C. pneumoniae* in atherogenesis found that exposure of human monocyte-derived macrophages to *C. pneumoniae* followed by the addition of low-density lipoprotein resulted in foam cell formation and the accumulation of cholesteryl esters (33). Foam cell formation is an early event in the atherosclerotic process.

Ultrastructural studies have demonstrated *C. pneumoniae* in ciliated bronchial cells in mice, in lung macrophages in mice and rabbits, and in human foam cells (which are macrophages) and smooth muscle cells that take up lipids (7,34,35); by double ICC staining with chlamydia-specific and cell-specific MAbs, *C. pneumoniae* was found in both smooth muscle cells and macrophages within aortic atheroma lesions (18). Similarly, in specimens removed from patients with symptomatic coronary artery disease, *C. pneumoniae* was found within macrophages in atherectomy tissue was also found in tissue removed at atherectomy, *C. pneumoniae* bacteremia, as determined by PCR positivity of buffy coat specimens, was found in 13% of patients with symptomatic coronary atherosclerosis; control specimens were negative (13,53). In mouse models of *C. pneumoniae* infection, *C. pneumoniae* was detected by culture and PCR in peripheral blood mononuclear cells but not in plasma after intranasal inoculation (36).

The ability of *C. pneumoniae* infection to induce production of proinflammatory and procoagulant activities was investigated to determine its putative role in eliciting immune responses consistent with atherosclerotic processes. *C. pneumoniae* infection of human vascular endothelial cells results in production of tissue factor, increased levels of monocyte chemotactic protein-1, and increased platelet adhesion to infected cells (37,38). Infection of endothelial cells also results in expression of adhesion molecules, including E-selectin, intercellular adhesion molecule-1, and vascular adhesion molecule-1, which are important in leukocyte adhesion (38,39). Lastly, infection of macrophages results in the production of proinflammatory cytokines, tumor necrosis factor alpha, interleukin-1 beta, interleukin-6, and interleukin-8 as well as in expression of CD14 molecules (38,40).

Animal Models

Although studies support a potential role for *C. pneumoniae* in atherogenesis, etiology can be established only through animal models or intervention studies. Rabbits and mice are susceptible to *C. pneumoniae* infection and provide well-defined models of atherosclerosis. Respiratory disease in both species is characterized by multifocal interstitial pneumonia. The disease is more severe and longer lasting in mice, and organisms are reisolated more readily from the lungs and aorta.

Rabbit models have been used to determine whether *C. pneumoniae* respiratory infection leads to vessel wall infection and inflammatory changes characteristic of atherosclerosis. In one study, New Zealand White rabbits received a single nasopharyngeal inoculation of *C. pneumoniae* at 1 month of age. Two of ten rabbits demonstrated atherosclerotic changes. The changes were observed 7 and 14 days postinoculation. One rabbit had an accumulation of foam cells in the aortic arch (characteristic of an early lesion) and focal perioaortitis in the abdominal aorta (41). A second rabbit demonstrated spindle cell proliferation of smooth muscles cells in the aorta. Both rabbits had bronchiolitis and pneumonitis (41). In another study, atherosclerotic-li ke changes were found in the aortas of six of nine New Zealand White rabbits 2 to 4 weeks after they received two inoculations of *C. pneumoniae* (42). In both studies, rabbits were fed normal diets, and atherosclerotic-like changes were not observed in any of the controls.

We used two mouse models, C57BL/6J and apolipoprotein E (apoE)-knockout, to test the hypothesis that following upper respiratory tract infection, lung macrophages are infected, disseminate to the aorta, and contribute to atherogenesis. C57BL/6J mice, the background strain of apoE-knockout mice, get atherosclerosis only if fed a diet high in fat and cholesterol. In contrast, apoE-knockout mice get atherosclerotic lesions, with some characteristics of human disease, spontaneously on a regular chow diet in a time- and age-dependent manner. Using C57Bl/6J mice, we demonstrated that after intranasal or intraperitoneal infection, *C. pneumoniae* infects alveolar and peritoneal macrophages, respectively (36). Additionally, the organism was found in blood monocytes and not in plasma cells, which indicates that a cell-associated bacteremia followed acute infection. Passive transfer by intraperitoneal inoculation of alveolar or peritoneal macrophages obtained from mice inoculated intranasally or intraperitoneally with *C. pneumoniae* resulted in dissemination of infection to the lung, thymus, spleen, and abdominal lymph nodes (36).

To determine whether infection disseminates to the aorta and is found within atherosclerotic lesions as in human disease,

apoE-knockout mice received single or multiple intranasal inoculations. *C. pneumoniae* was detected for up to 20 weeks postinfection in the aorta and within the lesion in apparent foam cells by ICC staining (43). When the aorta was positive, the percentage of *C. pneumoniae*–positive mice was 33% to 100%. Controls remained negative for *C. pneumoniae* (43). In contrast, 1 (0.8%) of 12 animals of the background strain (C57BL6/J mice) fed a nonatherogenic diet contained the organism in the aorta for up to (but not later than) 2 weeks postinfection. Like studies of human granulomatous tissues and tissues from different anatomic sites (27,43), these studies suggest that the organism has a tropism to atherosclerotic lesions.

Computer-assisted morphometric analysis of lesion size has been used to determine if *C. pneumoniae* infection alters disease progression. Comparisons of 10 infected and 10 uninfected mice at different times following three intranasal inoculations of *C. pneumoniae* suggest that infection significantly augments the progression of lesion size (44). Thus, early studies in rabbit and mouse models indicate that *C. pneumoniae* infection can induce inflammatory changes similar to those of atherogenesis and augment the progression of the atherosclerotic lesion.

Persistent Infection

Although *C. pneumoniae* antigen and DNA are often found in atheromas, isolation is rare. Similarly, in the mouse model of *C. pneumoniae* infection, lungs remain PCR positive and pathologic lesions persist after the organism can no longer be cultured. In the apoE model, the organism can be cultured from the lung and aorta in a few mice for up to 3 weeks postinfection but is detected by PCR and ICC up to 20 weeks postinfection at both sites. Does PCR positivity in the absence of culture positivity reflect persistent infection or undegraded DNA? Two lines of evidence strongly support the presence of viable organisms. Two independent studies using two different strains of mice have demonstrated that lung infection could be reactivated by treatment with cortisone (45,46), specifically after intranasal inoculation, when the organism could no longer be cultured from lungs. Animals were treated with cortisone or saline. With saline tratment, *C. pneumoniae* was not cultured, although animals were frequently PCR positive. In contrast, with cortisone treatment, reactivated infection was demonstrated by culture in 46% and 60% of infected mice. The second line of evidence came from mice acutely infected intranasally with live or UV-inactivated organisms; alveolar macrophages were isolated at various times postinfection. When live organisms were used, the organism could be cultured and detected by PCR up to 7 days postinfection. In contrast, when mice were inoculated with UV-inactivated organisms, the organism could only be detected by PCR immediately after inoculation in isolated macrophages (36). These experiments demonstrated that DNA from dead organisms is rapidly degraded, whereas live organisms survive within macrophages.

Drug efficacy studies of chlamydia in animal models or humans must be interpreted with caution because chlamydial infections can persist after antibiotic therapy. The efficacy of two antibiotic therapies was investigated in the mouse model of pneumonitis. After infection, mice were treated with a single dose of doxycycline each day for 3 days or with a single dose of azithromycin. After either treatment, when the organism could no longer be cultured, the infection appeared to be cleared. However, *C. pneumoniae* DNA could be detected by PCR isolation in 25% or 77% of mouse lungs, depending on the infecting dose. No differences were observed in the lungs of treated and untreated mice (47). These results suggest that the organism can persist after single-dose treatment regimens and that prolonged treatment may be needed.

Intervention Studies

Animal Models

Animal models of *C. pneumoniae* infection and atherosclerosis further define a causative role by determining whether intervention with antimicrobial agents can alter disease progression and by identifying successful treatment regimens. New Zealand White rabbits were fed a diet with 0.25% cholesterol and were inoculated intranasally three times with *C. pneumoniae* (48). Infected rabbits and controls were treated for 7 weeks with azithromycin. Three months

after the final inoculation, the maximal intimal thickness (MIT) of the thoracic aortas increased in infected rabbits but not in controls. The MIT of azithromycin-treated rabbits was less than that of untreated infected rabbits and similar to that of controls. However, the organism was detected by immunofluorescence in the aorta of treated rabbits as frequently as it was detected in the aorta of untreated rabbits (48).

Human Studies

Three small pilot studies on the potential use of antibiotics against *C. pneumoniae* have yielded promising results. The first intervention study focused on male patients at least 6 months after myocardial infarction. Titers were arbitrarily classified as seronegative (8), intermediately positive (>8, 32), or highly positive (64). Those who had serum antibodies that persisted 3 months later were treated with one or two courses of azithromycin (500 mg per day orally for 3 days). Treated patients had a fivefold decrease in cardiovascular events and a reduction in immunoglobulin (Ig)G titer (49).

The second study included 202 patients hospitalized with unstable angina or non-Q wave infarctions. The study was randomized with half of the patients receiving roxithromycin (150 mg twice a day for 30 days) and the other half receiving a placebo. Serology was not considered for inclusion or exclusion in the study. After 30 days, a statistically significant decrease in cardiovascular events was observed in treated patients, while those receiving the placebo were treated only when a combined endpoint of severe recurrent angina, acute myocardial infarction, or ischemic death was used (50).

In the third (randomized) study, 88 patients with percutaneous coronary revascularization procedures were treated with azithromycin (500 mg per day for 2 days and 250 mg per day for 28 days) or received placebo (51). After 6 months, patients receiving azithromycin had lower frequencies of both angiographically confirmed restenosis (9% versus 16%) and recurrent angina (40%) than patients receiving placebo (60%). No changes in antibody titers were observed after azithromycin treatment. Despite study limitations (52) and antiinflammatory effects of the antibiotic on atherosclerosis, the results are encouraging and warrant carefully designed larger-scale intervention studies with longer observation times.

Conclusions

A causative role of *C. pneumoniae* infection in cardiovascular disease has not yet been firmly established. However, the high frequency of infection found in human atherosclerotic tissue in comparison to normal tissue, the induction and progression of atherosclerotic-like inflammatory changes in infected animal models of atherosclerosis, and the early results from antichlamydial intervention studies in humans are consistent with a causative role of *C. pneumoniae* in the disease process.

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Bacterial Symbiosis in Arthropods and the Control of Disease Transmission

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Bacterial symbionts may be used as vehicles for expressing foreign genes in arthropods. Expression of selected genes can render an arthropod incapable of transmitting a second microorganism that is pathogenic for humans and is an alternative approach to the control of arthropod-borne diseases. We discuss the rationale for this alternative approach, its potential applications and limitations, and the regulatory concerns that may arise from its use in interrupting disease transmission in humans and animals.

For more than 80 years, insecticides have been one of the primary means of controlling insect-borne diseases. Recently, however, the control of insect pests and vectors of disease has become increasingly difficult for various reasons, including the emergence of insecticide resistance, changes in the environment, and reduction in public health interventions due to social and economic problems in countries where insect-borne diseases are endemic. According to the World Health Organization (WHO), approximately 125 arthropod species are resistant to at least one, and often two or more, insecticides (1). In many parts of the world where insect-borne diseases cause illness and death, insecticides are available; however, sustaining insecticide vector control long term can be extremely costly and may be unachievable.

Alternative Approaches to Controlling Disease Transmission by Arthropods

Concerns related to the use of insecticides for vector control have led to alternative approaches for reducing disease transmission by arthropods. One approach focuses on the use of transgenic methods, i.e., the insertion and expression of a gene derived from one organism in a second, heterologous organism. Major components of the transformation system include the identification of 1) potential DNA vectors (transposable elements or viral-transducing agents) for genome integration (2-8); 2) selectable phenotypic marker genes, such as eye color mutants or various enzymes (e.g., ß-galactosidase, neomycin phosophotransferase, or organophosphate-degrading enzyme), as indicators of stable germ line transformation (9-11); and 3) specific refractory genes that express the desired phenotype (i.e., a factor that would inhibit transmission of the pathogen) (12-14). Additional studies have focused on the identification of regulatory sequences (e.g., stage-specific, tissue-specific, and constitutive promoters [15-20]), vector population genetics (21-23), and the development of mathematical models that can be used for predicting the behavior of genes once they are introduced into wild populations (24,25). The accomplishments and future prospects of efforts to produce transgenic arthropods have been discussed extensively (26-31).

Another approach for reducing disease transmission by arthropods is to genetically modify symbiotic bacteria of arthropod vectors to prevent the arthropods from transmitting human pathogens. With this approach, the arthropod is not transformed, but the symbiotic bacteria that it harbors are (32). Such arthropods are called paratransgenic. This approach is guided by the following basic concepts: 1) many arthropods (especially those that throughout their entire developmental cycle feed on restricted food sources such as blood, cellulose, phloem, stored grains) harbor bacterial symbionts; 2) in some cases, these symbionts can be

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cultured and genetically transformed to express a gene whose product kills a pathogen that the arthropod transmits; 3) normal arthropod symbionts can be replaced with genetically modified symbionts, resulting in a population of arthropod vectors that can no longer transmit disease. While not applicable to all groups of arthropods, this approach has worked in three species of Chagas disease vectors and holds promise in a number of other arthropods.

Bacterial Symbionts and Control of American Trypanosomiasis

American trypanosomiasis (Chagas disease) is a parasitic illness that affects 16 to 18 million people in regions of South and Central America, according to current WHO estimates. Neither a cure for chronic Chagas disease nor a vaccine for preventing infection is available. The etiologic agent, the flagellate protozoon *Trypanosoma cruzi*, is transmitted by blood-sucking triatomine bugs, which become infected while feeding on an infected host. Transmission to humans occurs as the insect feeds when the engorged triatomine bug, while feeding, deposits on the skin a fecal droplet that contains infective trypanosomes, which then get rubbed either into the bite lesion or a mucous membrane (Figure 1).

Rhodnius prolixus is the chief vector of Chagas disease in certain regions of Central America and northern South America. *Rhodococcus rhodnii*, a soil-associated nocardiform actinomycete, residing extracellularly in the gut lumen of *R. prolixus* in close proximity to *T. cruzi* (33), is transmitted effectively from adult triatomid bugs to their progeny through coprophagy (ingestion of fecal material from other bugs). The vital role of *R. rhodnii* in the growth and development of *R. prolixus* has been demonstrated repeatedly under laboratory conditions (34-36). Aposymbiotic nymphs of *R. prolixus* (insects that have been cured of symbionts) do not reach the sexually mature adult stage; most deaths occur after the second developmental molt. Introduction of the bacteria to first or second instar nymphs permits normal growth and maturation.

Scientists have exploited this symbiotic association to introduce and express a series of foreign gene constructs in *R. prolixus* (37,38). DNA shuttle plasmids capable of replication in both *Escherichia coli* and in *R. rhodnii* have been constructed and used to genetically

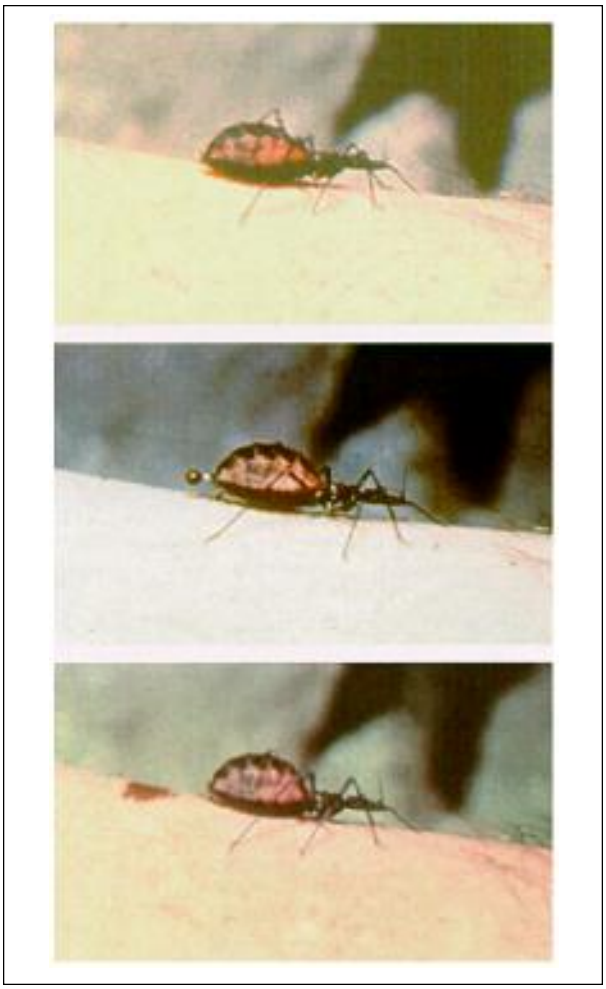

Figure 1. A triatomine bug vector of Chagas disease in the process of feeding. The fecal droplet contains infective trypanosomes and bacterial symbionts. (Photographs courtesy of Robert B. Tesh).

modify *R. rhodnii* (Figure 2). The genetically altered symbionts can then be introduced into aposymbiotic first instar nymphs of *R. prolixus,* where, like unmodified microorganisms, they allow normal growth and reproduction of the insect while expressing specific gene products of interest.

In our laboratory, a selectable genetic marker coding for resistance to the antibiotic thiostrepton was expressed by transgenic symbionts within the gut of insects colonized experimentally (37). The insects could be fed on blood that contained thiostrepton, and the bacteria survived and persisted throughout the insect's development to adulthood. Furthermore, we showed that when thiostrepton was omitted from the blood, the symbionts maintained

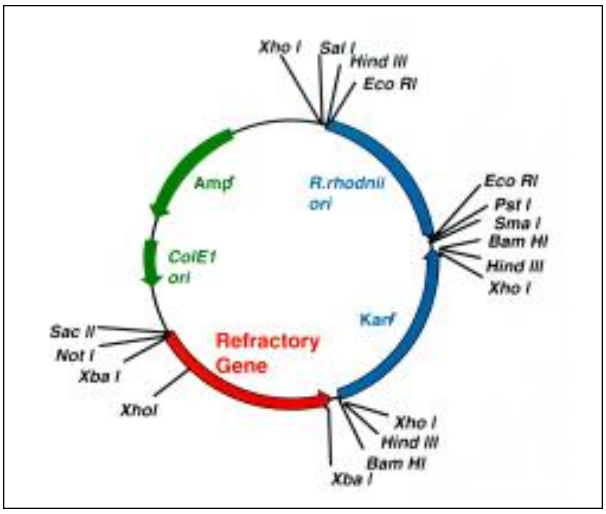

Figure 2. Shuttle plasmid for genetic transformation of *Rhodococcus rhodnii*.

resistance to the antibiotic, which indicates that the plasmid was stable in its bacterial host. By sterilizing the surface of the eggs with a topical iodine solution and colonizing the resulting aposymbiotic (sterile) insects with genetically modified symbionts (delivered to the insects orally), we developed a line of paratransgenic arthropods in which individual insects were refractory to infection with *T. cruzi* (38). Refractoriness was conferred after the antimicrobial peptide L-Cecropin A was expressed and secreted. The gene for this peptide was contained in a shuttle plasmid expression vector used to transform *R. rhodnii,* which subsequently expressed and secreted the gene product within the gut of experimentally colonized insects (38). Cecropin A, a 38–amino acid peptide, belongs to a family of small channel-forming peptides with potent antimicrobial activity; these peptides insert into biologic membranes, forming channels that ultimately lead to cell lysis and death (39-41). This family of peptides has been isolated from several insect and vertebrate tissues, which they defend against bacterial pathogens (41). Cecropin A has strong lytic activity against *T. cruzi* but negligible deleterious effects on *R. rhodnii* or gut tissues of *R. prolixus*. Laboratory colonies of *R. prolixus* that carried genetically altered *R. rhodnii* transformed to express mature Cecropin A were completely refractory to infection with *T. cruzi* strain DM28 in approximately 70% of the insects. In the remaining insects, numbers of *T. cruzi* were reduced to less than 1% of the numbers seen in control *R. prolixus*, which carried untransformed *R. rhodnii*.

These studies demonstrate that symbiotic bacteria of disease-transmitting triatomine bugs can be genetically modified to express biologically active molecules and then can be reintroduced into the host insect, expressing the desired phenotype (Figure 3). The genetically altered *R. rhodnii* allowed normal development and sexual maturation of the host insect.

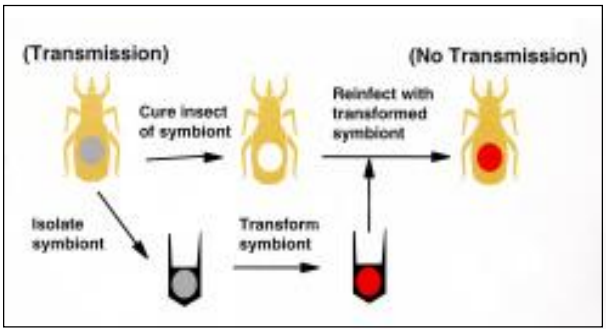

Figure 3. Symbionts can be genetically altered and used to replace native symbionts, resulting in insects that can no longer transmit disease. (Illustration courtesy of Mark Q. Benedict).

Delivery Mechanisms/Field Applications

For the bacterial symbiont approach to be used in a field intervention program, a mechanism must be developed for spreading the genetically altered bacteria through an insect population. The delivery system must allow dispersal of recombinant genetic material without adverse environmental effects. Using bacteria that have specialized symbiotic associations with specific insect hosts to spread transgenes greatly reduces the chance of unwanted gene spread; the natural insect-symbiont association can be used for this purpose. In *R. prolixus,* early instar nymphs acquire the symbiont *R. rhodnii* by coprophagy. When they first emerge, instar nymphs are transiently aposymbiotic; they pick up the required bacteria by probing the eggshell or fecal droplets of other bugs (Figure 1). Scientists have observed that triatomine bugs actively probe small dots of black ink on paper, apparently because they resemble the black fecal droplets shed by triatomine bugs after digestion of the bloodmeal. When live bacterial cultures and a small amount of India ink are added to an inert

carrier, a formulation of the genetically modified bacteria is produced that resembles bug feces and can be ingested by immature insects; in this way, modified bacteria can be established uniformly and effectively in laboratory colonies of *R. prolixus* (37,38). Studies to test this approach in the laboratory use a design that simulates a field application. Wooden frames composed of housing materials common in the rural tropics (primarily mud and thatch) are treated with the bacterial formulation. Field-collected *R. prolixus* are added to these frames, which are then enclosed in large plexiglass containers. The progeny of the field-collected insects are examined for colonization with the modified symbiont, ability of the symbiont to compete with native symbionts established in the field-collected specimens, and expression of the desired transmission phenotype.

A possible strategy for using vector-symbiont intervention for the control of Chagas disease transmission would require that in disease-endemic areas, individual houses likely to become infested with triatomines be treated with the bacterial formulation, either when they are new and uncolonized or after insecticide treatment that kills any resident insects, especially in corners and cracks where they are most likely to hide. In homes treated with the bacterial formulation, which likely contain wild-type symbionts, the genetically modified symbionts could be given a competitive advantage of being applied in greater concentrations than the native symbionts. As triatomine bugs from adjacent untreated areas reinfest the house, they would lay their eggs, which would hatch within days. The digestive tract of the new immature bugs would be colonized through coprophagy with genetically altered symbionts, which would keep them from subsequently becoming infected with the Chagas disease trypanosome. These progeny would then amplify and disperse the altered symbionts through natural coprophagic routes. Since triatomine bugs involved in domiciliary transmission must invade and become established in the homes, peridomestic or sylvatic habitats would not need to be treated to affect domestic transmission. Because of the labor and expense involved in repeated insecticide treatment, reinfestation is a potential major obstacle in Chagas disease control. Vector-symbiont intervention could play an important role in an integrated control approach that used a combination of insecticidal and molecular genetic interventions.

Bacterial Symbionts in Tsetse

Bacterial symbionts have also been evaluated in other insect disease vectors. The tsetse vectors of African sleeping sickness (trypanosomiasis) harbor as many as three distinct populations of bacterial flora (33,42-45). Like triatomine bugs, tsetse are obligate bloodfeeders, and at least one population of bacteria (found in uterine secretory cells) is presumed to be nutritional mutualist symbionts. The primary, or P-symbionts, are highly specialized intracellular bacteria apparently essential for the flies' survival (33,45). These bacteria have not been cultured or transformed. The secondary, or S-symbionts, comprise another population of gram-negative bacteria found in various tissues of tsetse, including the salivary glands, where they reside in large numbers, especially in older flies (S. Aksoy, pers. comm.). S-symbionts can be isolated from hemolymph and cultivated in vitro, where they have been shown to grow both intra- and extracellularly (Figure 4) (46-48). A potential transformation system has been developed for these bacteria with the recombinant plasmid pSUP204 (47). This DNA vector contains the broad host range replicon *oriV,* derived from a *Pseudomonas aeruginosa* plasmid, RSF1010, and ligated into the *E. coli* cloning vector pBR325 (49,50). Recent studies indicate that genetically transformed tsetse S-symbionts can be microinjected into recipient flies and express a reporter

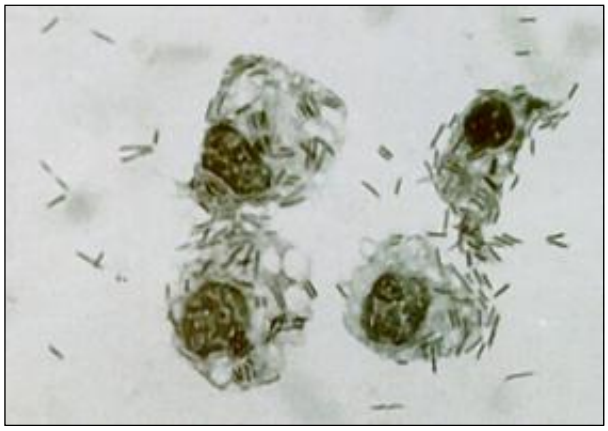

Figure 4. The tsetse secondary symbiont GP01 growing intracellularly and extracellularly in culture.

gene (S. Aksoy, pers. comm.). This area of research will likely yield new approaches for controlling tsetse transmission of trypanosomes.

Intracellular Insect Reproductive System Agents—*Wolbachia*

Obligate intracellular bacteria found in many species of arthropods (51,52), the *Wolbachia* are maternally transmitted from parent to offspring and are often involved in a variety of reproductive anomalies, such as cytoplasmic incompatibility (reproductive incompatibility due to maternal, nongenetic factors), sex ratio determination and distortions, and parthenogenesis (53-56). Although extremely fastidious, these microbes could be used in the transgenic alteration of arthropod vectors in two ways (32): 1) direct transformation and subsequent expression of a gene product by the *Wolbachia* in the arthropod, and 2) use of *Wolbachia*-induced cytoplasmic incompatibility to drive a second, maternally inherited factor into a population of vectors. The first approach entails the use of either episomal plasmids or DNA integration vectors for transformation of the *Wolbachia*. Although theoretically possible, such a transformation system (i.e., a suitable DNA vector and methods for introducing the DNA and selecting for transformants) does not now exist. Recent successful genetic transformation of rickettsial agents, however, suggests that this limitation may soon be overcome (57).

Since *Wolbachia* are frequently observed in reproductive tissues of arthropods (Figure 5), one potential approach that they might be used to disrupt transmission of a pathogen is suggested by studies performed using a viral transduction system in the mosquito *Aedes aegypti* (14). In these experiments, antisense DNA sequences corresponding to a membrane protein of the dengue type 2 virus were expressed somatically by using a recombinant Sindbis virus vector. Mosquitoes that were coinfected with the two viruses were shown not to be able to transmit dengue virus. Similar work has been done with *Aedes triseriatus* mosquitoes, which are potential vectors of the LaCrosse virus (58). These types of experiments could be done by using genetically modified *Wolbachia*, with the goal of blocking transovarial transmission of an arboviral agent, such as La Crosse encephalitis virus or Rift Valley Fever virus, which is dependent on vertical transmission from the adult mosquito to its progeny for maintenance of the virus in nature. Genetically transformed *Wolbachia* could be utilized in a manner similar to that of viral transducing agents to express antisense DNA sequences that interfere with replication of transovarially transmitted virus pathogen.

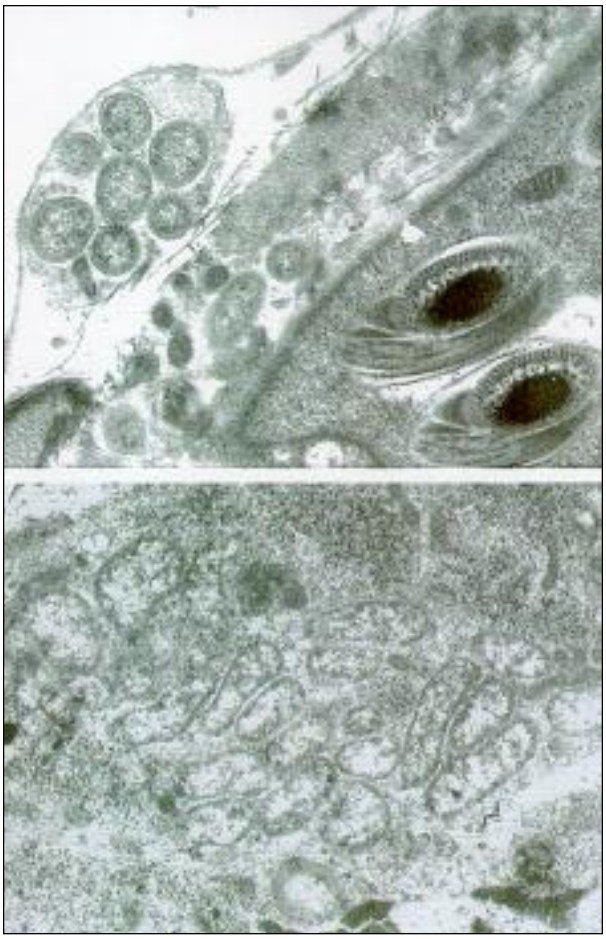

Figure 5. *Wolbachia*-like organisms in insect reproductive tissues.

The disruption of transovarial transmission is an application that could potentially be adapted for use in a number of species of mosquitoes, ticks, and mites in which transovarial transmission of pathogens is important. Very recent studies report the distribution of *Wolbachia* throughout somatic and germ-line tissues in various insects. These observations suggest that potential transmission-blocking applications using genetically modified *Wolbachia* would not be limited simply to transovarially transmitted agents but potentially applicable to microbial pathogens that reside and/or replicate

in other insect tissues as well, such as the hemolymph or salivary glands (59).

Alternatively, *Wolbachia* could be used to render a population of arthropod disease vectors incapable of transmitting a disease agent: *Wolbachia*-mediated cytoplasmic incompatibility could spread a second maternally inherited gene into the vector population. In its simplest form (Figure 6), cytoplasmic incompatibility results when a *Wolbachia*-negative female mates with a *Wolbachia*-positive male; no offspring are produced from such an incompatible cross. Since the reciprocal cross between an infected female and an uninfected male results in *Wolbachia*-infected progeny, the net effect in matings between the two strains, other things being equal, is that *Wolbachia*-infected progeny will be more prevalent than *Wolbachia*-free progeny. This phenomenon has been observed in natural populations of *Wolbachia*-infected and -uninfected *Drosophila simulans* in California, demonstrating the rapid spread of *Wolbachia* and the corresponding cytoplasmic incompatibility phenotype across broad geographic regions (61). How this phenomenon might be utilized has been discussed in great detail (32,60).

As *Wolbachia* spread through the population by cytoplasmic incompatibility, other maternally inherited organisms (or organelles) in the *Wolbachia*-infected strains are transmitted. "Cage studies," using mosquitoes with different mitochondrial haplotypes, have found that haplotype frequencies changed so quickly that within two generations, one haplotype was completely replaced by the other as a result of *Wolbachia*-driven cytoplasmic incompatibility (62).

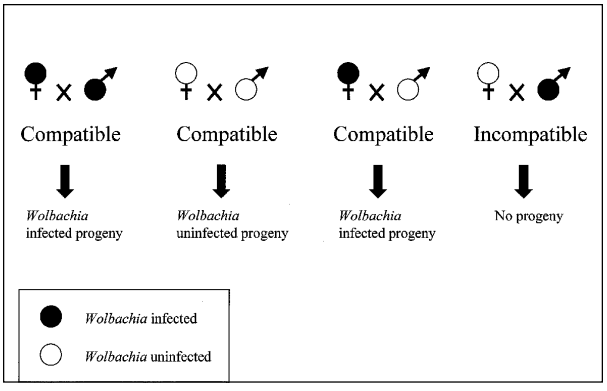

Figure 6. *Wolbachia*-mediated cytoplasmic incompatibility.

In an actual scheme for genetic modification of a vector population, the gene blocking pathogen transmission could be carried by a second, nonpathogenic species of bacteria, such as the tsetse S-symbiont (32,63) or a rickettsialike commensal that is maternally inherited (64), or even possibly an organelle such the mitochondrion. The transformed agent could be introduced into a *Wolbachia*-infected laboratory colony, and the females could be released. Over time, because of the driving mechanism of cytoplasmic incompatibility, a gene product critical for transmission of an arthropod-borne disease agent could be blocked by a second product expressed and secreted by the genetically transformed, maternally inherited microorganism, resulting in the modification of the vector population's capacity to transmit the disease agent.

Limitations

The application of these methods is limited primarily by the occurrence of suitable microorganisms in specific arthropod vectors. In general, many arthropods (e.g., ticks, mites, triatomine bugs, bed bugs, lice, some species of fleas, and tsetse) that feed on single food sources throughout their entire developmental cycle contain microbial symbionts that produce nutritional supplements lacking in the restricted diet (so called nutritional mutualists) (33,65,66). Mosquitoes and many other dipterans, however, do not fall into this category because they do not feed on blood strictly as a source of nutrition for basal metabolism; rather, the females alone feed on blood, which is used directly to produce progeny. Although other symbionts may be present in mosquitoes, nutritional mutualists are altogether lacking.

Reported from numerous and diverse species of arthropods (51), *Wolbachia* may occur naturally in more than 15% of all insect species (67). *Wolbachia* have been transferred in the laboratory from one arthropod species to another, successfully conferring the cytoplasmic incompatibility phenotype to the recipient species (68,69). Other "passenger" microorganisms, such as tsetse S-symbionts (33,42) and *Rickettsia*-like organisms (70,71), are common in arthropods, and although the nature of their symbiotic relationship with the arthropod host is often unclear, their consistent association

suggests their potential as vehicles for expressing a transgene in the arthropod.

Other limitations to the use of arthropod bacteria include the development of appropriate transformation methods and reagents. Because many nonpathogenic microorganisms in arthropods have been poorly studied and characterized, accurate identification of the agent is a prerequisite to genetic transformation studies. These microorganisms can be characterized either through traditional bacteriologic characterization or by a polymerase chain reaction–based approach that uses common eubacterial primers to amplify the 16S rRNA locus that can be sequenced and used for identifying the microbe (72,73). DNA vectors and transformation methods have been developed for diverse groups of both gram-positive and -negative microorganisms; these tools and approaches can often be adapted for use with a bacterial symbiont or passenger. We have efficiently transformed several species of coryneform bacteria, common agents of various triatomine bugs that transmit Chagas disease, by using transformation vectors and regulatory sequences developed for use in *Mycobacterium* spp.

Release of Paratransgenic Insects: Regulations and Safety

In addition to questions regarding science and efficacy, numerous safety and regulatory concerns pertaining to laboratory and field research with genetically modified arthropod vectors of human disease must be addressed. These concerns include potential environmental and ecologic hazards associated with release; potential public health risks; and overall public perception, which can determine the future of entire programs (74,75).

In the United States, federal guidelines and regulations address a broad range of issues pertaining to research, transport, and release of microorganisms, plants, and insects. The U.S. Department of Agriculture Animal and Plant Inspection Service (APHIS) regulates the release of transgenic arthropods of agricultural importance. None of these guidelines or regulations, however, directly address genetically modified arthropod vectors of human disease agents. Nevertheless, the work that has been done and the policy that is in place set a precedent.

The basic principles of containment regarding research with transgenic and paratransgenic insect disease vectors are similar to those regarding human pathogens and biologic insecticide agents (insects and microorganisms). Guidelines that discuss established standards for work with human disease agents, typically classified as biosafety level (BSL)-2, BSL-3, or BSL-4, on the basis of the assessment of virulence and transmissibility have been published (76), and a permitting process oversees the import and transport of these agents into and throughout the United States. The Environmental Protection Agency (EPA) and USDA-APHIS share oversight with regard to the development and use of biologic insecticides (77). USDA-APHIS also regulates the use of insects and arthropods that are predators of insect pests. The containment procedures and facilities associated with the use of these organisms in new geographic regions in the United States might be adapted as the minimum standards for work with transgenic arthropods (75).

With respect to regulation of the release of transgenic arthropods of agricultural importance and risk assessment, USDA-APHIS has formed an interagency "virtual" committee charged with the responsibility of evaluating proposals to release transgenic arthropods that could affect plants and animals (excluding humans); efforts are under way to form an interagency committee with the authority to regulate the release of arthropods that could affect humans. Information on the program that APHIS has assembled, including details regarding permits and current applications that have been submitted under several plant pest statutes, can be accessed at http://www.aphis.usda.gov/biotech/arthropod/; risk assessment information is also available at this site.

Assessment of the environmental risks of releasing transgenic and paratransgenic arthropods shares principles with environmental impact analysis of biologic insecticides (78). The same arguments used to promote the potential benefits of recombinant organisms are equally effectively used to argue the potential hazards (79). To assess risk, Hoy (74) addresses four basic concerns: 1) the attributes of the unmodified organism; 2) attributes of the genetic alteration, i.e., only of the gene transferred; 3) phenotype of the modified organism compared with the unmodified organism; and 4) attributes of the accessible

environment. These concerns can guide formulation of research plans to assess environmental risks associated with the release of transgenic arthropods.

Transgenic and paratransgenic research with arthropods raises several public health concerns. Because the arthropods may transmit human disease agents, ethical issues must be considered. For example, consider that a transgenic or paratransgenic insect is released under the assumption that its ability to transmit disease has been demonstrated to be significantly decreased or eliminated, as determined by laboratory studies in carefully defined conditions. Then suppose that when pilot field studies are conducted, for some reason, it is shown that under true field conditions the ability to transmit disease is not reduced as much as had been anticipated on the basis of the controlled laboratory studies. The result is that the human population present where the release takes place could actually be at increased risk for disease due to the release of large numbers of competent or partially competent vectors. The assessment of this type of risk is extremely important, as is the related human subjects issue of informed consent and how it would be obtained in a study where the population that is potentially at risk cannot be accurately determined.

Public perception, frequently not considered until a potential product is ready for field testing, is extremely important (e.g., public response to genetic control studies in India in the 1970s, including the release of sterile mosquitoes [80] and to the ongoing controversy surrounding the labeling of foods that are, or contain, the products of genetic engineering in agriculture [81-83]). The most effective biotechnology programs are those in which the public is informed and brought into discussions at the earliest possible time (75,84).

The efficacy of a biologic agent to prevent or reduce the impact of a disease is of paramount importance, yet most of this information can be obtained definitively only after the method has been tested on a large scale and under different environmental conditions. On the other hand, information on safety, as well as on the behavior of the agent in the field, is required before we know how well it works and what other effects it has on the environment. Thus, the scientist developing a new agent must make an honest, imaginative leap into the future and try to predict any possible dangerous consequences—the responsibility for risk assessment must be shouldered by the scientist, together with the appropriate regulatory agencies. Because the safety questions are numerous, those of the greatest potential significance, both in the short term and in the long term, should be examined first. The risks associated with the intervention must be assessed against the risk for disease, the risk to health and the environment from pesticides, and the risk of doing nothing.

Conclusions

Decreasing effectiveness of controlling diseases transmitted by arthropods has led to evaluation of new control strategies, including the use of transgenic methods. A strategy that relies on producing paratransgenic arthropods has been applied with initial success to the triatomine bugs that transmit Chagas disease, resulting in vector strains incapable of being infected with the pathogenic agent *T. cruzi*. This approach may also prove feasible for a number of other vector-borne diseases, using either nutritional symbionts of arthropods that feed exclusively on blood, *Wolbachia* that have been successfully transferred between species in the laboratory, or other microbial passengers consistently associated with their arthropod host. As the tools and methods that allow broader applications of this approach are developed, and actual products arrive at the point of field testing, permitting and regulation will be required.

The regulatory process must begin with the individual investigators, who must thoroughly evaluate the product being developed and work with regulatory agencies and others to ensure that if and when genetically modified organisms are used for the control of insect borne diseases, they will be both safe and efficacious.

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Genetic Epidemiology of Infectious Diseases in Humans: Design of Population-Based Studies

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The spread and clinical manifestations of an infection in human populations depend on a variety of factors, among them host genetics. Familial linkage studies used in genetic epidemiology to identify host genes test for nonrandom segregation of a trait with a few candidate chromosomal regions or any regions in the genome (genomewide search). When a clear major gene model can be inferred and reliable epidemiologic information is collected (e.g., in schistosomiasis), parametric linkage studies are used. When the genetic model cannot be defined (e.g., in leprosy and malaria), nonparametric linkage studies (e.g., sibling-pair studies) are recommended. Once evidence of linkage is obtained, the gene can be identified by polymorphisms strongly associated with the trait. When the tested polymorphism is in strong linkage disequilibrium with the disease allele or is the disease allele itself (e.g., in HIV infection and malaria), association studies can directly identify the disease gene. Finally, the role of the detected polymorphism in causing the trait is validated by functional studies.

The profound influence of the host's genetic makeup on resistance to infections has been established in numerous animal studies (1,2) in which disease phenotypes, environmental factors, and crosses can be controlled. Furthermore, recent developments (e.g., use of gene knockout or mutant and transgenic mice) allow genetic analysis of complex traits involved in susceptibility or resistance to infectious pathogens (2,3). As a result of these new developments, the *Lsh/Ity/Bcg* gene was isolated on mouse chromosome 1, which controls innate early susceptibility to several *Mycobacterium* species, as well as other intracellular pathogens (e.g., *Salmonella* Typhimurium, *Leishmania donovani*) (2,4), and was further identified and designated natural resistance-associated macrophage protein 1 (Nramp1) (5). Involvement of a gene in an experimental infection does not imply that differences in susceptibility or resistance to that infection in human populations can be accounted for by polymorphisms in the human homologue of this gene. Genetic epidemiology studies (6,7) combine epidemiologic and genetic information to identify the genes that influence substantially the expression of human complex phenotypes, such as infectious disease-related traits. Epidemiologic information includes measured risk factors that could influence the trait under study (e.g., contamination by the infectious agent, age). Genetic information is derived from familial relationships between study participants (collection of families) or from the typing of genetic markers. Recent maps of the human genome established on the basis of highly polymorphic markers (8) are a fundamental tool for studies involving genetic markers, and two strategies can be used in this context. The first, the candidate gene method, is the typing of a few markers in a limited number of chromosomal regions containing genes related to the phenotype under study. The second is a random search along the whole genome (genomewide search) for chromosomal regions that could be involved in the control of the phenotype.

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The genetic epidemiology of human infectious diseases differs from the genetic study of other complex phenotypes in three ways. 1) Environmental factors influencing the risk for infection are generally known and when accurately measured, can be included in the analysis; 2) Choice of candidate genes is strongly determined by the gene's function and response to the studied pathogen or by mouse-human chromosome tests that exploit the identification of murine resistance loci; and 3) Major genes involved in the response to a given pathogen can be identified by characterizing phenotypic response to pathogen exposure, such as clinical response, biologic response (intensity of infection), and immunologic response (levels of antibodies or cytokines). The role of genetic factors in the control of these phenotypic responses is generally suggested by twin studies, by strong ethnic differences, or by the great variability of individual phenotypes within their familial aggregation. Specific statistical methods are used to identify these genetic factors and to distinguish them from environmental factors causing the familial resemblance. All these statistical methods search for one or more genes that influence the studied phenotype and are classically divided into parametric and nonparametric. Parametric, or model-based, methods (segregation analysis and linkage analysis by the classical lod-score method) require defining the model and specifying the relationship between the phenotype and factors (mainly a putative gene and environmental covariates) that may influence its expression. Nonparametric or model-free methods (nonparametric linkage analysis and association studies) study the genetic factors influencing a phenotype without specifying the model. Each method has advantages and disadvantages; however, the two methods complement each other. The choice of a design for a particular study depends on several factors related to the phenotype (e.g., nature, frequency), population, accurate measurement of environmental factors, and known genetic background. Both methods have led to successful gene localizations and identifications in the analysis of several infectious disease phenotypes (9,10).

Parametric (Model-Based) Studies

Parametric studies require explicit specification of the model, i.e., the definition of the relationship between the observed phenotype and the putative genotype. In a simple monogenic disease due to a diallelic gene (D,d), the model is specified by the frequency of the deleterious allele (D for example) and the three probabilities for a person to have the disease, given the presence of genotype DD, Dd, or dd (penetrances). For complex instances, such as susceptibility/resistance, the susceptibility (or the resistance) depends not only on a putative genotype but also on environmental factors that may influence exposure. In such cases, the phenotype/genotype model includes, in addition to the frequency of the deleterious allele, all the parameters that describe and quantify the relationship between susceptibility and the relevant genetic and environmental factors. This relationship can be mathematically expressed in several ways, most recently regression methods that define model parameters in terms of regression coefficients. Furthermore, regression methods could be used to analyze binary (11) as well as quantitative (12) phenotypes. In quantitative phenotypes, the effect of a genotype is defined in terms of three different phenotypic means depending on the genotypes of the study participants. Parametric methods are based on two kinds of complementary analyses, segregation analysis and linkage analysis by the classical lod-score method (13). Both require epidemiologic information (i.e., the measure of the phenotype and of all relevant environmental factors) for each family member. Linkage analysis needs the typing of genetic markers.

Parametric Segregation Analysis

Segregation analysis is the first step in determining from family data how a given phenotype was inherited. Familial aggregation of infection-related phenotypes can result from genetic relationships, shared environment, and cultural habits. The goal of segregation analysis is to discriminate between these factors, primarily to test for the existence of a single gene, called a major gene. The major gene is not the only gene involved in the expression of the phenotype; rather, of all involved genes, this one has an effect important enough to distinguish it from the others. For a binary clinical phenotype (affected/unaffected by the disease), this effect can be expressed in terms of relative risks, e.g., the ratio of the probability for being infected given a DD genotype to the probability of being infected given a dd genotype. For a quantitative

phenotype, this effect is measured by the proportion of the phenotypic variance explained by the major gene (heritability due to the gene). Primarily, segregation analysis uses maximum likelihood methods to test whether the observed familial distributions of the phenotype fit the distributions expected under different hypotheses of familial transmission (in particular the segregation of a major gene). When evidence indicates a major gene, segregation analysis estimates the measurements for the phenotype/genotype model, which are required for parametric linkage analysis.

Parametric Linkage Analysis

Linkage analysis by the classical lod-score method (13) confirms and locates the gene, detected by segregation analysis (denoted as the phenotype locus). Linkage analysis tests whether, in families, the phenotype locus is transmitted with genetic markers of known chromosomal location. The lod score is a likelihood ratio testing the hypothesis of linkage (against the hypothesis of no linkage) for different genetic distances (or recombination fractions) between the phenotype locus and the marker locus (14). Classically, two conclusions can be reached with a lod-score analysis: 1) linkage between the two loci when the lod score is above a given threshold, and 2) exclusion of linkage between the two loci when the lod score is below a given threshold. Linkage with the phenotype locus can be tested marker by marker (two-point analysis) or by a set of linked markers (multipoint analysis). In linkage, as in segregation analysis, all inferences for individual genotypes at the phenotype locus are made from individual phenotypes and the specified phenotype/genotype model; the lod-score method is most powerful when this model is well defined. A mispecification of the phenotype/genotype model, however, can lead to both inability to detect linkage (and therefore to false exclusion of the region containing the phenotype locus) and to a bias in the recombination fraction estimate (i.e., the genetic distance) between the phenotype locus and the marker locus (15). Nevertheless, such a mispecification does not affect the robustness of the method; i.e., it does not lead to false conclusions in favor of linkage, as long as only one phenotype/genotype model is tested. Correction for multiple testing should accompany the use of several phenotype/genotype models. Similar problems occur when several markers are tested, and guidelines have been proposed to adapt lod-score thresholds to the context of genomewide search (16). Another problem arises when marker data are missing for some family members. In this case, linkage analysis also depends on marker allele frequencies; mispecification of these frequencies can affect both the power and robustness of the method. Multiple marker testing and mispecification of marker allele frequencies are also common problems to the nonparametric methods.

Model-Based Studies and Infectious Diseases

Leprosy Studies

Several segregation analyses have been performed in infectious diseases; some suggest that a recessive major gene may play a role in leprosy subtypes (lepromatous or nonlepromatous) (17-19). A recessive major gene was also found to influence leprosy regardless of the clinical defined subtype, in pedigrees of large families from a small Caribbean island (17); the frequency of the deleterious allele was estimated to be 0.3 (9% of homozygous persons predisposed to leprosy); by age 60, the penetrance was approximately 0.6 for predisposed homozygous, whereas it remained below 0.02 for others. Lod-score analysis could not find any linkage between this leprosy susceptibility locus and five markers (including HLA) that were typed in this population (20).

Malaria Studies

In malaria, segregation analyses have focused on a quantitative phenotype measuring the intensity of infection, i.e., parasitemia levels. Although one study showed the role of a recessive major gene controlling levels of parasitemia (21), two subsequent studies found evidence of a more complex genetic mechanism (22,23). The discrepancies in these results can be explained by several factors related to the host, the parasite, and mosquito transmission. However, all studies showed correlations between siblings and between age and infection (children becoming more often infected than adults). Further genetic analyses such as sibling-pair (sib-pair) study designs should focus on infection in young children.

Schistosomiasis Studies

Model-based studies have been particularly successful in finding susceptibility genes in

schistosomiasis. Several reports indicated that infection intensity was largely determined by the susceptibility/resistance of infected persons (24). In a Brazilian population, segregation analysis showed that the intensity of infection by *Schistosoma mansoni* was controlled by a major gene (25). This gene, SM1, accounts for 66% of the infection intensity variance that remains after other covariate effects (water contact levels, age, gender) have been taken into account. Under this major gene model, approximately 3% of the population is homozygous and predisposed to very high infection levels, 68% is homozygous resistant, and 29% is heterozygous with intermediate levels of resistance (Figure 1). Parametric linkage analysis using the model estimated from segregation analysis was used to locate the gene. A genomewide search was carried out, and SM1 was mapped to human chromosome 5q31-q33, a genetic region that contains several genes encoding molecules that control T-lymphocyte differentiation (26). More recently, a study in a Senegalese population confirmed the presence of a locus influencing *S. mansoni* infection levels on chromosome 5q31-q33 (27). Furthermore, this region has been linked with loci related to immunoglobulin E (IgE) and eosinophilia production, i.e., a locus regulating IgE levels (28,29), a locus controlling bronchial hyperresponsiveness in asthma (30), and a locus involved in familial hypereosinophilia (31). This genetic localization, together with observations that human resistance to schistosomiasis is regulated by lymphokines characteristic of Th2 subsets (32) and that resistant homozygotes mount a Th0/2 response while susceptible homozygotes exhibit a Th0/1 response against schistosomes (V. Rodrigues, A. Dessein, unpub. data), argues strongly that differences in human susceptibility to schistosomiasis are influenced by polymorphisms in a gene controlling T-lymphocyte subset differentiation. In this regard, a segregation analysis showed that interleukin 5 (IL-5) levels are also under the control of a major gene in the same Brazilian population used in the study on infection intensity (33), raising the possibility that IL-5 might play a critical role in resistance, a view consistent with the known role of IL-5 in the defense against schistosome infections.

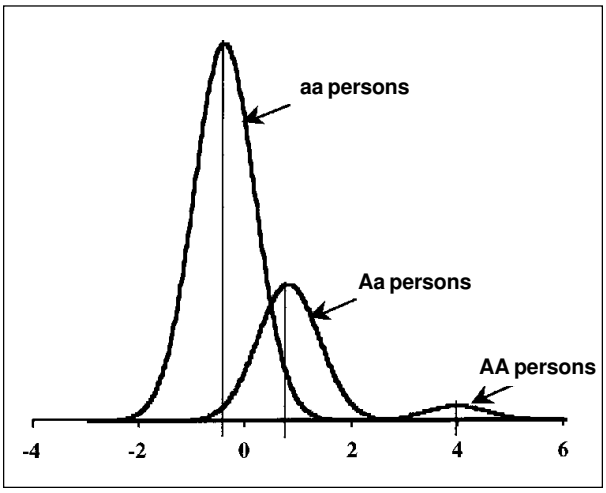

Figure 1. Distribution of the adjusted standardized infection intensities by *Schistosoma mansoni* predicted by the major gene model obtained from segregation analysis and used for linkage analysis. The frequency of allele A predisposing to high infection levels was estimated at 0.16 (70% of aa, 27% of Aa, and 3% of AA persons), and the three means (corresponding to vertical lines) were -0.43, 0.78, and 3.96 for aa, Aa, and AA persons, respectively, with a residual variance equal to 0.33.

Another trait of interest in schistosomiasis is the phenotype of severe hepatic fibrosis due to *S. mansoni* infection for which the role of genetic factors has been suggested. Segregation analysis conducted in a Sudanese village found evidence of major gene involvement in severe hepatic periportal fibrosis (A. Dessein, L. Abel, unpub. data). Whether this gene and SM1 are one and the same is under investigation.

Nonparametric (Model-Free) Studies

Nonparametric or model-free studies (nonparametric linkage analysis and association studies) examine the genetic factors influencing a phenotype without specifying the phenotype/genotype model. These studies are strongly recommended when little is known about the relationship between the phenotype and a putative gene as in the study of complex traits (e.g., infectious disease-related traits) when either no segregation analysis has been performed or no clear major gene model can be inferred from segregation analysis. Nonparametric studies test whether or not the alleles of a given marker are distributed at random in persons having a certain phenotypic resemblance. Nonparametric linkage analyses study the distribution of marker alleles inherited from a same ancestor, i.e., alleles identical by descent

(IBD), in persons from the same family (e.g., siblings), whereas association studies examine the distribution of a given marker allele, e.g., HLA-DR2, in persons not from the same family.

Nonparametric Linkage Analysis

The most commonly used nonparametric linkage analysis is the sib-pair method. Two siblings can share 0, 1, or 2 parental IBD alleles of any locus, and the respective proportions of this sharing under random segregation are simply 0.25, 0.5, and 0.25 (Figure 2). When the phenotype under study is a clinical disease (affected/unaffected), the method tests whether affected sib-pairs share more parental alleles than expected under random segregation. This excess allele sharing can be tested by a simple chi-square, in particular when all parental marker data are known. Maximum likelihood methods have also been developed to analyze data from affected sib-pairs data, such as the maximum likelihood score (34) and a maximum likelihood binomial approach (35), and can lead to more powerful tests. When the phenotypic response under study is quantitative, the method tests whether siblings with close phenotype values share more IBD alleles than siblings with more distant values. This is the basis of the classical approach proposed by Haseman and Elston (36), which regresses the squared difference of the sib-pair phenotypic values on the expected proportion of alleles shared IBD by the sib-pair. Many recent studies have used other methods not detailed here (37-39). Some of these methods are implemented in popular packages, such as MAPMAKER/SIBS (40), which also allow multipoint analysis of sib-pair data. Sib-pair methods have the same problems as parametric linkage analysis with respect to missing parental marker data and testing with multiple markers; in particular, the number of comparisons made influences the significance levels of the tests, and suspected linkage should be confirmed by replication studies. However, affected sib-pair methods have been effective for several diseases, e.g., insulin-dependent diabetes mellitus (41,42), in genomewide searches for human susceptibility genes in a multifactorial phenotype.

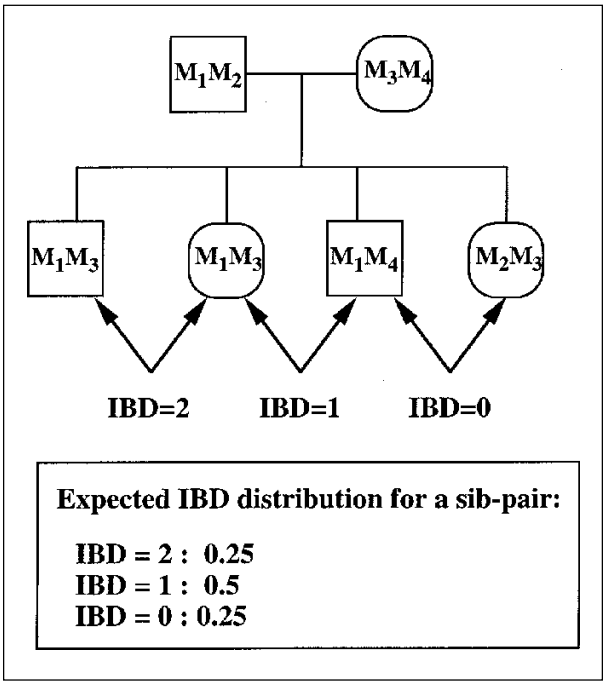

Figure 2. Principle of sib-pair analysis. Two siblings can share 0, 1, or 2 parental marker alleles identical by descent (IBD) at any locus with respective probabilities 0.25, 0.5, and 0.25 under random segregation.

Leprosy Studies

Sib-pair methods in infectious diseases have focused on candidate regions and have not yet resulted in published genome scans. In leprosy studies using the HLA complex, sib-pair analyses have shown a nonrandom segregation of parental HLA haplotypes in sets of children with tuberculoid leprosy and in siblings with lepromatous leprosy, respectively (18,43,44). However, the observed random segregation of HLA haplotypes in all leprosy patients and in healthy siblings in families with multiple cases of leprosy argued against any involvement of HLA-linked factors in susceptibility to leprosy (44,45). The human gene NRAMP1 (46), homologue of the mouse gene Nramp1, has provided an excellent candidate gene for the study of susceptibility to leprosy. A recent sib-pair study in Vietnam has found linkage between leprosy and NRAMP1 haplotypes consisting of six intragenic variants of NRAMP1 and four polymorphic flanking markers (47) and provided the first evidence that NRAMP1 could be a susceptibility locus for leprosy. Furthermore, this study, combined with segregation analysis performed in the same population (18), suggested genetic heterogeneity according to the ethnic origin of the families (Vietnamese or Chinese), which may explain, at least in part, the

results of two previous reports that showed no association between leprosy and distal chromosome 2q where NRAMP1 is located (48,49). Overall, these studies suggest genetic control on at least two levels: a first dependent on non–HLA-linked factors, among which NRAMP1 could play a role, and a second influenced by HLA-linked genes.

Malaria Studies

Two sib-pair studies focusing on candidate genes have been reported in malaria-related phenotypes. In one (50), nonrandom segregation of the MHC region was found in pairs of dizygous twins with mild clinical malaria. In another (51), the 5q31-q33 region, previously shown to be linked to *S. mansoni* infection levels (26), may be involved in the control of parasitemia due to *Plasmodium falciparum,* although the sample size was too small for definitive conclusion; larger studies are ongoing.

Mycobacterium Studies

The recent demonstration that mutations in the interferon γ receptor 1 (IFNγR1) gene cause disseminated infection due to weakly pathogenic mycobacteria (52,53) was first based on homozygosity mapping (54), a nonparametric linkage method, which locates a rare recessive mutation in consanguineous families by searching for chromosomal regions for which all affected family members are homozygous IBD; i.e., they have received two copies of the same ancestral mutation. In consanguineous infected children from two families, two groups located the genetic defect on chromosome region 6q22-q23 and identified mutations in the IFNγR1 gene leading to the absence of expression of the receptor at the cell surface (52,53). In vitro experiments established the causative relationship between the presence of two mutated IFNγR1 alleles and impaired response to IFN by the cells of these patients (55). Although inherited IFNγR1 deficiency was found in additional families, IFNγR1 mutations were not found in other families with infected patients (J.L. Casanova, pers. comm.), which suggests that other genetic defects may be involved.

Association Studies

Classic association studies are population-based case-control studies that compare the frequency of a given allele marker in unrelated persons with the phenotype and controls without the phenotype (6,7). G is the disease locus influencing the trait, and M is the marker locus under consideration; G is assumed to be diallelic (D,d) with D being the deleterious allele, and M has several alleles (M_1, M_2, ..., M_n). Association studies examine the role of a particular allele of M. As an example, M_1 is said to be associated with the disease under study if it is found at a significantly higher or lower frequency in case-patients than in controls by a simple 2 x 2 contingency table. The simplest explanation for the association is that allele M_1 is the deleterious allele D itself. Another explanation is that M_1 has no direct effect on the phenotype but is in linkage disequilibrium with allele D. Linkage disequilibrium means two conditions: 1) linkage between locus M and locus G (generally close linkage) and 2) preferential association of allele M_1 with allele D; i.e., the DM_1 haplotype is more frequent than expected by the respective frequencies of D and M_1 (e.g., many present cases are due to one D allele from an ancestor bearing the DM_1 haplotype). Even very close linkage alone (only the first condition is fulfilled) does not lead to association, and therefore, the absence of association does not exclude linkage. On the basis of these two explanations, association studies best use the candidate gene approach when they consider markers that are either within or in close linkage with a gene that is related to the phenotypic response. A final explanation for association is the existence of an artifact due to population admixture. For example, a case-control study conducted in a mixture of two subpopulations of which one has a higher disease prevalence and a higher M_1 frequency than the second will show a positive association of allele M_1 with the disease. To avoid population admixture, family-based association methods have been developed (56), such as the transmission disequilibrium test (TDT) (57). The sampling unit in these methods consists of two parents with an affected child; parental alleles not transmitted to affected children are used as controls. More specifically, the TDT considers affected children of parents heterozygous for M_1, e.g., M_1M_2, and simply tests whether these children have received M_1 with a probability different from 0.5, the value expected under random segregation (Figure 3). The TDT is a very efficient method of detecting the effect of allele M_1 when M_1 is the deleterious allele D itself

(58). Under this hypothesis that the tested allele M_1 is the deleterious allele, TDT was more powerful than even the sib-pair method in the context of a genomewide search involving 500,000 diallelic polymorphisms (5 polymorphisms per gene for an assumed 100,000 genes) (58). However, in the more common situation where M_1 is different from D, the power of TDT is highly dependent on the respective frequencies of M_1 and D and the strength of the linkage disequilibrium between M_1 and D (59). These results indicate that linkage methods are still useful for identifying genes involved in infectious diseases, at least until molecular resources become available for full genomic screening of human genes.

Leprosy Associations

Most reported associations between leprosy and different HLA alleles could be due to population admixture and statistical problems (multiple testing); therefore, replication studies are very important. In tuberculoid leprosy, the most consistent associations were found with HLA-DR2 (43,45). With HLA molecular typing, a recent study (60) associated Indian tuberculoid leprosy patients and alleles DRB1*1501, DRB1*1502 (both DR2 alleles), and DRB1*1404,

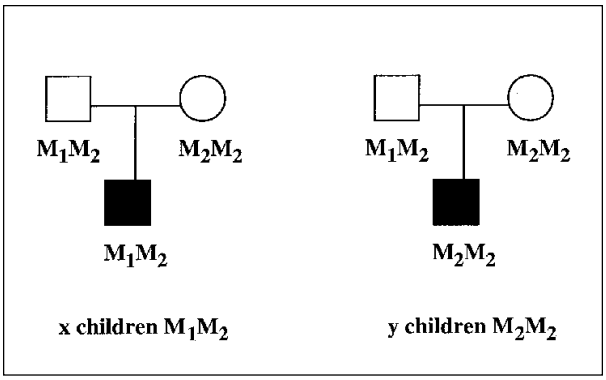

Figure 3. Principle of the transmission disequilibrium test (TDT) for investigating association between a disease and allele M_1. The sample consists of x+y families with one affected child and two parents. For ease of presentation, we assume that only one parent is heterozygous for M_1 (e.g., M_1M_2), although the second parent could be used for the test if he were himself heterozygous for M_1. There are x affected children who have received allele M_1 from their M_1M_2 parent and y who have received M_2. The TDT statistic is simply $(x-y)^2/(x+y)$, which is distributed as a chi-square with one degree of freedom.

which are characterized by arginines at position 13 or 70-71. Lepromatous leprosy was associated with HLA-DR3 in several studies (43,45). One report (44) analyzed the transmission of the parental DR3 allele to lepromatous children by a method (similar to TDT) presented several years later (57).

Malaria Associations

In malaria, population-based association studies have been used to test the hypothesis that certain genetic red cell defects, found more frequently in malaria-endemic areas than in nonendemic-disease areas, had a protective effect against severe malaria (cerebral malaria, severe anemia); the results supported the hypothesis that persons with certain abnormal hemoglobins (61) or glucose-6-phosphate-deshydrogenase deficiency (62) had a reduced risk of developing severe malaria. More recently, a study in Gambia (63) showed that an HLA class I antigen and an HLA class II haplotype were independently associated with protection from severe malaria when a two-stage strategy was used to avoid the problem of multiple testing. In the same population, persons homozygous for a variant of the TNF-α gene promoter, denoted as TNF2, were found to have an increased risk (independent of their HLA alleles) for cerebral malaria (64). A recent work showing that TNF2 is a much stronger transcriptional activator than the more common allele TNF1 (65) indicates that TNF2 affects TNF-α expression and may be directly responsible for the reported association of TNF2 with cerebral malaria. These genetic findings are consistent with immunologic reports showing high TNF-α blood levels in cerebral malaria. Although these genetic polymorphisms (genetic defects of the red cell HLA-TNF polymorphisms) have certainly played a role in selection among populations exposed to malaria infection (61,63), they cannot entirely explain the large interindividual variable responses to the parasite; likely only a minority of genes influencing malaria resistance have been identified (66). This view is supported by a recent report that a coding polymorphism in the intercellular adhesion molecule-1 (ICAM-1), a molecule that affects adherence of infected red blood cells to small vessel endothelium, is associated with an increased susceptibility to cerebral malaria (67).

HIV Associations

A major advance in the involvement of host factors in HIV-1 infection came when infection status (seropositive/seronegative) was associated with the gene encoding the CC-chemokine receptor 5 (CCR5), the coreceptor of macrophage-tropic HIV-1 strains (68). Two persons exposed many times to HIV-1, yet uninfected, were shown to be homozygous for a defective CCR5 allele containing an internal 32 base-pair deletion (Δ32) (69), and several large cohort studies found HIV-1 infected patients not to be CCR5Δ32 homozygous, whereas exposed HIV-1 seronegative persons did have the defective allele (70-72). Subsequent reports showed that this protection was not complete since some CCR5Δ32 homozygous persons were found to be HIV-1 infected (10). Furthermore, several studies in HIV-1 infected persons found CCR5Δ32 heterozygous status may protect against disease progression (71,72), depending on virus strain (73). However, it is clear that CCR5Δ32 does not alone explain HIV-1 infection status, especially in African populations where Δ32 is absent (70,74), and the search for other host genes involved in susceptibility/resistance to HIV infection will be of major interest.

Conclusions

Recently developed genetic epidemiology methods and dense human genetic maps, together with the growing availability of candidate genes, are essential for identifying genes that influence human infectious diseases. Nevertheless, investigating the role of genetic factors in a given phenotypic response depends on many different factors related to the phenotype, population, accurate measurement of environmental factors, and previous knowledge; no unique optimal design can be applied for most phenotypic responses related to infectious agents. Among possible study designs, familial linkage studies search for a chromosomal region showing a nonrandom segregation with the phenotype by either focusing on a few candidate regions or using a genomewide search. The main goals of the genome approach are to ensure that all major loci involved in the control of a phenotype are identified and to provide the opportunity to discover new major genes (and consequently physiopathologic pathways) involved in phenotypic responses. Parametric linkage studies are powerful when a clear major gene model can be inferred from segregation analysis. Nonparametric linkage studies are strongly recommended when little is known about the relationship between the studied phenotype and a putative gene, and sib-pair studies have led to successful gene localizations in the analysis of several complex traits, including infectious disease-related traits. Once evidence for linkage is obtained, fine genetic and physical mapping is performed to narrow down the genetic interval. The next step is the search, by molecular methods, of polymorphisms in candidate genes located within the identified interval. These candidate genes are selected from gene databanks or are obtained by a systematic characterization of the genes of the region (positional cloning). On the other hand, association studies performed with candidate genes can directly identify the disease gene when the tested polymorphism is in strong linkage disequilibrium with the disease allele or is the disease allele itself. Finally, evidence for an association should be completed by functional analysis, which will test whether the detected polymorphism modifies the gene expression or the gene product in a manner that can affect susceptibility to the disease.

Progress in the genetic dissection of infectious diseases will also come from the integrated analysis of different phenotypic responses (clinical response, intensity of infection, immunologic response), which can all contribute to the pathologic process, as illustrated in malaria and schistosomiasis studies. The identification of host genes in human infectious diseases will provide new understanding of disease pathogenesis. How this genetic information will modify our approach to prevention and treatment of infectious diseases cannot yet be fully appreciated. However, the identification of susceptibility/resistance genes in schistosomiasis, mycobacterial, and HIV infections has already opened new avenues for the screening of genetically predisposed persons and the development of vaccines.

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Insecticide Resistance and Vector Control

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Insecticide resistance has been a problem in all insect groups that serve as vectors of emerging diseases. Although mechanisms by which insecticides become less effective are similar across all vector taxa, each resistance problem is potentially unique and may involve a complex pattern of resistance foci. The main defense against resistance is close surveillance of the susceptibility of vector populations. We describe the mechanisms of insecticide resistance, as well as specific instances of resistance emergence worldwide, and discuss prospects for resistance management and priorities for detection and surveillance.

Many new and reemerging diseases are transmitted by arthropod vectors. Mosquitoes transmit malaria (1,2), dengue-dengue hemorrhagic fever (DHF) (3-5), yellow fever (6), Venezuelan equine encephalitis (3,7), and filariasis (8); sand flies transmit leishmaniasis (7); ticks transmit Lyme disease and ehrlichiosis (9,10); fleas and lice transmit *Bartonella* (11); and fleas, lice, and ticks transmit various rickettsioses (12-14). Resistance to insecticides has appeared in the major insect vectors from every genus. As of 1992, the list of insecticide-resistant vector species included 56 anopheline and 39 culicine mosquitoes, body lice, bedbugs, triatomids, eight species of fleas, and nine species of ticks (15). Other insects of public health importance, such as certain flies and cockroaches, show resistance in all genera.

Resistance has developed to every chemical class of insecticide, including microbial drugs and insect growth regulators. Despite decades of international efforts, a detailed practical description of insecticide resistance that would allow control strategies to be adjusted to specific needs remains the exception rather than the rule.

Insecticide resistance is expected to directly and profoundly affect the reemergence of vector-borne diseases (1), and where resistance has not contributed to disease emergence, it is expected to threaten disease control (15). However, careful scrutiny of current information about vector resistance (e.g., the World Health Organization [WHO] resistance database and records of control programs) shows that the full effect of resistance on control efforts is not known. Many instances of resistance reported for vector species and their regional or countrywide distribution are based on single datasets from a single point within a country and may be years, if not decades, old. Researching every resistance problem and every application of vector control is not practical. Yet control measures have to be selected for use, often at times of emergency. Although alternatives to vector control with insecticides are available, drug resistance problems (e.g., malaria) or vaccine cost and availability (e.g., Japanese encephalitis) make vector control an important option (1,16). Shrinking availability of insecticides as a result of resistance is exacerbated by removal from the market of insecticides no longer registered for public health use, especially in the past decade; the cost to keep certain compounds on the market is higher than can be recouped from such use. In addition, insecticide use is also monitored and restricted by regulatory agencies.

The potential of resistance to interfere with emergency use of insecticides first became apparent in 1993 when flooding in nine midwestern states increased the threat over the next 2 years of arboviral disease transmission (17). Most of the nine states affected had no public health entomologic or vector control

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resources, and none had susceptibility data for their vector mosquitoes. Preliminary data showed that resistance to the insecticides proposed for emergency use was widespread throughout the Midwest. As a result of these findings, a resistance surveillance laboratory was established at the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. Data collected by this laboratory in the last 3 years confirm that states vary enormously in their resources to deal with insecticide resistance. At present, 26 states participate in the Emerging Infectious Disease insecticide resistance surveillance project.

We provide an update on resistance of disease vectors to insecticides, use specific instances of emerging resistance to illustrate this complex, worldwide problem, and offer strategic priorities for combating it.

Resistance Mechanisms

Insecticide resistance mechanisms (as opposed to insecticide avoidance behaviors important in the control of malaria vectors) have a biochemical basis (Figure 1). The two major forms of biochemical resistance are target-site resistance, which occurs when the insecticide no longer binds to its target, and detoxification enzyme-based resistance, which occurs when enhanced levels or modified activities of esterases, oxidases, or glutathione S-transferases (GST) prevent the insecticide from reaching its site of action. An additional mechanism based on thermal stress response has been proposed (18), but its importance has not been assessed.

Target-Site Mechanisms

Alterations of amino acids responsible for insecticide binding at its site of action cause the insecticide to be less effective or even ineffective. The target of organophosphorus (OPs) (e.g., malathion, fenitrothion) and carbamate (e.g., propoxur, sevin) insecticides is acetylcholinesterase in nerve synapses, and the target of organochlorines (DDT) and synthetic pyrethroids are the sodium channels of the nerve sheath. DDT-pyrethroid cross-resistance may be produced by single amino acid changes (one or both of two known sites) in the axonal sodium channel insecticide-binding site (19,20). This cross-resistance appears to produce a shift in the sodium current activation curve and cause low sensitivity to pyrethroids (21). Similarly, cyclodiene (dieldrin)

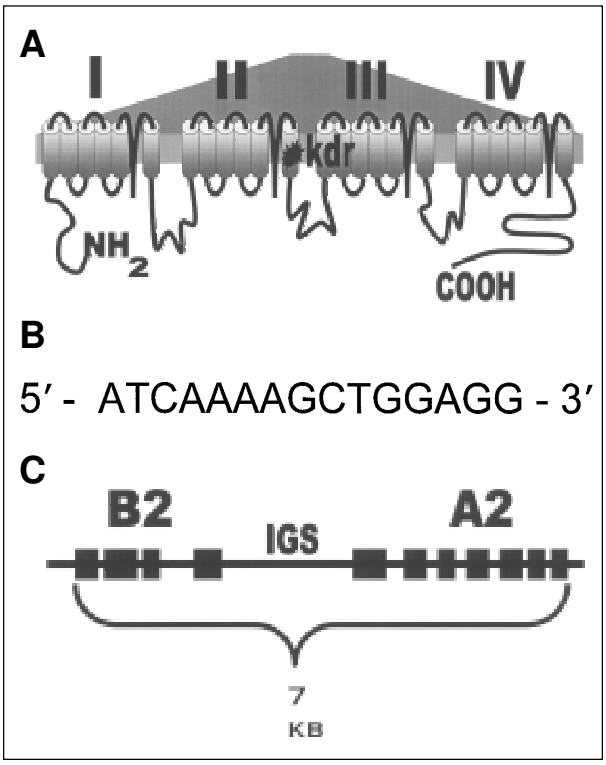

Figure 1. Examples (drawn from references cited in the text) of biochemical resistance mechanisms on the molecular level. A. Single amino acid mutation in the IIS6 membrane-spanning region of the sodium channel gene that confers target-site DDT-pyrethroid resistance in *Anopheles gambiae*. The same mutated codon produces resistance in insects as diverse as mosquitoes, cockroaches, and flies. B. Regulatory element (found upstream of coding sequence) termed the "Barbie Box" that allows induction of insecticide detoxifying oxidase and esterase resistance genes. Many such putative control elements have been found associated with vector resistance enzymes. C. Esterase A2-B2 amplicon. These resistance esterase genes lie 5' end to 5' end within the same amplification unit. More than 100 copies of this amplicon may be present in a single mosquito. This is one example of a family of amplified esterase genes.

resistance is conferred by single nucleotide changes within the same codon of a gene for a γ-aminobutyric acid (GABA) receptor (22). At least five point mutations in the acetylcholinesterase insecticide-binding site have been identified that singly or in concert cause varying degrees of reduced sensitivity to OPs and carbamate insecticides (23).

Detoxification Mechanisms

The enzymes responsible for detoxification of xenobiotics in living organisms are transcribed

by members of large multigene families of esterases, oxidases, and GST. Perhaps the most common resistance mechanisms in insects are modified levels or activities of esterase detoxification enzymes that metabolize (hydrolyze ester linkages) a wide range of insecticides. These esterases comprise six families of proteins belonging to the α/ß hydrolase fold superfamily (24,25). In Diptera, they occur as a gene cluster on the same chromosome (26,27). Individual members of the gene cluster may be modified in instances of insecticide resistance, for example, by changing a single amino acid that converts the specificity of an esterase to an insecticide hydrolase (28) or by existing as multiple-gene copies that are amplified in resistant insects (the best studied examples are the B1 [29] and A2-B2 [30] amplicons in *Culex pipiens* and *C. quinquefasciatus*).

The cytochrome P450 oxidases (also termed oxygenases) metabolize insecticides through O-, S-, and N-alkyl hydroxylation, aliphatic hydroxylation and epoxidation, aromatic hydroxylation, ester oxidation, and nitrogen and thioether oxidation (31). The cytochrome P450s belong to a vast superfamily. Of the 62 families of P450s recognized in animals and plants, at least four (families 4,6,9,18) have been isolated from insects. The insect P450 oxidases responsible for resistance have belonged to family 6, which, like the esterases, occur in Diptera as a cluster of genes (32). Members of the cluster may be expressed as multiple (up to five) alleles (33). Enhanced levels of oxidases in resistant insects result from constitutive overexpression rather than amplification (34,35). The mechanisms of oxidase overproduction in resistance are under extensive investigation and appear to result from both cis- and trans-acting factors, perhaps associated with the phenomenon of induction (36-38).

Most organisms possess multiple GST from two or more classes (39). GST implicated in DDT insecticide resistance exist as clusters of genes that have been further shuffled through the genome by recombination (40). A number of resistance GST genes, including multiple forms in the same insect, have been characterized in vectors (41-43).

Resistance to Growth Regulators, Ivermectins, and Other Microbial Agents

Because of their more recent introduction to vector control programs, we discuss growth regulators, ivermectins, and other microbial agents as a group. The initial mechanisms that conferred resistance to insect growth regulators were oxidase-based (44). Resistance to ivermectins has resulted from a number of factors, including oxidase, conjugation, and altered target-site mechanisms (45). Vectors have not yet demonstrated resistance to these compounds in the field.

Microbial agents such as *Bacillus sphaericus* and *B. thuringiensis* are considered insecticides because the principal active agents are crystal toxins produced by the bacteria. The mechanisms of resistance to *B. sphaericus* are not yet defined (46,47), but more than one mechanism seems to be involved (48). Resistance to *B. thuringiensis* has resulted from reduced binding of the toxin to the brush border in the lumen of the insect gut (49,50) or by enhanced digestion of toxin by gut proteases (51). The six different toxin types in the *B. thuringiensis israelensis* strain used for vector control were expected to retard or prevent development of a comprehensive resistance mechanism; however, multitoxin resistance to *B. T. israelensis* has already appeared (52,53).

Detecting and Monitoring Resistance

The initial step in identifying a potential problem is to detect changes in the susceptibility of a population of vectors—through bioassay, biochemical assay, or molecular assay (Figure 2).

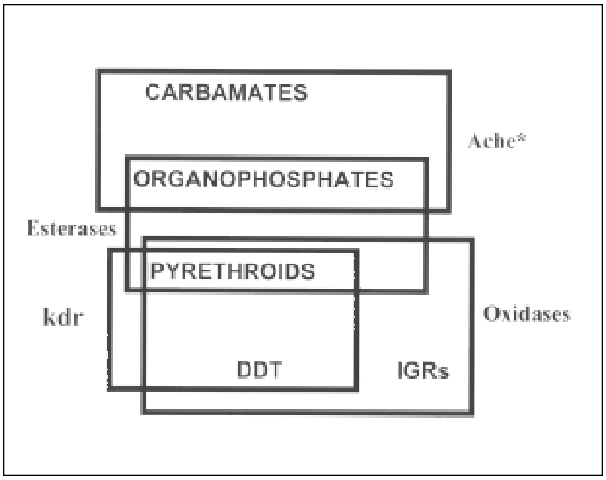

Figure 2. Cross-resistance relationships of commonly used classes of insecticides.

Bioassays

WHO has developed susceptibility bioassay tests (available in kit form for purchase from WHO) for mosquitoes, lice, bedbugs, reduviid bugs, cockroaches, blackflies, houseflies, ticks, and fleas (15). Our laboratory found that time-mortality bioassays were more sensitive than dose-mortality bioassays in detecting changes in susceptibility and had better correlation with microplate-based biochemical assays for resistance mechanisms (54,55). Time-based bioassays have been further modified through the use of insecticide-coated glass bottles and solutions of standard-grade insecticides or synergists; this approach simplifies the bioassay process and increases the amount of information that can be obtained from a limited pool of mosquitoes (56).

Biochemical and Molecular Assays

Biochemical and molecular methods can detect resistance mechanisms in individual insects; therefore, they can confirm resistance with the use of only a small number of insects. Identification of resistance mechanisms helps determine the cross-resistance spectrum (Figure 3), facilitates the choice of alternative insecticides, and allows detailed mapping of areas with resistant populations. Specific biochemical assays have been developed for all known resistance mechanisms, except the modified sodium and GABA receptor mechanisms (57-59).

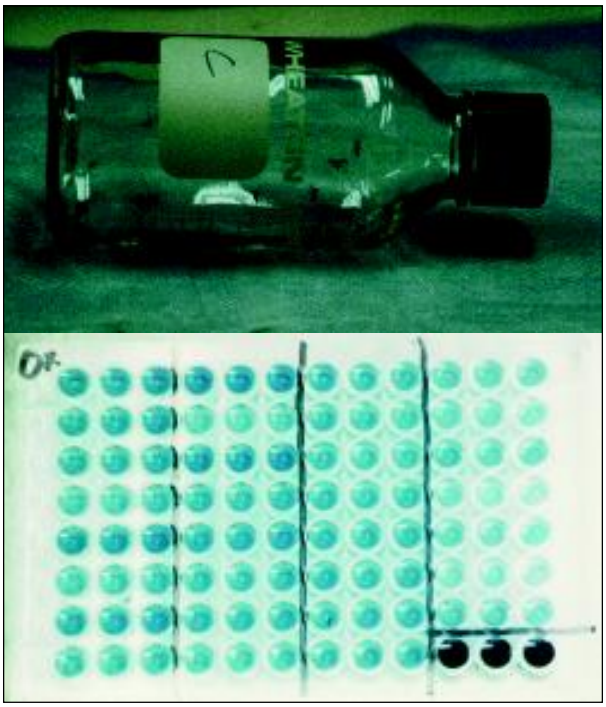

Figure 3. Examples of simple diagnostic assays for insecticide resistance include bioassays run in treated bottles (upper) and biochemical detection and measurement of resistance enzyme activity in microplates (lower).

Molecular information on resistance mechanisms will increasingly be incorporated into resistance diagnostic procedures. One type of mechanism that will become much easier to detect will be the point mutations that cause target-site resistance or changes in detoxification enzyme specificity. Thus far, target-site mechanisms have been detected by polymerase chain reaction-restriction enzyme (PCR-REN) and PCR amplification of specific alleles (60,61).

Features of Resistance Emergence

Innumerable genetic, biologic, and operational factors influence the development of insecticide resistance. In many respects, resistance is a chaotic problem, with different outcomes possible in a particular area, depending on the influence of diverse factors on initial conditions. Even so, certain factors affect resistance development throughout the world. We discuss major resistance characteristics and show why each manifestation of resistance is potentially unique and therefore must be independently evaluated.

Focal Nature of Resistance

Vector control personnel frequently assume that resistance in a particular species occurs throughout their control area, but in reality, insecticide resistance is focal. In Guatemala, sampling sites for *Anopheles albimanus* only a few kilometers apart varied not only by presence or absence of resistance, but also by level of resistance and by dominant mechanism responsible for resistance (55).

Surveillance data (Brogdon and McAllister, unpub. data) from the United States show that resistance to OPs in *Culex* mosquito species is focal in a number of states—generally high in urban areas and absent in rural sites. The higher levels of resistance are in areas of ongoing control activities. When resistance levels in adjacent counties were compared, levels were higher in areas of intensive mosquito control.

While the relative importance of agricultural versus public health use of insecticides to resistance development has been widely argued

(62), resistance has been associated with both uses. Agricultural use caused resistance in Central American *An. albimanus* (55). However, in Haiti (54) and in Sudan (62), the impact of public health spraying on development of resistance is clear. In Sri Lanka, resistance in one vector, *An. cuilifacies*, is characteristic of public health spraying, while resistance in another, *An. nigerrimus*, has a profile that indicates agricultural chemicals (62). Resistance appears rapidly in areas where bed nets are used to control mosquitoes (Western Kenya) or sandflies (Colombia) (63; Brogdon and McAllister, unpub. data).

Resistance and Disease-Endemic Areas

Vector control is affected not only by the focal nature and distribution of resistance but also by disease incidence. Resistance detection could actually interfere with disease control programs if adequate surveillance data are not also collected. In Ecuador (57) we observed OPs resistance in the malaria vector *An. albimanus* in the central agricultural provinces of Guayas, Manabi, and Los Rios, the sources of the country's resistance surveillance data. However, in the northern province of Esmeraldas, where insecticide use had been limited and an epidemic of *Plasmodium falciparum* malaria was under way, populations were completely susceptible. Lack of insecticide use does not preclude immigration of resistance genes (e.g., the movement of esterase resistance to OPs in *C. pipiens* into certain areas of France [64]).

Resistance and Disease Control

To compromise insecticide vector control, the level of resistance must be high enough to adversely affect disease transmission. In many cases, vector control may not be affected by the level of resistance. For example, an activity may be controlling only 75% of the vector population. If, for example, the level of resistance is lower than 10%, resistance will not affect disease control efforts; in this situation, increasing surveillance and monitoring level and frequency of resistance would be sufficient. No change in control methods would be needed. Western Kenya is a good operational example of the coexistence of resistance and disease control. Pyrethroid resistance appeared soon after bed nets were introduced (64). After 2 years, the resistance level had not changed significantly, possibly because of the continual massive introduction of susceptible genes (65). Other reasons may explain why the presence of insecticide resistance genes in vectors in a control area does not mean that effective control is not being achieved. For example, resistance genes may not be expressed, they may be expressed in an alternative stage of development to that being controlled by insecticide, or the gene detected may be a member of an alternative gene subfamily to one that can affect the compound being used. We have observed in *An. albimanus* and *An. gambiae* that resistance enzymes, especially esterases and GST, may be expressed only in freshly emerged adult anophelines and may be absent in older mosquitoes, those potentially infective for malaria (Brogdon and McAllister, unpub. data).

Pyrethroid Resistance

Pyrethroid resistance is emerging despite early optimism that because of its rapid toxicologic action this newest large class of insecticides would not produce resistance (66). Resistance is not evolving through unique new mechanisms; rather, existing mechanisms are being enhanced, and cross-resistance is occurring. In Guatemala, pyrethroid resistance was first reported in an *An. albimanus* population resistant to fenitrothion. When deltamethrin was used, the esterase conferring fenitrothion resistance was enhanced by selective pressure to produce deltamethrin cross-resistance (67). Additionally, we are now finding DDT-permethrin cross-resistance due to oxidase cross-resistance in the same mosquito. A similar pattern of cross-resistance has been documented for *C. pipiens* in Ohio. Multiresistance (two or more resistance mechanisms in the same insect) is becoming widespread as control programs make sequential use of one chemical class after another.

A far more threatening development in pyrethroid resistance is the appearance of target-site resistance (also termed knockdown resistance) to pyrethroids in several important vectors in multiple locations. We have detected the knockdown resistance mechanism in the dengue and yellow fever vector *Aedes aegypti* from Puerto Rico and Indonesia and in the encephalitis vector *C. quinquefasciatus* from Louisiana. French researchers have detected the mechanism in *An. gambiae*, the primary African vector of malaria, in several countries of West

Africa (68,69). This resistance mechanism may be a legacy of similarities in the site of action of pyrethroids and DDT.

Prospects for Resistance Management

A National Research Council report (70) on strategies and tactics for pesticide resistance management described insecticide susceptibility as a resource and resistance surveillance as an essential step in resistance management. Resistance surveillance has three objectives: 1) Provide baseline data for program planning and pesticide selection before the start of control operations; 2) Detect resistance at an early stage so that timely management can be implemented (even detection of resistance at a late stage can be important in elucidating the causes of failure of disease control; however, in such cases, any management other than replacement of the pesticide may not be possible); 3) Continuously monitor the effect of control strategies on resistance.

Resistance to insecticides—even to pyrethroids—in disease vectors is widespread. With the availability of more sensitive and easy-to-use surveillance techniques (56,58,59), the means for managing resistance are at hand. A challenge to resistance management is that efforts to control vector-borne diseases are becoming more diversified; the global effort to control malaria is a case in point. In the 1950s, WHO mounted a global effort to eradicate malaria. Failure of this effort was caused by many factors, including insecticide resistance. Now the best prospect for a worldwide malaria vector control strategy appears to be the use of pyrethroid-impregnated bed nets, because they are less expensive than spraying walls with residual insecticide, are effective in reducing child deaths, and can be better administered through a horizontal, community-based program (71). This type of vector control means that resistance surveillance will also have to be handled and interpreted locally. Decisions will have to be made within local programs by the end user. Moreover, as urbanization increases around the world, some horizontal programs are better able financially to contract out control and surveillance activities. In the United States, local control programs use diverse methods for vector control to meet their specific, often unique needs (72). Diverse methods will likely become the characteristic approach to vector control worldwide, given the prohibitive economics of mounting a worldwide disease control campaign based on vertical programs and the availability of entrepreneurs that increasingly contract for vector control on a city-by-city basis internationally (73-75). In vertical programs, resistance surveillance was a component more in theory than in fact. The challenge will be to maximize the exposure of vector control personnel and entrepreneurs to management principles and to make widely available the surveillance tools required.

Priorities for the Future

Sufficient means exist to detect and manage resistance at a higher level and with greater effectiveness. A number of training courses and consultations have been conducted to broaden the base of resistance surveillance in the United States. However, the need for resistance surveillance information is global. Perhaps the most cost-effective way to disseminate this information will be the Internet.

Internationally, the increasing diversity of vector control measures will require continued development of simple and informative methods. How can we use the most sensitive and informative molecular and biochemical methods in concert with bioassay techniques? How can these be best simplified so that relatively untrained personnel can use them?

While initial detection and field surveillance for resistance will likely continue to be based upon simple bioassay, biochemical, and molecular tools, the deeper understanding of how resistance arises and maintains itself in populations requires molecular genetics studies.

The most complete understanding of insecticide resistance mechanisms in disease vectors has come from studies with mosquitoes. Much more attention is needed for resistance detection and surveillance methods in other vector groups, such as sand flies, triatomids, lice, fleas, and ticks. Most important, however, is that resistance detection be made an integral part of all control programs. The resources for vector control, even under emergency situations, are limited and must be used as effectively as possible.

Dr. Brogdon is a research entomologist with the National Center for Infectious Diseases, CDC. His research interests include insecticide resistance in disease vectors; vector biochemistry, physiology, and molecular biology; and mosquito behavior.

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Mutators and Long-Term Molecular Evolution of Pathogenic *Escherichia coli* O157:H7

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It has been proposed that an increased mutation rate (indicated by the frequency of hypermutable isolates) has facilitated the emergence of *Escherichia coli* O157:H7. Analysis of the divergence of 12 genes shows no evidence that the pathogen has undergone an unusually high rate of mutation and molecular evolution.

Escherichia coli O157:H7, a highly virulent organism first linked to infectious disease in 1982 (1) and now found worldwide, has caused serious foodborne epidemics in the United States, Japan, and Europe (2). One hypothesis for the emergence and rapid spread of this organism is that strong mutator alleles enhance genetic variability and accelerate adaptive evolution (3). LeClerc et al. (3) found that more than 1% of O157:H7 strains had spontaneous rates of mutation that were 1,000-fold higher than those of typical *E. coli*. These mutator strains were defective in methyl-directed mismatch repair (MMR) as a result of deletions in the intergenic region between the *mutS* and *rpoS* genes (3). According to the mutator hypothesis, a pathogen able to enter a transient hypermutable state could overcome the fitness costs of deleterious mutations by accruing new genetic variation at times critical for survival and colonization of new hosts.

Adaptive evolution by transient or prolonged states of hypermutation can cause neutral mutations to rapidly accumulate throughout the genome. To detect possible elevation in the rate of molecular evolution in the emergence of *E. coli* O157:H7, we compared 12 genes with housekeeping functions (Figure) that have been sequenced in both *E. coli* O157:H7 and *E. coli* K-12 (a commensal organism), as well as in an outgroup species, *Salmonella enterica* serotype Typhimurium. The evolutionary distance (expressed in point mutations per 100 sites) between Typhimurium and K-12 is shown against the distance between Typhimurium and O157:H7 for synonymous and nonsynonymous sites separately (Figure). The line indicates equal rates of evolution in the two

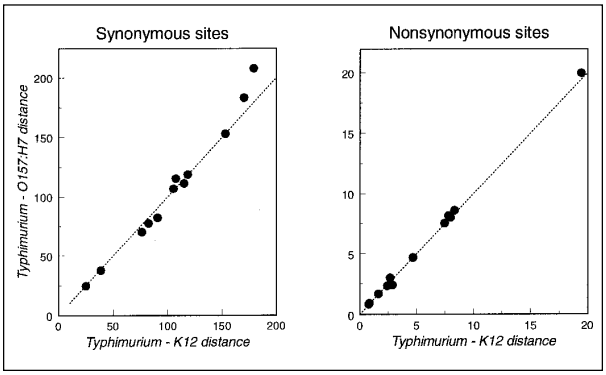

Figure. Evolutionary distance in terms of synonymous and nonsynonymous changes per 100 sites (4) for 12 genes sequenced from *Escherichia coli* O157:H7, *E. coli* K-12, and *Salmonella enterica* Typhimurium. The points for synonymous sites are (left to right): *gap, crr, mdh, icd, fliC* (conserved *5′* and *3′* ends), *trpB, putP, aceK, mutS, trpC, tonB,* and *trpA*. Under the mutator hypothesis, the genetic distance between the pathogenic O157:H7 strain (or the closely related strain ECOR37) and the outgroup (Typhimurium) is expected to exceed the distance between the commensal K-12 and the outgroup. Prolonged periods of enhanced mutation rate should drive the points above the dotted line marking equal rates of molecular evolution. Two loci (*tonB* and *trpA*) show departure from the equal rate line, but neither has evolved differently from that expected by the molecular clock. The sequences of 12 genes were obtained from GenBank or the original sources as follows: *aceK* (5), *crr* (6-8), *fliC* (9), *gap* (10), *icd* (11), *mdh* (12), *mutS* (13,14), *putP* (15), *tonB* (16,17), *trp* (17-20).

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lineages. An elevated mutation rate in O157:H7 over evolutionary time should result in greater divergence from Typhimurium than from K-12 and in the distribution of points above the equal-rate line. For both synonymous and nonsynonymous sites, most genes fall below or very near the equal-rate line with only two exceptions: *tonB* and *trpA* deviate in the direction expected under the mutator hypothesis. To test the significance of these deviations, we compared the observed degrees of divergence of K-12 and O157:H7 from Typhimurium and the expectations of the molecular evolutionary clock hypothesis (21). The basis of this test is that a constant rate of mutation results in equal numbers of substitutions in two sequences from an outgroup (21). Considering synonymous and nonsynonymous changes together with Typhimurium as an outgroup, we found that 11 of the 12 loci, including *tonB* ($m_1 = 0$, $m_2 = 3$, $X^2 = 3.00$, $p > 0.05$) and *trpA* ($m_1 = 4$, $m_2 = 10$, $X^2 = 2.57$, $p > 0.05$), did not deviate significantly from a uniform rate of evolution predicted by a molecular clock. Only *mdh* exhibited a significant departure from the molecular clock ($m_1 = 14$, $m_2 = 5$, $X^2 = 4.26$, $p < 0.05$); however, the direction was away from that predicted by the mutator hypothesis (the Typhimurium–K-12 distance exceeded the Typhimurium-O157:H7 distance).

Our findings do not conflict with the observation that MMR defects occur in relatively high frequency in emerging pathogens; however, the findings indicate no evidence of a genomewide elevation of the mutation rate in pathogenic *E. coli* O157:H7. The uniform rate of divergence of O157:H7 and K-12 suggests several possibilities. One is that the mutator state is transient and so brief that the impact on long-term rates of evolution is undetectable. This possibility is consistent with the view that mutators may generate favorable mutations in periods of intense selection and then revert to a nonmutator phenotype (22,23). Another possibility is that all bacterial populations experience brief episodes of adaptive evolution driven by hypermutation. Matic and co-workers (24) found equivalent frequencies of mutators among strains of commensal bacteria and both emerging and classical pathogenic *E. coli*.

Finally, defects in MMR that produce the mutator phenotype also relax the normal barriers to recombinational exchange between bacterial species (25). The enhanced recombination that accompanies the mutator phenotype may explain why *E. coli* O55:H7, the immediate ancestor of O157:H7 (26) that also carries the same defective MMR allele (3), harbors such an extraordinary variety of plasmid and chromosomal virulence factors (27). Together with our finding of clock-like divergence of *E. coli* O157:H7 housekeeping genes, these observations indicate that the main evolutionary benefit of the mutator phenotype is the enhanced ability to acquire useful foreign DNA (3), not an increased rate of point mutation over the long term.

Dr. Whittam is professor of biology at the Institute of Molecular Evolutionary Genetics, Pennsylvania State University. His research focuses on understanding how evolutionary forces operate to determine the amount and organization of genetic variation in natural populations of bacteria. Specific studies include the evolution of pathogenic forms of *Escherichia coli* associated with intestinal and extraintestinal infections, the evolution of virulence and resistance in a host-parasite interaction using an amoeba-*Legionella* system, and the ecologic determinants and evolution of host specificity in *Rhizobium*-legume associations.

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Increasing Hospitalization and Death Possibly Due to *Clostridium difficile* Diarrheal Disease

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This study calculated yearly estimated national hospital discharge (1985 to 1994) and age-adjusted death rates (1980 to 1992) due to bacterial, viral, protozoal, and ill-defined enteric pathogens. Infant and young child hospitalization (but not death) rates in each category increased more than 50% during 1990 to 1994. Age-adjusted death and hospitalization rates due to enteric bacterial infections and hospitalizations due to enteric viral infections have increased since 1988. The increases in hospitalization and death rates from enteric bacterial infections were due to a more than eightfold increase in rates for specified enteric bacterial infections that were uncoded during this period (ICD9 00849). To identify bacterial agents responsible for most of these infections, hospital discharges and outpatient claims (coded with more detail after 1992) were examined for New Mexico's Lovelace Health Systems for 1993 to 1996. Of diseases due to uncoded enteric pathogens, 73% were due to *Clostridium difficile* infection. Also, 88% of Washington State death certificates (1985 to 1996) coded to unspecified enteric pathogen infections (ICD0084) listed *C. difficile* infection.

Infectious diarrhea remains a major cause of death worldwide (1). In the United States, enteric pathogens are estimated to cause 25 to 99 million episodes of diarrhea and vomiting each year, resulting in 2.2 million physician visits (2). U.S. residents at highest risk for severe illness or death from diarrhea are young children (3-5) and the elderly (5). Race, socioeconomic status, and residence in a nursing home are also risk factors for death due to diarrhea (1-6).

Increasing concern over waterborne transmission of enteric pathogens (7-10) prompted this study. Since hospitalization or death from infectious diarrhea is uncommon, state- or hospital-specific studies are unlikely to include enough cases to accurately estimate the incidence or describe the temporal trends in hospitalization or death rates. Therefore, this study focused on national hospitalization and death data to determine if the incidence of hospitalizations or deaths due to infectious diarrhea has changed during the past decade and, if so, to identify specific pathogens responsible for the changes.

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Data Sources

Computerized data on U.S. death rates (1980 to 1992), which included coded underlying cause of death, and National Hospital Discharge Survey (NHDS) data (1985 to 1994) were obtained from the National Center for Health Statistics (NCHS). NHDS data came from yearly surveys of hospital discharges conducted by NCHS through a multistage sampling scheme (11). Yearly national estimates of discharges by diagnosis, age, and gender were obtained by using multipliers provided by NCHS. Yearly midyear Bureau of the Census population estimates were used for calculating national death and hospitalization rates.

Deaths Due to Diarrhea, 1980–1992

The number of diarrhea deaths in 1980 to 1992 was determined by using the coded

underlying causes of death. Deaths are coded to only four digits of the International Classification of Disease 9th revision (ICD9). Causes of death were grouped into four categories according to type of pathogen: bacterial, parasitic, viral, and ill-defined (Table 1).

Hospitalizations Due to Diarrhea, 1985–1994

Hospitalizations due to diarrhea (coded to five digits of the ICD9) were ascertained for each year from 1985 to 1994 by selecting the same groups of ICD9 codes from the first seven discharge diagnoses recorded for each hospital discharge. For the few hospital discharges with multiple infectious diarrhea codes, priority was given to the code that appeared first.

Hospitalization and Death Rates Adjusted by Age

Age-adjusted hospitalization and death rates for each year, standardized to the 1990 U.S. population, were calculated for each of the four ICD9 enteric pathogen categories (bacterial, parasitic, viral, and ill-defined). A ratio of deaths to hospitalizations for each category was calculated by dividing the number of deaths caused by pathogens in each category by the number of hospital discharges with the first infectious enteric disease discharge diagnosis included in that category.

Hospitalizations and Deaths–Bacterial Causes

Hospitalizations and deaths due to bacterial causes (including cholera, typhoid and paratyphoid fever, other salmonella infections, shigellosis, other food poisoning bacteria, and infections due to other specified bacteria) were also analyzed (Table 1). Age-adjusted death rates (each year) from "other specified enteric bacteria" (ICD9 0084) and age-adjusted hospitalization rates (each year) due to "uncoded but specified enteric bacteria" (ICD9 00849) were calculated. Additional cause codes were added in 1992 (ICD9 00843-00847 for hospital ICD coding, including 00845 for *Clostridium difficile* (Table 1). However, these codes were not yet incorporated for this analysis of national hospital discharge data. During this time, cause of death was only coded to the fourth ICD9 digit.

Table 1. International Classification of Disease (ICD9) Categories

Bacterial	
001[a]	Cholera
002[a]	Typhoid and paratyphoid fevers
003[a]	Other salmonella infections
004[a]	Shigellosis
005[a]	Other food poisoning bacterial
008[a]	Intestinal infections due to other organisms
0080	*Escherichia coli*
0081	Arizona group of paracolon bacilli
0082	*Aerobacter aerogenes* (Enterobacter)
0083	Proteus
0084	Other specified bacteria
0085	Bacterial enteritis (unspecified)
041[a]	Bacterial infection in conditions classified elsewhere and of unspecified site
For hospital coding	
00841	Staphylococcus
00842	Pseudomonas
00849	Other
After 1992	
00843	Campylobacter
00844	*Yersinia enterocolitica*
00845	*Clostridium difficile*
00846	Other anaerobes
00847	Other gram-negative bacteria
Viral	
074[a]	Specified disease due to coxsackie virus
045[a]	Acute poliomyelitis
047[a]	Meningitis due to enterovirus
048	Other enterovirus diseases of central nervous system
0086	Enteritis due to specified virus
0088	Other viral organism not elsewhere classified
0700[a]	Viral hepatitis A with hepatic coma
0701[a]	Viral hepatitis A without mention of hepatic coma
Parasitic	
006[a]	Amebiasis
007[a]	Other protozoal intestinal diseases
127[a]	Other intestinal helminthiases
128	Other and unspecified helminthiases
129[a]	Intestinal parasitism, unspecified
Other	
009[a]	Ill-defined intestinal infections

[a] Diarrhea codes selected for study.

Deaths (1985–1996) and Hospitalizations (1993–1996)

To make use of enhanced ICD9 coding available after 1992, inpatient health-care records for 1993 to 1996 from the Lovelace Health Systems in Albuquerque, New Mexico, were reviewed to identify bacterial pathogens responsible for most hospitalizations that would have been coded (before 1992) to specified but uncoded bacterial pathogens. Since death records are only coded to the fourth digit, Washington State deaths (with any multiple cause of death code of ICD9 0084) occurring between 1985 and 1996 were also reviewed to identify the specific pathogen that resulted in this ICD9 code assignment.

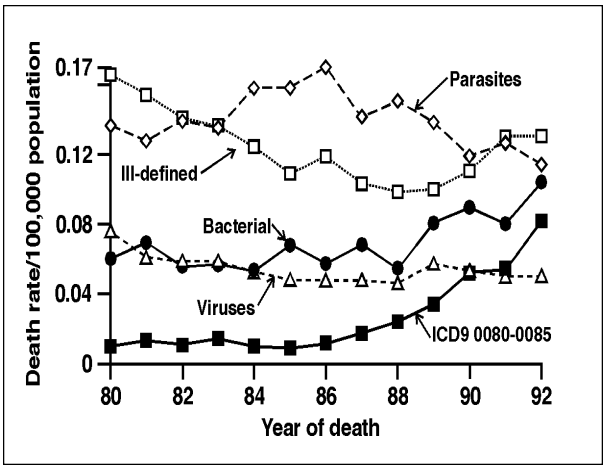

Figure 1. Age-adjusted death rates per 100,000 population by grouped underlying cause of death for selected enteric pathogens, United States 1980-92. (Standardized to the 1970 U.S. population).

Findings

Death Rates

Age-adjusted death rates for protozoal, viral, and other causes remained relatively stable from 1980 to 1992 (Figure 1), but age-adjusted death rates for bacterial causes increased from 0.060 per 100,000 population in 1980 to 0.104 per 100,000 in 1994, more than 60% ($p < 0.00001$) (Figure 1). To ascertain the specific bacterial agents responsible for the increase, deaths were initially categorized by each three- and four-digit ICD9 code included in the bacterial category. The age-adjusted death rate due to other specified bacterial pathogens (ICD9 0080-0084) increased from 0.0102 per 100,000 population to 0.0821 per 100,000 population ($p < 0.000001$), more than eightfold (Table 2). This increase accounted for the overall increase in the age-adjusted death rate for deaths due to enteric bacterial pathogens, as the age-adjusted death rate for other enteric bacterial causes remained stable or declined.

Most of the increase in the age-adjusted death rate for ICD9 0080-0084 was due to a statistically significant ($p < 0.00001$) increase in the death rate of persons age 45 years and older

Table 2. Age-adjusted death rates for bacterial causes of diarrhea

	ICD9 0080-0084[a]				Remainder of bacterial causes[b]			
Year	AARATE[c]	<45 yrs[d]	≥45 yrs[d]	(Deaths)	AARATE[3]	<45 yrs[d]	≥45 yrs[d]	(Deaths)
1980	0.0102	0.0013	0.0090	(21)	0.0500	0.0133	0.0368	(107)
1981	0.0135	0.0035	0.0100	(29)	0.0559	0.0107	0.0453	(120)
1982	0.0107	0.0022	0.0085	(23)	0.0463	0.0087	0.0365	(100)
1983	0.0144	0.0040	0.0104	(32)	0.0426	0.0092	0.0334	(94)
1984	0.0101	0.0026	0.0075	(23)	0.0433	0.0068	0.0365	(98)
1985	0.0092	0.0016	0.0075	(21)	0.0587	0.0131	0.0456	(135)
1986	0.0119	0.0029	0.0090	(28)	0.0485	0.0108	0.0347	(105)
1987	0.0175	0.0029	0.0146	(41)	0.0506	0.0097	0.0409	(120)
1988	0.0241	0.0008	0.0232	(57)	0.0305	0.0071	0.0234	(74)
1989	0.0342	0.0016	0.0326	(83)	0.0465	0.0111	0.0354	(114)
1990	0.0520	0.0043	0.0478	(129)	0.0374	0.0070	0.0303	(93)
1991	0.0547	0.0027	0.0520	(139)	0.0254	0.0055	0.0190	(65)
1992	0.0821	0.0046	0.0775	(213)	0.0220	0.0049	0.0171	(57)

[a] Causes include *Escherichia coli* (0080), Arizona group (0081), *Aerobacter aerogenes* (Enterobacter) (0082), *Proteus mirabilus* and *morganii* (0083), other specified enteric bacterial infections (0084).
[b] Causes include cholera (001), typhoid and paratyphoid fever (002), other salmonella infections (003), shigellosis (004), bacterial enteritis, unspecified (0085), bacterial infection in conditions classified elsewhere (041).
[c] Age-adjusted death rates per 100,000 population, adjusted to the 1990 U.S. population.
[d] Contribution to age-adjusted death rate from persons younger than age 45 years and from persons age 45 years and older.

(Table 2). For both older and younger age groups, the increase was most apparent from 1988 to 1992. During this time, approximately 96% of U.S. deaths coded to ICD9 0080-0084 were coded to ICD9 0084, which includes other specified but uncoded enteric bacterial pathogens.

Hospitalization Rates

The estimated number of hospitalizations in the United States coded to infectious enteric agents increased from 131,252 to 337,178 from 1985 to 1994, a 2.5-fold increase. Age-adjusted hospitalization rates for viral causes increased more than twofold, whereas rates for bacterial causes increased more than fourfold (Figure 2). Hospitalization rates for protozoal and ill-defined causes remained stable or fluctuated from year to year (Figure 2). In 1985, bacterial causes accounted for 21% of all hospitalizations for infectious enteric agents, while in 1994, they accounted for 38% (Figure 2). A large increase was apparent in the age-adjusted hospitalization rate for other specified bacterial pathogens (ICD9 00800-00849) (Figure 2).

The increased age-adjusted rate of hospital discharge coded to "other specified bacterial pathogen" infections (ICD9 0080-00849) was statistically significant ($p < 0.00001$) (Table 3). The age-adjusted hospitalization rate for bacterial causes, other than ICD9 0080-00849, remained stable (Table 3). As with deaths, the increase in hospitalizations for bacterial causes

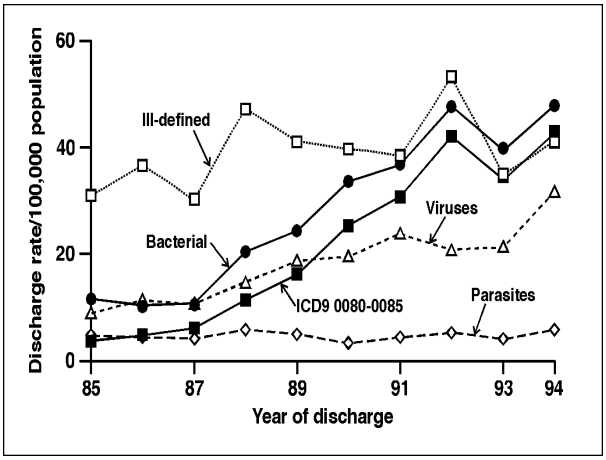

Figure 2. Age-adjusted hospital discharge rates per 100,000 population by grouped discharge diagnosis for selected enteric pathogens, United States 1985-94. (Standardized to the 1970 U.S. population). Discharges were included if a selected enteric pathogen was among the first seven discharge diagnoses. The pathogen group was assigned according to the first pathogen listed.

appears to have begun in 1988, but unlike the increase in death rates, hospitalization rates increased for persons both younger and older than 45 years of age ($p < 0.001$) (Table 3). For discharges coded to ICD9 0080-00849, 88% were coded to ICD9 00849, which includes "other uncoded but specified enteric bacterial pathogens." Discharge rates due to bacterial causes of diarrhea increased in each age group, with

Table 3. Age-adjusted hospitalization rates for bacterial causes of diarrhea

	ICD9 00800-00849[a]				Remainder of bacterial causes[b]			
Year	AARATE[c]	<45 yrs[d]	≥ 45 yrs[d]	(Discharges)	AARATE[c]	<45 yrs[d]	≥45 yrs[d]	(Discharges)
1985	3.72	1.71	2.02	(8,716)	7.76	4.06	3.70	(18,286)
1986	4.79	2.01	2.74	(11,316)	5.56	2.60	2.96	(13,174)
1987	6.05	1.95	4.10	(14,433)	4.55	2.14	2.41	(11,037)
1988	11.36	3.20	8.14	(27,492)	9.07	6.04	3.02	(22,219)
1989	16.09	5.64	11.05	(38,894)	8.10	4.96	3.14	(19,931)
1990	25.23	6.40	18.82	(62,750)	8.28	4.29	4.00	(21,037)
1991	30.67	7.55	23.12	(78,100)	6.05	3.52	2.53	(15,620)
1992	41.96	9.71	32.25	(107,874)	5.56	3.15	2.42	(14,568)
1993	33.91	8.32	25.60	(88,715)	5.51	3.54	1.98	(14,454)
1994	42.66	10.48	30.18	(113,411)	5.22	3.44	1.78	(13,944)

[a]Causes include *Escherichia coli* (0080), Arizona group (0081), *Aerobacter aerogenes* (Enterobacter) (0082), *Proteus mirabilus* and *morganii* (0083), *Staphylococcus* (00841), *Pseudomonas* (00842), *Campylobacter* (00843), *Yersinia enterocolitica* (00844), *Clostridium difficile* (00845), other anaerobes (00846), other gram-negative (00847), other (00849). Codes 00843-00847 were added in 1992.

[b]Causes include cholera (001), typhoid and paratyphoid fever (002), other salmonella infections (003), shigellosis (004), bacterial enteritis, unspecified (0085), bacterial infection in conditions classified elsewhere (041).

[c]Age-adjusted discharge rates per 100,000 population, adjusted to the 1990 U.S. population.

[d]Contribution to age-adjusted discharge rate from persons younger than age 45 years and from persons age 45 years and older.

greater increases seen in children and persons more than 45 years of age (Table 4).

Infant hospitalization rates increased for bacterial, viral, protozoal, and ill-defined conditions ($p < 0.001$) (Table 4). Hospitalization rates also increased for children ages 1 to 4 years of age for bacterial, viral, and ill-defined conditions ($p < 0.001$) (Table 4); however, no increases in children's death rates were observed.

Death-to-Discharge Ratio

The ratio of deaths to hospitalizations for protozoal and ill-defined causes of diarrhea remained stable between 1985 and 1992. However, despite the increasing incidence of enteric bacteria-caused deaths, the ratio of deaths to hospitalizations declined from 6.6 per 1,000 to 2.7 per 1,000 hospitalizations ($p < 0.001$). The ratio of hospitalizations to deaths in the viral cause category declined from 6.0 per 1,000 to 2.9 per 1,000 hospitalizations ($p < 0.001$).

Clostridium difficile Infections

Additional disease codes for hospital discharge coding were added in 1992, reducing the number of discharges coded to ICD9 00849. Examination of all computerized health-care billing records (1993 to 1996) with an ICD9 of 00843-00849 from the Lovelace Hospital and Lovelace Health Care Systems in Albuquerque, New Mexico, found 94 inpatients with these codes; *C. difficile* (ICD9 00845) accounted for 73%. This suggests that *C. difficile* infection was likely to have been the most common pathogen previously coded to ICD9 00849. Eighty-six percent of patients diagnosed with *C. difficile* were younger than age 60 with 65% younger than age 40. In addition, the records of 22 (88%) of 25 Washington State deaths occurring between 1985 and 1996 with a multiple cause of death code ICD9 0084 cited *C. difficile.*

Conclusions

Infectious diarrhea remains an uncommon cause of hospitalization and accounted for almost the same number of deaths in 1992 as in 1980. Increases in death rates for bacterial causes offset stable or declining death rates for viral, parasitic, and ill-defined causes of diarrhea. The increase in death rates for "other specified enteric bacteria" was due to increases in deaths associated with ICD9 code 0084, "uncoded but specified bacterial pathogens." Increases in

Table 4. Age-specific rates of hospitalizations (estimated discharges) by diagnostic group

	Discharge rate/100,000 population (discharges)	
Age	1985-89	1990-94
Bacterial causes[a]		
1	57.5 (10,374)	100.8 (19,536)
1-4	7.5 (5,416)	21.5 (16,696)
5-14	4.6 (7,988)	8.2 (14,988)
15-24	7.6 (14,739)	10.6 (19,216)
25-34	13.0 (26,955)	13.5 (28,583)
35-44	10.1 (17,089)	18.5 (36,871)
45-54	11.1 (13,551)	26.4 (36,152)
55-64	18.1 (19,302)	41.7 (43,774)
65-74	34.9 (30,182)	95.4 (87,902)
75	65.9 (39,903)	211.8 (145,965)
Protozoal causes[b]		
1	3.8 (680)	9.1 (1,767)
1-4	3.0 (2,157)	2.6 (2,006)
5-14	1.4 (2,508)	1.3 (2,426)
15-24	3.3 (6,337)	1.8 (3,228)
25-34	4.3 (8,934)	5.6 (11,941)
35-44	5.7 (9,723)	5.0 (10,044)
45-54	4.8 (5,902)	2.8 (3,894)
55-64	4.5 (4,786)	3.4 (3,611)
65-74	9.0 (7,783)	5.0 (4,640)
75	12.4 (7,507)	6.6 (4,561)
Viral causes[c]		
1	167.6 (30,234)	387.7 (75,120)
1-4	8.9 (6,400)	67.1 (52,021)
5-14	8.3 (14,566)	16.3 (29,617)
15-24	10.4 (20,142)	13.5 (24,577)
25-34	15.3 (31,694)	17.4 (36,913)
35-44	8.8 (14,837)	11.4 (22,707)
45-54	6.7 (8,209)	8.3 (11,312)
55-64	10.6 (11,231)	5.4 (5,658)
65-74	10.4 (9,020)	8.1 (7,481)
75	18.8 (11,390)	15.8 (10,893)
Ill-defined diarrheal causes[d]		
1	178.9 (32,272)	283.9 (55,006)
1-4	27.0 (19,452)	62.8 (48,694)
5-14	13.3 (23,301)	17.3 (31,594)
15-24	24.4 (47,242)	18.2 (32,966)
25-34	30.3 (62,701)	21.7 (45,966)
35-44	25.7 (43,618)	19.4 (38,516)
45-54	29.5 (35,972)	28.9 (39,459)
55-64	46.8 (49,773)	32.6 (34,245)
65-75	75.5 (65,336)	50.2 (46,257)
75	114.7 (69,476)	93.1 (64,163)

[a]Discharges with an enteric bacteria diagnosis (ICD9 001-005, 0080-0085, 041).
[b]Discharges with an enteric parasite diagnosis (ICD9 006-007, 127, 129).
[c]Discharges with an enteric virus diagnosis (ICD9 0086, 0088, 045, 047, 048, 0700, 0701, 074).

hospitalization rates for these "uncoded but specified bacterial pathogens" correspond temporally to the increase in death rates coded to ICD9 0084. A review of recent hospitalization records from a New Mexico health maintenance organization and death records from Washington State show that *C. difficile* was the most common pathogen in this coding group. These findings suggest that *C. difficile* was likely to have been responsible for the increase in both age-adjusted hospitalization and death rates from enteric bacterial pathogens.

Increases in death and hospitalization rates due to bacterial causes may simply indicate improved ICD9 coding for both hospitalization and death or increased diagnostic accuracy. New kits for detection of *C. difficile* toxins in stool samples may have resulted in increased *C. difficile* diagnoses. If so, the increase in enteric bacterial infections, likely due to *C. difficile,* may not be a true increase in illness from this pathogen. If this were the case, however, one might predict a corresponding reduction in hospitalizations and deaths from ill-defined diarrheal causes. Death rates for ill-defined causes of diarrhea presumed to be infectious actually rose somewhat during 1990 to 1992, and hospitalization rates have remained stable. Further studies are needed to determine if this increased age-adjusted hospitalization and death rate is due to increased *C. difficile*–associated disease and, if so, to identify risk factors for infection and disease.

Additional information about the causes of increased hospitalization and death from enteric bacterial pathogens could be provided by a review of computerized health-care delivery records. *C. difficile* can be associated with almost any antibiotic therapy, but it has been particularly associated with aminopenicillins, cephalosporins, and clindamycin, which have greater effects on the intestinal flora (12). Other factors trigger *C. difficile* toxin–associated colitis (13). Records from health maintenance organizations containing prior diagnoses and pharmaceutical treatments may provide better understanding of the risk factors.

In this study, data from one hospital system in one state and deaths occurring in one state suggest that a likely cause of the national increase in hospitalizations and deaths due to enteric bacteria may be *C. difficile* infection. It is possible that *C. difficile* is a relatively more important cause of hospitalization at Lovelace Health Systems in New Mexico and of death in Washington State than elsewhere in the United States.

The age-specific rates of hospital discharge coded to enteric bacterial, viral, and ill-defined conditions increased for children under 5 years of age. Reasons for these increases merit further inquiry. No increases in death rates from these pathogens were observed in these age groups. However, since adverse outcomes of infectious diarrhea that requires hospitalization may indicate problems with access to health care, information on the socioeconomic characteristics of the families of these children would be of interest.

Dr. Frost is an epidemiologist and the director of the Southwest Center for Managed Care Research in Albuquerque, New Mexico. His research interests include the study of waterborne diseases and the use of health-care data for promoting public health programs within managed care organizations.

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Introduction of *Aedes albopictus* into a La Crosse Virus–Enzootic Site in Illinois

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In late summer and fall 1997, *Aedes albopictus* mosquitoes were found in Peoria, Illinois, a long recognized focus of La Crosse virus transmission. Larvae were found in tires and other artificial containers, biting adults were recovered, and eggs were collected in oviposition traps within a 25-ha area. One chipmunk trapped <0.25 km from the infested area tested positive for neutralizing antibodies against La Crosse virus.

Aedes albopictus (Skuse) was probably introduced into the United States in used tires from northern Asia (1) and has spread throughout much of the southern United States, often displacing *Ae. aegypti* (L.) (2,3). Although *Ae. albopictus* mosquitoes have a short flight range (200 m to 600 m), they are readily transported in containers and vehicles (3,4).

In laboratory studies, *Ae. albopictus* was a more efficient vector of La Crosse (LAC) virus than the natural vector, *Ae. triseriatus* (Say) (5), and could readily transmit LAC virus to chipmunks (6). In field and laboratory studies of larval competition in tires and artificial containers, *Ae. albopictus* outnumbered *Ae. triseriatus* (7,8). LAC virus has not been isolated from field-caught *Ae. albopictus* females in an endemic-disease area.

LAC virus infection, the most common cause of pediatric arboviral encephalitis in the United States (9), is endemic in several midwestern and mid-Atlantic states (10). In Illinois, human clinical cases of LAC virus encephalitis are concentrated around Peoria, often near discarded tires and containers (11,12). LAC virus encephalitis is one of the main threats associated with the introduction of *Ae. albopictus* to the midwestern United States (1,2).

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Mosquito Surveillance

Peoria City/County Health Department and Illinois Department of Public Health personnel annually conduct surveillance of container-breeding mosquitoes in Peoria County by searching for and monitoring artificial containers, collecting adult females, and monitoring oviposition activity (13).

During an investigation of a suspected LAC virus encephalitis case, *Ae. albopictus* larvae and adults were collected on August 20, 1997, from approximately 10 tires and a steel box. During annual surveillance in August and September 1997, *Ae. albopictus* larvae and pupae were collected from tires and other containers. Adult mosquitoes were collected near most breeding sites and in several additional locations (Figure). Approximately 160 *Ae. albopictus* and one *Ae. triseriatus* adult females were collected. All artificial containers with mosquito larvae were treated with Abate larvicide. During the first week of October, larvae were also collected from a small boat containing water, and adults were collected in the immediate vicinity.

Nine oviposition traps were placed in the infested site starting August 21, 1997 (Figure). The *Aedes* eggs found in seven traps were hatched, and the larvae were reared to adults. *Ae. albopictus* were identified from four traps, with two traps each yielding more than 100 mosquitoes. *Ae. triseriatus* were identified from

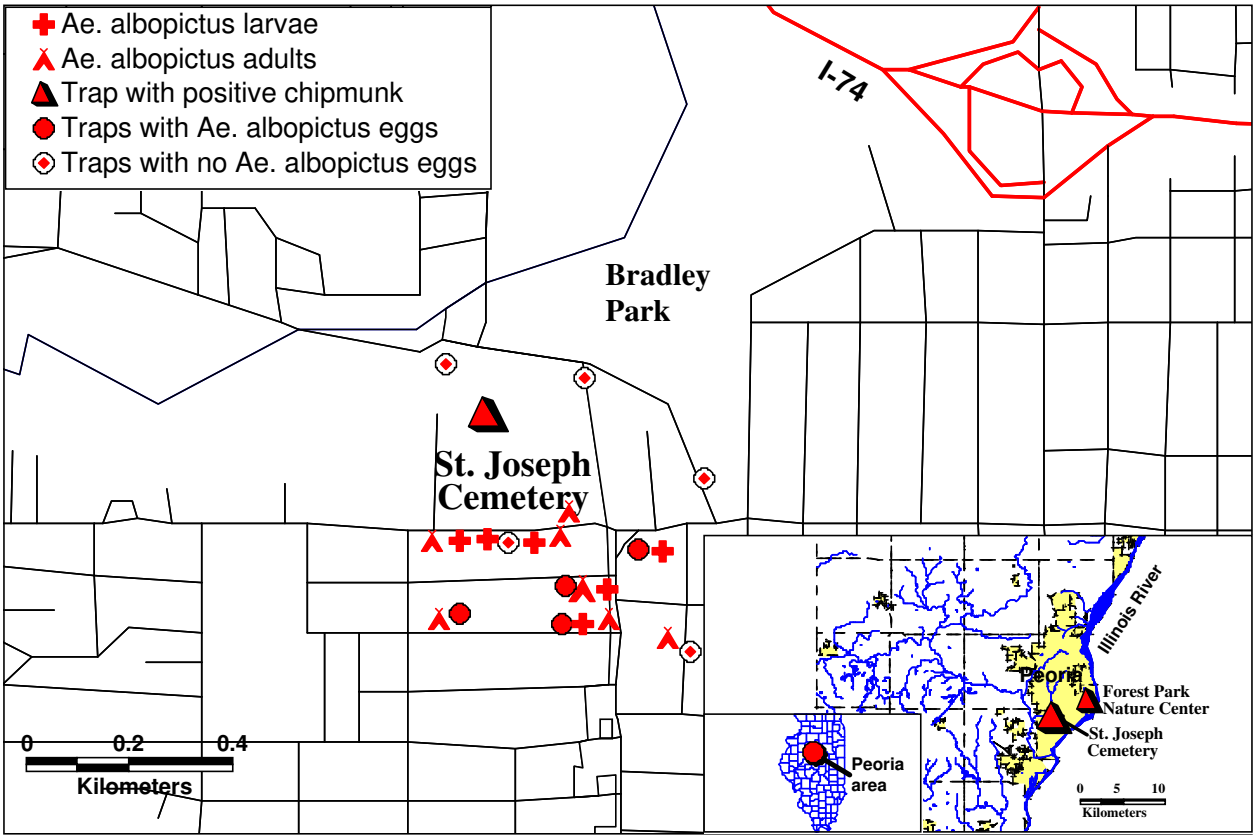

Figure. Locations in Peoria, Illinois, where *Aedes albopictus* larvae and adults were found and where a La Crosse–positive chipmunk was trapped. Location of oviposition traps are shown, including traps where no *Ae. albopictus* eggs were deposited. Insets show location of Peoria and of the study sites.

four traps, with the total numbering less than half of *Ae. albopictus*. No LAC virus was isolated from these field-collected mosquitoes.

Reservoir Host Trapping and Sampling

Fifteen Tomahawk live traps (model 102/rat: 40.6 x 12.7 x 12.7 cm.; Tomahawk Live Trap Company, Madison, WI) were placed 5 to 15 m apart in an east-west transect in St. Joseph Cemetery, Peoria, Illinois, on September 20, 1997 (Figure). To attract chipmunks and squirrels, traps were baited with sunflower seeds and small pieces of peanut butter sandwiches. Trapped animals were processed and released after recovering from anesthesia. Animal trapping and handling procedures were conducted in accordance with a protocol approved by the Laboratory Animal Care Advisory Committee at the University of Illinois (protocol #V6R131).

In St. Joseph Cemetery, 10 chipmunks (*Tamias striatus*) were caught in 15 traps opened one morning (Figure). This 67% trapping success rate is high compared with the rate at Forest Park Nature Center (Figure), a long-recognized LAC virus–enzootic transmission focus in Peoria (11), where on the same date, chipmunks and squirrels (*Sciurus carolinensis* and *S. niger*) were collected in 15 (30%) of 50 traps.

LAC Antibody Detection

Blood (0.2 ml) was taken from anesthetized chipmunks by a suborbital sinus puncture behind the right eye with a 100-µl capillary tube (14) and absorbed onto two Nobuto (Toyo Roshi Kaisha, Tokyo, Japan) filter strips, which were tested for antibody at the University of Notre Dame. Neutralizing (N) antibodies against LAC virus were detected by a cell culture assay and the serum-dilution neutralization test (SDNt) (15). Titers, calculated by the method of Reed and Muench (16), were expressed as the highest dilution showing <50% cytopathic effects. Homologous (LAC virus) and heterologous

(Jamestown Canyon, Trivittatus, eastern equine encephalitis, St. Louis encephalitis virus) mouse hyperimmune ascitic fluids served as positive and negative N antibody controls, respectively.

Of the 10 chipmunks trapped in St. Joseph Cemetery, 1 (10%) was positive (titer = 8) for N antibodies against LAC virus. Two (22%) of nine chipmunks trapped on the same date in Forest Park Nature Center were N-antibody positive (titers 4, 16). Low titers may be associated with the differing amounts of sera present in the Nobuto strips (all sera were eluted in the same amount of media and then diluted as if all were equal). Low titers may also be caused by animals having been infected earlier in the season. Because little serum was available, we conducted only SDNt, our most sensitive and specific serologic procedure for detecting California serogroup positives.

Mapping

The spatial distribution of mosquito collections (adults and larvae), locations and catches of oviposition traps, and locations of chipmunk traps were overlaid on a street map of the city of Peoria (Figure). The LAC virus–seropositive chipmunk caught in St. Joseph Cemetery was trapped approximately 150 m from sites where *Ae. albopictus* eggs, larvae, and adults were collected (Figure). These data were compared with data on human cases (with known addresses) (12); all epidemiologic and entomologic data were stored in a geographic information system (MapInfo GIS, Troy, NY) as part of an ongoing study. The system allows for ready management of georeferenced data, continuous updating of entomologic and epidemiologic data, and production of custom maps.

Conclusion

The detection of *Ae. albopictus* mosquitoes, coupled with the trapping of an LAC virus–seropositive chipmunk within 150 m of larval and adult *Ae. albopictus* collection sites (well within the flight range of *Ae. albopictus*), is noteworthy. Human cases were reported within 1.5 km of these sites in the 1970s and in 1994. Even within Peoria, the spatial distribution of enzootic foci and human cases is patchy. These findings are the first evidence that *Ae. albopictus* has been introduced into the heart of an urban and suburban area where LAC virus has long been endemic and has caused disease in humans. A focus in rural southeastern Indiana has been described (17) in a highly LAC virus-endemic area of the state (18).

Studies in Indiana and an estimate of the northern limits of the distribution of *Ae. albopictus* (19-21) suggest that *Ae. albopictus* may become entrenched in central Illinois, especially after mild winters. *Ae. albopictus* eggs stored outdoors at the University of Notre Dame in northern Indiana were hatched successfully in late February 1998, following several days of record warm temperatures, and in mid-April after cold weather had passed. In addition, *Ae. albopictus* have long been detected in Indianapolis, Indiana, at a latitude close to that of Peoria, Illinois (M. Sinsko, Indiana State Board of Health, pers. comm.).

The detection of this *Ae. albopictus* focus marks the beginning of a natural experiment to test the ability of *Ae. albopictus* to overwinter in central Illinois, displace *Ae. triseriatus* from artificial and natural containers, and survive intensive control efforts. If this population of *Ae. albopictus* reemerges in 1998, its vertebrate host feeding preference and its ability to act as a vector for enzootic and endemic transmission of LAC virus should also be evaluated.

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Mycobacterium canettii, the Smooth Variant of *M. tuberculosis*, Isolated from a Swiss Patient Exposed in Africa

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An exceptionally smooth and glossy morphotype of *Mycobacterium tuberculosis* complex was isolated from a 56-year-old Swiss patient with mesenteric tuberculosis. Direct 16S rRNA sequence analysis of the hypervariable signature gene regions revealed a 100% homology to the specific *M. tuberculosis* complex sequence. Spoligotyping and restriction fragment length polymorphism analyses using the insertion sequences IS*6110* and IS*1081* and the polymorphic GC-rich sequence as additional genetic markers identified the isolate as the novel taxon *M. canettii*. Like a Somali child with a similar case, this patient probably contracted the infection in Africa, which raises questions about the geographic distribution of *M. canettii*.

A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex was described (1) in 1997 in a highly exceptional strain, designated So93, isolated in 1993 from a 2-year-old Somali child with lymphadenitis. Although on the basis of biochemical tests and the 16S rRNA gene sequence this isolate was clearly a member of the *M. tuberculosis* complex, it exhibited an unusually smooth and glossy colony morphology. Genetically, strain So93 differed from all known *M. tuberculosis* complex strains (nearly 2,000 *M. tuberculosis* isolates have been examined) (1). It contained a single copy of the insertion sequence IS*1081*, had a direct repeat region with an exceptional composition, and differed in the sequence of the *recA* gene. In 1969, Canetti had isolated from a French patient an *M. tuberculosis* strain with a smooth colony morphology. Daffé et al. (2) had demonstrated that this particular strain differed from the commonly rough strains by having large amounts of lipooligosaccharides. Isolate So93 and the Canetti strain from the 1960s shared virtually all phenotypic and genetic characteristics. Therefore, these two smooth *M. tuberculosis* variants were designated '*M. canettii*' (1).

We report here a third tuberculosis (TB) case caused by *M. canettii*. The mycobacterial isolate NZM 217/94 was cultured from pus aspirated from the ileocecal region of a 56-year-old male patient born in Switzerland. Phenotypic and genetic characteristics were compared with both the isolate So93 and the Canetti strain.

The patient had been working for an international organization since 1963, in Uganda for 20 years, then in Kenya for 1 year. HIV infection was detected in this patient in 1993. That same year, he contracted pneumonia of unknown etiology, which was successfully treated with cotrimoxazole. Two short episodes of severe diarrhea followed in 1994. The patient was transferred from Kenya to the University Hospital in Geneva, where a partial occlusion of the ileum caused by an extrinsic compression was diagnosed. A right hemicolectomy was performed. An extensive inflammatory mass of mesenteric lymph nodes was strongly positive for acid-fast bacilli by direct microscopy. After as little as 1 week, culture (BACTEC 12B medium; Becton Dickinson Diagnostic Instrument Systems, Sparks, MD) yielded mycobacteria, which were identified as *M. tuberculosis* complex. The

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patient was already in stage C2 of AIDS, which favored a rapid development of mesenteric disease. No pulmonary signs of the disease were observed (3). Response to quadruple antituberculotic chemotherapy was excellent. However, within 3 months, extensive cutaneous lesions developed due to a generalized highly malignant non-Hodgkin lymphoma, another complication of AIDS, known as fulminant disease with poor prognosis. A cytostatic therapy was initiated. The patient returned to Kenya but did not follow any treatment regimens. He was hospitalized in Geneva in 1995 with severe cachexia and died 9 months later. The postmortem findings showed massive invasion of the abdominal cavity with malignant lymphoma but no further evidence of active TB, neither by histology nor by culture.

Although the isolate exhibited abundant smooth and glossy colonies on Middlebrook 7H10 agar (Figure 1), the *M. tuberculosis* complex gene probe (Accuprobe; Gen-Probe Inc., San Diego, CA) was positive. Growth was strictly aerobic. The strain reduced nitrate, but niacin production and 68°C catalase were negative. Analysis of cellular fatty acids was done by gas-liquid chromatography in conjunction with the Microbial Identification System (Microbial ID Inc., Newark, DE) (4) and yielded *M. tuberculosis* complex. Cellular fatty acid esters ranged from C12:0 to C20:0, the major components being C16:0 (35.5%), C18:1 (24.9%), C18:0 (11.2%), and tuberculostearic acid (10 Me-18:0 TBSA; 12.1%).

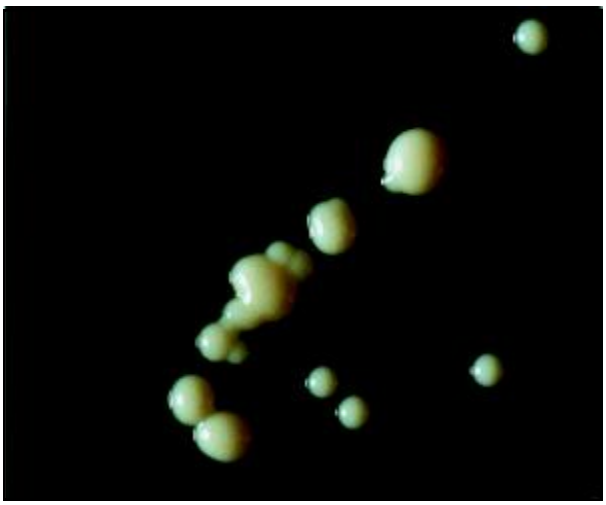

Figure 1. Colony morphology of strain NZM 217/94 on Middlebrook 7H10 agar.

Mycolic acids consisted of α, methoxy, and keto mycolates, which are typical for tubercle bacilli. Guanine plus cytosine content of the DNA was 66.4 mol%. The polymerase chain reaction/restriction enzyme pattern of the amplified 65-kDa heat shock protein gene fragment (65-kDa-HSP-PRA) (5) yielded bands of 245/125/80 bp and of 160/140/70 bp, respectively, following *Bst*EII and *Hae*III digests, a pattern compatible with *M. tuberculosis* complex (5). Direct 16S rDNA sequence analysis (6) showed that the hypervariable signature gene regions were identical to *M. tuberculosis* complex.

Strain NZM 217/94 was susceptible to *p*-nitro-α-acetylamino-ß-hydroxypropiophenone (NAP) (7) and thiophene carboxylic acid (T_2H) as well as to all primary antituberculotic drugs (isoniazid 0.2 and 1μg/ml, rifampin 0.2 μg/ml, ethambutol 2.5 μg/ml), except pyrazinamide (100 μg/ml). Susceptibility was observed against the second-line drugs streptomycin (10 μg/ml), ethionamide (5 μg/ml), rifabutin (1 μg/ml), sparfloxacin (1 μg/ml), ofloxacin (1 μg/ml), ciprofloxacin (1 μg/ml), and *p*-amino salicylic acid (2 μg/ml). Susceptibility testing of the primary antituberculotics was done radiometrically (BACTEC 460TB System, Becton Dickinson) (8). Second-line drugs have been tested by the method of proportion (indirect test) on Middlebrook 7H10 agar (9).

To determine whether the isolate from the Swiss patient represented the *M. canettii* genotype, restriction fragment length polymorphism (RFLP) analyses and spoligotyping were performed (1,10). The IS*6110* RFLP patterns of the Swiss (NZM 217/94) and the Somali isolates (So93) were identical (Figure 2a). As previously shown, the Canetti isolate contains an additional copy of this insertion sequence. Furthermore, the Swiss isolate contains a single copy of IS*1081* on a similarly sized *Pvu*II restriction fragment, as previously found in the So93 strain (Figure 2b). The patterns generated with the polymorphic GC-rich repetitive sequence (PGRS) of strains NZM 217/94 and So93 were identical; whereas for the Canetti strain, 7 out of 20 PGRS-containing *Alu*I restriction fragments were found at other positions (data not shown). By the recently developed spoligotyping technique (10) we could demonstrate that the Swiss isolate contained the same two spacer sequences found in both the Canetti strain and the So93 isolate (Figure 2c).

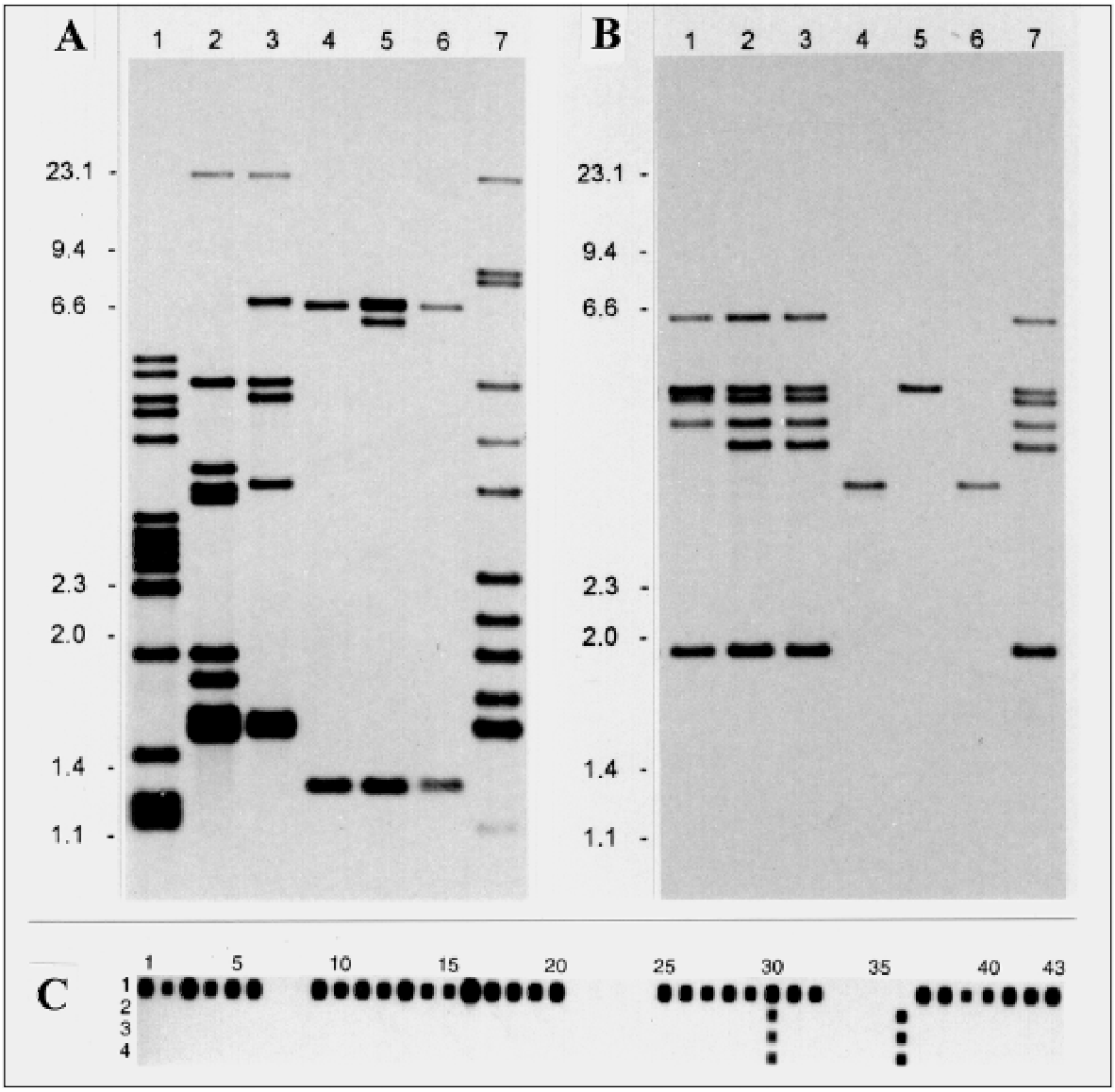

Figure 2. IS*6110* (a) and IS*1081* (b) hybridization patterns of *Pvu*II-digested genomic DNA. Lanes 1 to 3: clinical *M. tuberculosis* isolates; lane 4: the Somali isolate, So93; lane 5: the Canetti strain isolated in France in 1969; lane 6: the Swiss isolate, NZM 217/94; lane 7: *M. tuberculosis* Mt14323 (reference strain). Figure c represents spoligotyping patterns. Lane 1: *M. tuberculosis* H37Rv (reference strain); lane 2: the Canetti strain; lane 3: the Somali isolate, So93; lane 4: the Swiss isolate, NZM 217/94.

These data demonstrate the first TB case caused by *M. canettii* in a Swiss patient. RFLP analyses using IS*6110,* IS*1081,* and PGRS as probes as well as spoligotyping revealed the same DNA fingerprint patterns for the Swiss isolate as previously found for strain So93 isolated from a Somali child. With its highly exceptional smooth and glossy colony morphology and the unusual, abundant growth, the Swiss isolate belongs to the same taxon of the *M. tuberculosis* complex as the Somali isolate and the Canetti strain. The Canetti strain isolated in France about 30 years ago (from a patient of whom no further details are available) showed RFLP patterns that were highly similar but not identical to the ones of the Swiss and Somali isolates. Our data indicate 1) that the *M. canettii* taxon comprises strains with some degree of evolutionary divergence and 2) that this evolutionary lineage already existed but was

likely overlooked because the lack of adequate genetic characterization techniques.

Although the exact source of infection in this latest clinical case of *M. canettii* could not be established with certainty, the patient was repeatedly exposed to TB patients in East Africa. Since the patient had no known exposure to pets or wild animals, a human source of infection seems very likely. This report indicates that *M. canettii* may be of wider prevalence than previously thought; therefore, further studies are needed to determine its geographic distribution (i.e., its reservoir) and eventually the extent of genetic variation and presence of virulence factors associated with this particular taxon of the *M. tuberculosis* complex. In addition, this study demonstrates the value of applying a combination of several subtyping methods by which future studies can be directed to determine whether *M. canettii* is emerging as an important pathogen.

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Human Infections with Shiga Toxin-Producing *Escherichia coli* Other Than Serogroup O157 in Germany

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We investigated different types of Shiga toxin-producing *Escherichia coli* (STEC) not belonging to serogroup O157 for their role as human pathogens. Non-O157 STEC isolated from 89 human patients in Germany were characterized according to serotypes, virulence markers, and association with human illness. *Eae*A-positive STEC were isolated from 54 (60.7%) of the patients and were frequently associated with severe diarrheal disease, hemolytic uremic syndrome, and young age. *Eae*A-negative STEC were found in 35 (39.3%) of the patients and were more associated with clinically uncomplicated cases and adult patients. For pediatric patients, a serotype-independent diagnosis of STEC is recommended.

Certain strains of *Escherichia coli* belonging to different O-(LPS) and H-(flagellar) serotypes produce potent cytotoxins called Shiga toxins (Stx) (1). The natural hosts of Shiga toxin-producing *E. coli* (STEC) are farm and wildlife ruminants. In humans, STEC can cause disease, although the clinical picture may vary from uncomplicated diarrhea, to hemorrhagic colitis (HC), to hemolytic uremic syndrome (HUS) (2,3). Large outbreaks and cases of HC and HUS in humans were mainly associated with STEC strains belonging to serogroup O157. Human infections with STEC O157 are under nation-wide surveillance in a number of countries, but the detection of non-O157 STEC infections is often limited to a small number of specialized laboratories because STEC O157 colonies are more easily detectable on some culture media than non-O157 STEC types, which are thus often missed in laboratory diagnosis of stool specimens (4,5). However, humans are likely more exposed to non-O157 STEC because these strains are more prevalent in animals and as contaminants in foods than STEC O157 (4,6). Infections with some non-O157 STEC types, such as O26 and O111, are associated with illness in humans, but the role of other non-O157 STEC types in human disease needs further examination (3,7). In Germany, the number of clinical laboratories performing diagnosis of STEC from stool samples has increased since immunologic assays (Stx-enzyme-linked immunosorbent assay [ELISA]) specific for detection of Stx1 and Stx2 became commercially available. The Stx-specific ELISA kits are useful for detection of different serotypes of STEC; consequently, more non-O157 STEC-positive stool specimens and bacterial isolates were sent to our laboratory for confirmation and typing. In this study, we report on non-O157 STEC infections in 89 human patients infected during 1996 in Germany. We investigated the relationship between STEC serotype, STEC virulence factors (such as the *eae*A gene, production of enterohemolysin), Stx1 and Stx2, and the clinical signs in the patients.

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Isolation of Non-O157 STEC Strains

Of 89 non-O157 STEC isolates from 89 patients, 46 were isolated in the collaborating laboratories and 43 in our laboratory from stool samples. STEC isolates or stool specimens were sent together with patient data (age, gender, and illness) from 37 private, hospital, and public health laboratories from rural and urban areas of Germany. In our laboratory, a small sample of stool was injected into 5 ml of Tryptic Soy Broth and incubated overnight at 37°C (8). Aliquots from the grown stool culture were tested for toxicity and for Stx-specific DNA sequences by polymerase-chain reaction (PCR) (9,10). STEC were isolated from Stx-positive stool cultures by

combined use of enterohemolysin agar and the STEC-RPLA test (8). Enterohemolysin-negative STEC were identified by testing different coliform colonies with the STEC-RPLA assay. All stool cultures were additionally plated on Sorbitol MacConkey Agar (SMAC) for identification of sorbitol-nonfermenting STEC O157 strains.

Most of our collaborating laboratories in Germany used Stx-specific ELISA for routine examination of stool samples. Examination for STEC was performed in cases of diarrhea, HUS, or on special request; diagnostic approaches differed among laboratories. Some supplied only Stx-ELISA-positive stool specimens for further examination; others attempted to isolate STEC colonies by using combinations of commercially available diagnostic tools such as Stx-ELISA, STEC-RPLA, agglutinating rabbit antisera for enteropathogenic *E. coli* (EPEC) O-groups including serogroup O157, enterohemolysin agar, and SMAC. Of the 37 collaborating laboratories, two used their own methods, such as the Stx-toxicity test or an Stx-specific PCR for identification of STEC from stool.

In our laboratory, all 89 non-O157 STEC isolates were tested for cytotoxicity by the Stx test and for production of Stx1 and Stx2 by the STEC-RPLA assay. The enterohemolytic phenotype was determined on enterohemolysin agar (8). The presence of the *eae*A gene was determined by PCR using two different primer pairs specific for the conserved region of the *eae*A gene (11,12). Serotyping of O- and H-antigens of *E. coli* strains was performed as described (13).

Characterization of Non-O157 STEC Strains

Only one STEC serotype was isolated from each of the 89 patients except for one patient with HC who excreted simultaneously STEC types O157:H7 and O26:H11. Of the 89 STEC strains, 69 (77.5%) could be typed to 15 different O-groups, and 20 were O-untypable or O-rough (Table 1). The STEC strains varied in H-antigens, which were conserved within some of the O-groups, and 31 STEC strains (34.8%) were nonmotile. Most isolates (n = 59) produced only Stx1 (66.3%) and 11 (12.4%) only Stx2. Production of Stx1 and Stx2 was found in 19 (21.3%) strains and was associated with strains of O-groups 113 and 145. Fifty-four (60.7%) of the STEC strains were found *eae*A-positive. The *eae*A-gene was present in all STEC strains belonging to some common serogroups (O26, O103, O111, O118, and O145) and in 8 of 17 STEC O-rough strains. The enterohemolytic phenotype was expressed in 82 strains (92.1%) and was highly associated with *eae*A positive STEC (53 [98.2%] of 54 were positive). All STEC strains were positive for fermentation of sorbitol.

Relationship Between STEC Types, Virulence Markers, and Clinical Status of the Patients

Fifty-one (60.7%) of the patients were female, and 33 (39.3%) were male; the gender of five patients was not reported. Of 85 patients whose ages were known, 46 (54.1%) were 3 months to 6 years of age, 10 (11.8%) were 6 to 15 years of age, and 29 (34.1%) were older than 15 years (Table 2). Patients were divided into four groups according to case history (Table 1). Six cases (6.7%) of asymptomatic excretors of STEC were detected by routine screening of stool specimens. Nonbloody diarrhea, in some cases accompanied with abdominal pain and vomiting, was reported for 72 (80.9%) patients. Bloody diarrhea was reported in 7 (7.9%) and HUS in 4 (4.5%) cases. Episodes of protracted, nonbloody diarrhea for 2 weeks or more were reported for 11 (12.4%) patients. Double infections with STEC and other diarrheal pathogens were not reported except for 6 (6.7 %) patients who were additionally infected with *Salmonella enterica* sp. (Table 1).

The clinical disease was not associated with the Stx types produced by the infecting strains. In contrast, the *eae*A-gene was closely associated with severe illness and young age (Tables 1, 2). Thus, 9 (81.8%) of the 11 patients with bloody diarrhea or HUS were infected with *eae*A-positive STEC of serogroups O26, O103, O111, O118, or O145; 8 (72.7%) of 11 patients with protracted, nonbloody diarrhea carried *eae*A-positive STEC of serogroups O118, O145, O163, and O-rough. Of 54 patients with *eae*A-positive STEC isolates, 41 (75.9%) were younger than 6 years of age with a peak age of 1 to 3 years (Table 2) and a mean age of 8 years and 9 months. In contrast, *eae*A-negative STEC were more prevalent in adults and were isolated from 35 patients; age was known in 31 (Table 2). Only five (16.1 %) were younger than 6 years of age, and the mean age in this group was 27 years and 10 months.

Most of the non-O157 STEC infections were sporadic, and the sources of infection

Table 1. Non-O157 STEC infections in human patients in Germany, 1996

STEC serotypes		No. of STEC strains with properties					Clinical status			
O-group[a] (no. of strains)	H-types[b]	Stx1	Stx2	Stx1+ Stx2	*eae*A	Entero-hemo-lytic	Asympto-matic	Non-bloody diarrhea	Bloody diarrhea	HUS[c]
O5 (1)	NM	0	0	1	0	1	0	1	0	0
O18 (1)	H15	0	0	1	1	1	0	1[d]	0	0
O26 (20)	H11, NT, NM	16	4	0	20	19	1	17	2[d,e]	0
O76 (3)	H19	3	0	0	0	3	0	3	0	0
O78 (1)	NM	0	1	0	0	1	1	0	0	0
O84 (1)	NT	1	0	0	1	1	0	1[d]	0	0
O91 (4)	H14, NM	3	0	1	0	4	0	4	0	0
O103 (7)	H2, H7, NT	6	1	0	7	7	0	5[d]	1	1
O111 (3)	NT	1	1	1	3	3	1	1	0	1
O113 (3)	H32, NT	0	0	3	0	3	0	3	0	0
O118 (8)	H12, H16 NT	7	1	0	7	7	0	6[d]	1	1
O128 (5)	H2, NM	3	0	2	0	5	1	4	0	0
O145 (6)	H28, NM	1	0	5	6	6	0	5	0	1
O146 (5)	H21, H28	3	1	1	0	5	0	4	1[d]	0
O163 (1)	H19	0	1	0	0	1	0	1	0	0
ONT (3)	ND	2	0	1	1	3	1	2	0	0
O-rough (17)	ND	13	1	3	8	12	1	14	2	0
Total (89)		59	11	19	54	82	6	72	7	4

[a]ONT= O-antigen not typable; O-rough= rough LPS, O-antigen not typable.
[b]NM= nonmotile; ND= H-antigen not determined; NT= H-antigen not typable.
[c]HUS= hemolytic uremic syndrome.
[d]A case of double infection with *Salmonella enterica* sp.
[e]A case of a double infection with STEC O26:H11 and O157:H7.

were not identified. However, outbreak investigations were not routinely performed, and a number of these infections could be part of unidentified outbreaks. In four cases, stool specimens from persons who were in contact with the patients were examined, and two outbreaks in families with diarrheal episodes were detected. One outbreak occurred in a family living in Berlin; diarrheal symptoms developed in the parents and their two children after they returned from vacation on a farm in Northern Germany. STEC O145:H- (Stx2 and *eae*A positive STEC) could be isolated from two family members. The second outbreak was detected in a family living in the region of Augsburg (Bavaria). The parents had no diarrheal symptoms, but their children had diarrhea over a period of 6 weeks. STEC O-rough, Stx1, and *eae*A-positive STEC were isolated from stool samples of both infants. All patients from both outbreaks recovered.

Conclusions and Recommendations

Human pathogenicity of *E. coli* strains belonging to the STEC group varied according to serotypes, virulence attributes, and other unknown factors (7). Besides Shiga toxins, the *eae*A gene product intimin contributed to diarrheal disease in humans, and typical human virulent STEC (e.g., enterohemorrhagic *E. coli* O157 strains) were found to carry genes for Shiga toxin(s), intimin, and enterohemolysin (7). Two major groups of non-O157 STEC strains were isolated from humans. Group I consisted of 54 strains (60.7%), which were all positive for *eae*A and with one exception for enterohemolysin, and group II of 35 (39.3%) STEC strains lacking the *eae*A gene. Group I STEC strains were more frequently associated with severe diarrheal disease, HUS, and with young age than group II strains, which were more frequently found in clinically uncomplicated cases (asymptomatic carriers, abdominal pain, uncomplicated diarrhea) and in older patients. These findings indicate that some or all of the group II strains are less virulent or nonpathogenic for humans, although they can colonize the human intestine. Many *eae*A-negative non-O157 STEC strains isolated from healthy animals have been found to adhere to HEp-2 cells in culture without carrying DNA sequences specific for diffuse adherence (*daa*), local adherence (*eaf*), or for enteroaggregative

Table 2. Age of patients and STEC type (*eae*A)

Patient age (years)[a]	No. of patients with STEC-isolate	
	*eae*A-positive	*eae*A-negative
0-1	6	1
1-2	13	1
2-3	11	0
3-4	5	2
4-5	2	1
5-6	4	0
6-10	2	2
10-15	2	4
15-20	0	4
20-30	2	2
30-40	3	5
40-50	4	2
50-60	0	6
60-72	0	1
Total	54	31

[a]Patient's age was known in 85 cases. The youngest patient was 3 months of age; the oldest, 72 years of age.

E. coli (14). Characterizing the colonization mechanisms of *eae*A-negative STEC could help to further define their role as human pathogens.

Our finding that infections with *eae*A-positive non-O157 STEC were more frequent in young infants resembles EPEC infection-related finding. EPEC cause gastroenteritis in very young children and occur rarely in adults. Humans exposed to EPEC show an immune response against bacterial antigens such as LPS, intimin, and fimbriae and may thus develop protective immunity to these pathogens (15). Similarly, a Canadian study has shown that non-O157 STEC-infections at young ages may confer protective immunity to subsequent infections in humans (16). It is possible that EPEC infections in early childhood confer cross-reacting protective immunity against STEC types that share common antigens (such as LPS and intimin) with classical EPEC strains. This could explain why infections with *eae*A-positive STEC occur less frequently in older patients.

Our data support previous findings that non-O157 STEC types are more frequently involved in nonbloody diarrhea than STEC-O157, whereas the latter are more frequently associated with HC and HUS (7). In 1996, 89 non-O157 STEC and 33 STEC O157 from 121 human patients were investigated in our laboratory. The proportion of patients with HUS or HC was 45.5% in the group of STEC O157 infected compared with 12.4% in the group of non-O157 STEC-infected patients. Only one of the 33 STEC O157 strains from human patients belonged to the sorbitol-fermenting, ß-glucuronidase-positive type of O157:H- strains reported in Germany (17).

Humans of all age groups can be infected with STEC belonging to different serotypes. Because the association between serotype and human pathogenicity is not always certain, a serotype-independent diagnosis of STEC is required. Although it is not possible for economic reasons to examine all patients with diarrhea for STEC, these organisms should be sought in pediatric patients, who are at higher risk for serious infection with these pathogens.

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New *Orientia tsutsugamushi* Strain from Scrub Typhus in Australia

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In a recent case of scrub typhus in Australia, *Orientia tsutsugamushi* isolated from the patient's blood was tested by sequence analysis of the 16S rDNA gene. The sequence showed a strain of *O. tsutsugamushi* that was quite different from the classic Karp, Kato, and Gilliam strains. The new strain has been designated Litchfield.

Scrub typhus has been recognized as an important cause of fever among inhabitants of tropical Queensland (northeast Australia) for the past 60 years (1-6). All three groups of rickettsial diseases occur in Australia (Figure). 1) Spotted fever group (SFG): i) *Rickettsia australis* (Queensland tick typhus) occurs along the eastern side of the continent to the east of the Great Dividing Range (7,8); ii) *R. honei* (Flinders Island spotted fever) is only now recognized on Flinders Island—a small island off the southeast corner of Australia (9,10); iii) an unidentified SFG rickettsial infection in Tasmania, a large island state to the southeast of the Australian mainland, has been identified through serologic evidence, but no isolate has been obtained. 2) Typhus group (TG): *R. typhi* (murine typhus) occurs throughout Australia, with foci recognized in southwest Australia, southcentral Australia (first described by Hone [11] in Adelaide, South Australia), and central Queensland (northeast Australia). 3) Scrub typhus: *Orientia tsutsugamushi* infection, well recognized in tropical coastal Queensland, has probably long been endemic in this area. As Europeans gradually settled the area, the etiologies of numerous febrile illnesses were determined. Serologic evidence (the Weil-Felix serologic test) of scrub typhus became available (1-3) and later isolates of *O. tsutsugamushi* were made by inoculating mice with patients' blood (4). A serologic analysis of the Queensland strains of *O. tsutsugamushi* suggested that most strains were Karp-like (12). (The Karp strain was isolated from Papua New Guinea [13].) The geographic distribution of scrub typhus in the rest of Australia is less clear. The disease was not considered to occur west of coastal Queensland. However, in the tropical region of the Northern Territory, six possible cases from 1937 to 1939 and one in 1957 were reported (all presumptively diagnosed). Since 1990 , however, nine cases of scrub typhus have been reported in Litchfield Park, a discrete area of rain forest in the

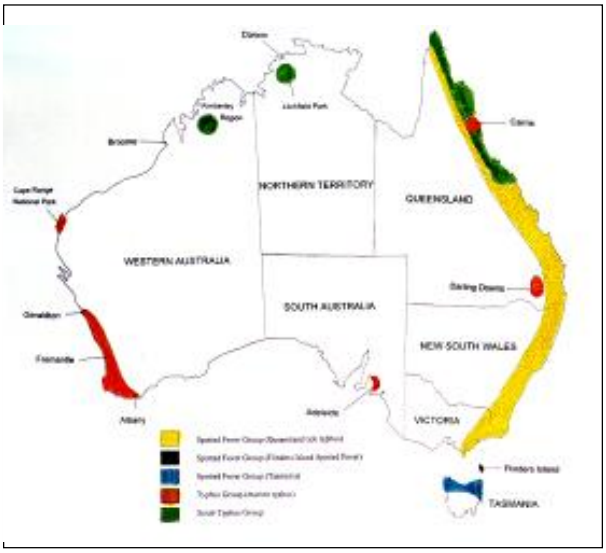

Figure. Geographic distribution of rickettsial diseases in Australia.

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Northern Territory opened as a park in 1986 (16). All patients had eschars, and all cases were confirmed by specific *O. tsutsugamushi* serologic tests. A case of scrub typhus was also reported from a similar rain forest pocket in the tropical Kimberley region of northwestern Australia (17).

Case Report

In mid-August 1996, a 38-year old man working on the construction of a tourist path in the rain forest fringe of Litchfield Park (15) became ill with fever, sweats, headache, sore throat, cough, lethargy, and confusion during his second week on the job. His condition worsened over at least a week. He eventually received amoxycillin and clavulanate from a local medical practitioner but contracted diarrhea and was admitted to Royal Darwin Hospital with fever, rigors, but no evident focus of infection. A diagnosis of septicemia was made, and he was started on ceftriaxone and gentamicin.

Over the next day, he became increasingly confused, and his fever persisted. He became hypotensive, hypoxemic, and oliguric and was transferred to the intensive care unit. On day 3 after admission, he required fluid loading, inotrope therapy, intubation, and ventilation. At that time, his work history was obtained from relatives, and a 6-mm sore with a necrotic dark center was noted on his upper right buttock. Scrub typhus was suspected, and he was given intravenous doxycycline. However, his condition continued to deteriorate with hypotension, renal failure, and adult respiratory distress syndrome. Mucosal and gastrointestinal bleeding developed, and the patient died 6 days after admission.

Laboratory Investigation

Rickettsial Serologic Tests

Paired sera, 6 days apart, showed a greater than fourfold rise in antibody titer to *O. tsutsugamushi* by microimmunofluorescence (15). Sera did not react to SFG rickettsiae or TG rickettsiae. All three classic strains of *O. tsutsugamushi* (Karp, Kato, Gilliam) reacted with the patient's serum, at titers of >1/1024 (negative <1/64).

Mouse Inoculation

Three female outbred white mice were inoculated with blood taken from the patient 4 days before he died. The mice were inoculated intraperitoneally with 0.1 ml, 0.2 ml, and 0.5 ml of whole blood in EDTA. The mice were housed in a flexible film isolator in a high security microbiology laboratory at the Australian Animal Health Laboratory, Geelong, Australia. None of the mice died.

Subsequently three additional mice were inoculated with very concentrated preparations of the *O. tsutsugamushi* strain isolated from the patient; none died. This strain of *O. tsutsugamushi* appears not to be virulent for mice despite being highly virulent for the patient. No attempt was made to isolate the strain from mouse organs.

Seroconversion of the inoculated mice to *O. tsutsugamushi* was confirmed by microimmunofluorescence.

Rickettsial DNA Detection by Polymerase Chain Reaction (PCR)

One ml of the patient's blood, taken 4 days before death, was lysed; DNA was extracted (18); and part of the 56kDa-type specific antigen (an outer membrane protein) gene was amplified by PCR. Primers 'a' (5'-TACATTAGCTGCGGGTATGACA-3') and 'b' (5'-CCAGCATAATTCTTCAACCAAG-3') were used as previously described (19), except that a nested procedure was not used. The PCR product was electrophoresed on agarose gel, stained with ethidium bromide, and detected by UV fluorescence. Positive and negative controls were run with the unknown samples. The PCR product from the patient's blood had the same molecular weight as a PCR product from a known *O. tsutsugamushi* strain.

Tissue Culture Infection

Three 25-cm^2 flasks containing a confluent monolayer of Vero cells were infected with whole blood (in EDTA) from the patient (0.1 ml, 0.5 ml, and 1.0 ml, respectively). After sitting at 35°C for 4 hours without centrifugation, the blood was removed, and the monolayers were washed twice with Hank's balanced salt solution. The medium used for culture was RPMI 164O with 10% fetal calf serum (heat-inactivated) at 35°C (without CO_2). Cultures were examined weekly for abnormalities, and the pH was adjusted as necessary. On day 60 postinfection, a 3+ cytopathogenic effect was observed in one flask, with most Vero cells floating in the supernatant. These cells were tested for bacterial contamination (Gram stain–negative; growth on horse

blood agar–negative) and for rickettsiae (positive for Giminez stain; positive for scrub typhus, as determined by immunofluorescence staining with convalescent-phase human serum; and positive as determined by PCR for 56 kDa antigen gene). The isolated strain was subcultured and grown for DNA extraction.

16S rDNA Sequencing

The 16S rDNA was amplified by PCR with primers A and H*(20), and the products were cloned into pGEM-T (Promega). The complete nucleotide sequences of both strands of several clones were determined by using internal primers (20,21); sequencing reactions were analyzed on an ABI 310 Genetic Analyser by using the ABI Big-Dye chemistry. The sequence has been submitted to GenBank under the accession number AF062074.

Comparison with the databases confirmed that the sequence from the novel isolate was *O. tsutsugamushi* but indicated that it differed greatly from the four type strains (Karp, Kuroki, Gilliam, Kato). The novel isolate has five unique base substitutions in the 16S rDNA and thus differs more from the reference strains than they do from each other. On this basis, it was designated a new strain and was called Litchfield.

The Litchfield strain of *O. tsutsugamushi* is the first isolated in culture from the Northern Territory of Australia, although previous isolates have been made from Queensland (4,12). *O. tsutsugamushi* has probably been present in the mites and native mammals of northern Australia for millennia. One of the most common mite vectors, *Leptotrombidium deliense,* has been detected on several species of native rat in Litchfield Park (22), although *O. tsutsugamushi* has not yet been isolated from native mammals from the Northern Territory. In Queensland, however, *O. tsutsugamushi* has been isolated from native mammals and mites (23-25).

The emergence of scrub typhus in Litchfield Park in the Northern Territory of Australia is probably due to increasing contact between humans and this scrub and rain forest environment and its animals. Tourism is a major industry in Australia, and increasing numbers of visitors are traveling to remote parts to experience the tropical "out-back." Thus, public health officials and local health providers need to be aware of foci of endemic diseases in remote parts of Australia. One problem with rickettsial diseases is that they do not respond to ß-lactam antibiotics (e.g., penicillins and cephalosporins) because of their unusual bacterial cell wall or to aminoglycoside antibiotics (e.g., gentamicin) because of their intracellular location. Treatment of patients with undiagnosed sepsis in Australian hospitals is very likely to involve the use of a cephalosporin plus gentamicin, a combination that is not effective against scrub typhus or any other rickettsial disease. The travel history of a febrile patient is essential if tropical infections are to be included in the differential diagnosis of febrile illness.

The 16S rDNA evolves relatively slowly. Hence, despite geographic proximity, the key difference between the sequences from the Karp strain (isolated from a patient in New Guinea) and the Litchfield isolate implies a long period of isolation between these strains. Few general conclusions can be drawn from one sequence for a single Australian isolate. However, the prediction is that at least some Australian strains of *O. tsutsugamushi* will differ substantially from type strains.

Dr. Odorico is a molecular biologist with a broad range of interests, including molecular phylogenetics. His present research involves the functional analysis of eukaryotic promoters by use of site-directed mutagenesis and reporter gene techniques.

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Streptococcus parasanguinis: New Pathogen Associated with Asymptomatic Mastitis in Sheep

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We describe two unusual cases in sheep of subclinical mastitis caused by *Streptococcus parasanguinis*. This bacterium has been associated with the development of experimental endocarditis; its presence at relatively high concentrations in apparently healthy sheep milk may pose a health risk in persons with predisposing heart lesions.

Ovine mastitis represents a major sanitation and economic problem for both milking sheep farmers and the sheep-milk cheese industry because it reduces milk production and quality. Many microorganisms produce asymptomatic, or subclinical, mastitis in milking sheep. Coagulase-negative staphylococci, particularly *Staphylococcus epidermidis*, are most prevalent (1,2,3), although other bacterial groups have recently emerged as clinically important (4). Streptococci are also responsible for a significant proportion of cases; however, while a large number of species of staphylococci cause subclinical mastitis, the number of streptococci species found to do so is limited, usually to *Streptococcus agalactiae*, *S. uberis,* and *S. dysgalactiae* (3,5).

We report two unusual cases of subclinical mastitis in sheep involving *S. parasanguinis* (6,7). This atypical viridans *Streptococcus* bacterium has been isolated from humans (throat, blood, and urine) (7) but has not been associated with mastitis. The viridans group streptococci are the most common pathogens associated with native valve endocarditis, a disease of increasing medical importance in industrialized countries (8). In patients with this disease, eating and teeth brushing cause low-grade bacteremia, which allows circulating bacteria to adhere to the damaged endocardium (9). Although *S. parasanguinis* has not yet been established as a human pathogen, it has been clearly associated with the development of experimental endocarditis (10). Therefore, the excretion of *S. parasanguinis* at relatively high concentrations in milk from apparently healthy sheep is of concern. Indeed, its presence in certain dairy products, such as nonpasteurized handmade cheeses, may pose a health risk in persons with predisposing heart lesions.

The two isolates in our study were recovered in 1997 from two sheep of an Assaf flock during a bacteriologic survey for determining the prevalence of subclinical mastitis in Madrid (central region of Spain). Affected sheep did not show signs of clinical mastitis or milk abnormalities but had California Mastitis Test (CMT) scores of 2+. CMT estimates the degree of inflammation of the mammary gland by detecting increased numbers of leukocytes in milk (11). The bacteriologic counts of the milk samples were 1 x 10^4 CFU/ml in one of the sheep and 1.7 x 10^3 CFU/ml in the other. Mammary glands with these characteristics (no clinical abnormalities, apparently normal milk secretion, positive for CMT, and bacteriologically positive) are routinely considered to have subclinical mastitis (1,12). Milk samples were collected and analyzed as described previously (13). After 48 hours of incubation at 37°C under aerobic conditions on Columbia blood agar, pure cultures of α-hemolytic colonies were recovered from both milk samples.

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The isolates are clinically important because both sheep fit the criteria for subclinical mastitis and the isolates were recovered in pure culture.

Both strains were catalase-negative gram-positive cocci, and biochemical identification was attempted with the commercial rapid ID 32 Strep system (Bio-Mérieux S.A.). Both isolates had an identical biochemical profile, which did not correlate with any of the species identified with the ID 32 multisubstrate strip. Sequencing of 16S rRNA genes provided a definitive identification of the streptococci isolates as *S. parasanguinis*. This molecular technique is a powerful method for determining the identity of bacteria that cannot be properly identified by conventional physiologic or biochemical approaches and has proven powerful for describing new bacterial pathogens causing mastitis (13,14). The 16S rRNA gene of each isolate was amplified by polymerase chain reaction (PCR) and was sequenced to determine genotypic identity (13,14). The determined sequences consisted of more than 1,400 nucleotides (representing more than 95% of the total 16S rRNA gene) and were compared with the sequences of other streptococcal species available in the European Molecular Biology Laboratory Database Library. The 16S rRNA gene sequence analysis showed both isolates to be genotypically identical and to display 99.7% sequence similarity with the strain 85-81 of *S. parasanguinis*, which on the basis of DNA-DNA hybridization studies was shown to be a true member of this species (7).

The two *S. parasanguinis* isolates represented 7% of our total streptococci isolated from cases of subclinical mastitis. According to these data, only sporadic cases of mammary gland infections due to *S. parasanguinis* would be expected. However, because many streptococcal isolates are usually identified only to the genus level (2,3,11,15), the diversity of species involved and the incidence of infection due to particular streptococcal species are difficult to know. The same difficulty is true with corynebacteria (4).

The recognition of *S. parasanguinis* as a new animal pathogen causing subclinical mastitis in sheep and the recent recognition of new *Corynebacterium* species associated with this disease (13,14) suggest the need for epidemiologic surveys to determine the range of species diversity, other than coagulase-negative staphylococci, involved in subclinical sheep mastitis. According to recent results, this range appears higher than previously considered. Particular attention should be paid to species potentially pathogenic for humans.

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Introduction of HIV-2 and Multiple HIV-1 Subtypes to Lebanon

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HIV genetic variability, phylogenetic relationships, and transmission dynamics were analyzed in 26 HIV-infected patients from Lebanon. Twenty-five specimens were identified as HIV-1 and one as HIV-2 subtype B. The 25 strains were classified into six *env-C2-V3* HIV-1 subtypes: B (n = 10), A (n = 11), C (n = 1), D (n = 1), G (n = 1), and unclassifiable. Potential recombinants combining parts of viral regions from different subtypes $A^{env}/D^{pol}/A^{gag}$, G^{env}/A^{pol}, and the unclassifiable-subtype^{env}/ unclassifiable-subtype$^{pol}/A^{gag}$ were found in three patients. Epidemiologic analysis of travel histories and behavioral risks indicated that HIV-1 and HIV-2 subtypes reflected HIV strains prevalent in countries visited by patients or their sex partners. Spread of complex HIV-subtype distribution patterns to regions where HIV is not endemic may be more common than previously thought. Blood screening for both HIV-1 and HIV-2 in Lebanon is recommended to protect the blood supply. HIV subtype data provide information for vaccine development.

Understanding the global genetic diversity of HIV is important for monitoring the spread of infection and developing effective vaccines. Currently, two principal genetic groups, designated M (main) and O (outlier), have been identified. M, which is highly prevalent, is further classified into 10 established envelope subtypes, A through J (1). Mosaic forms, which combine the genetic material from two distinct subtypes, have also been identified (1,2). HIV-1 subtype B predominates in Europe and the Americas, whereas HIV-1 non-subtype B strains dominate in sub-Saharan Africa. In contrast to HIV-1 infection, which is spread through all continents, HIV-2 is primarily re-stricted to West Africa and to population move-ments from or through this region. HIV-2 strains are classified into five subtypes, A through E; only subtypes A and B viruses are predominant (3).

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HIV-subtype distribution patterns in regions where HIV is not endemic are not well known (4). Information about these distribution patterns is important for 1) implementing proper diagnostic assays to protect the blood supply, 2) providing basic information on the prevalence of viral subtypes for vaccine development, and 3) monitoring trends of viral spread. Two distribution patterns of HIV subtypes have been recognized in areas where the disease had not been endemic. The first pattern consists of a restricted number of HIV-1 viral subtypes or their combination with HIV-2. Examples include HIV-1 subtypes E and B in Thailand (5) and HIV-1 subtype C and HIV-2 in India (6). In contrast, the second pattern includes a large number of only HIV-1 subtypes as documented in Cyprus (A, B, C, F, and I) and the Philippines (A, B, C, D, and E) (7,8). However, information is limited as to how common the spread of this HIV-subtype pattern is to areas in which the disease is not endemic.

Lebanon was chosen for a genetic variability study for three reasons. First, HIV is not endemic

in Lebanon, but its presence is slowly increasing (9,10). From 1984 (when the first case of AIDS was identified) (11) to 1996, 400 cases of HIV infection were reported to the Lebanon National AIDS Control Program (J.E. Mokhbat, pers. comm.). Second, Lebanon has a central geographic location, strong historic links, and frequent population movements to and from other countries. Third, the potential for introduction of multiple HIV subtypes is present given the large number of Lebanese expatriates who return to Lebanon after living and working in HIV-endemic regions such as Africa (mainly West Africa).

Study Population

With informed consent, interviews and blood samples were obtained from 26 HIV-seropositive Lebanese citizens (17 men and 9 women) living in Lebanon at the time of the study. They were referred (in 1996) from both urban and rural areas to the Medical Center of the American University of Beirut or the Saint George Hospital in Beirut, Lebanon.

Study Design

HIV-1 antibodies were discriminated from HIV-2 antibodies by a synthetic slot immunoblot composed of recombinant viral proteins and synthetic peptides (Genelavia Mixt, Peptilav 1-2, HIV Blot 2.2-Diagnostic Biotechnology and New LAV Blot II, Sanofi Diagnostic Pasteur, France).

Double-stranded viral DNA of nested polymerase chain reaction (PCR)-amplified *env* (C2-V3), pol (*protease-prot*), and *gag* (*p24* fragment) from peripheral blood mononuclear cells were direct sequenced (12,13). Comparative phylogenetic analysis of two or three independent viral regions enables identification of a possible mosaic viral genome (2). The sequences were aligned by use of the CLUSTAL multiple-sequence alignment program (14). After regions containing gaps were eliminated, the aligned sequences were analyzed with the maximum likelihood method by using the fastDNAml program, which uses randomized data input and global rearrangement (15). Additionally, the neighbor-joining method (PHYLIP package version 3.5c [16] or TREECON software [17]) was used, with or without bootstrapping. The stability of the tree's topology was tested by pruning, which consists of removing one species from the alignment and rerunning the phylogenetic analysis. SIV-cpz sequences were used as outgroup. All specimens were also screened for HIV-2 DNA by nested-PCR amplification of viral HIV-2 *prot* gene. The outer PCR primers were DP20: 5′ GACAGAGGACTTGCTGCA; nucleotide position: 2068-2083, HIV-2_{ROD}, and DP21: 5′ GGCCATTGTCTCAGTTTTGG; nucleotide position: 2435-2454. The inner primers were DP26a: 5′ CACCTCAATTCTCTCTTTGGA; nucleotide position: 2082-2102; and DP27: 5′ TAGATTTAATGACATGCCTAA; nucleotide position: 2360-2380. Analysis based on HIV-2 *pol* region is highly specific and allows classification of HIV-2 sequences into phylogenetic subtypes (3,18,19).

Molecular Characteristics of HIV Infection

Of 26 patients, 25 were HIV-1 PCR-positive; the remaining patient (LE25) was HIV-2 PCR-positive and was classified as having subtype B infection (Figure 1). No dual infections with HIV-1 and HIV-2 strains were found.

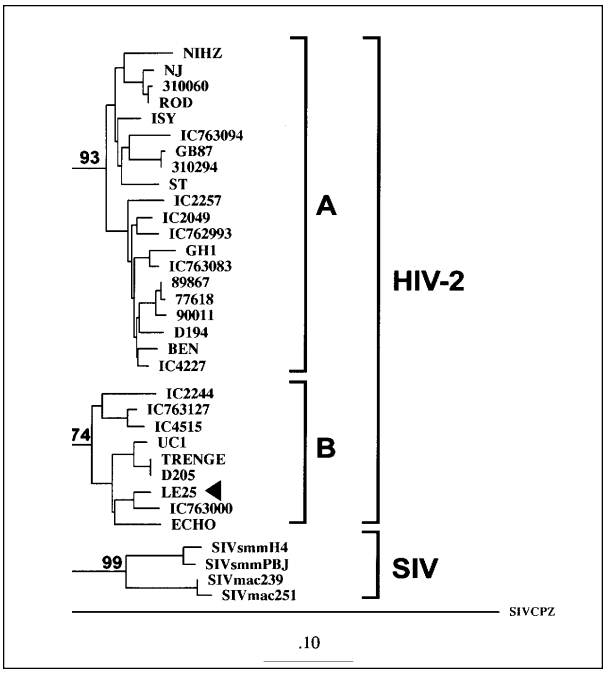

Figure 1. Phylogenetic classification of HIV-2 sequence from Lebanese patient LE25 (arrow) basing on the *prot* gene (GenBank accession no. AF026912). The tree was generated by the maximum likelihood method. Numbers at the branch nodes connected with subtypes indicate bootstrap values. The distinct HIV-2 subtypes are delineated. The scale bar indicates an evolutionary distance of 0.10 nucleotides per position in the sequence. Vertical distances are for clarity only.

The comparative phylogenetic analysis of 25 HIV-1 specimens identified 23 cases with the same subtype assignment for the *env* and *pol* sequences and classified 22 as known subtypes A (n = 10), B (n = 10), C (n = 1), D (n = 1) (Figure 2). In contrast, the remaining specimen (LE7) consisted of distinctively divergent *pol* and *env* sequences (Figure 2a, b) as they failed to cluster with known standard HIV-1 sequences, including divergent *env* subtypes I and J (Figure 2c). Intragene recombination within *pol* and *env* sequences of specimen LE7 was not observed when nucleotide signature patterns in HIV-1 subtypes were compared. Thus, this strain might represent a potential novel HIV-1 subtype. However, *gag* sequence of this virus grouped into HIV-1 subtype A, and this association was supported by the bootstrap value of 90% (Figure 2d). Taken together, these results indicate that LE7 viral genome has a mosaic pattern involving elements of an unclassifiable-divergent strain and subtype A (unclassifiable^{env}/ unclassifiable^{pol}/ A^{gag}). In addition, potential recombinant viruses combining viral *env*, *pol,* and *gag* from different subtypes were identified in two other specimens. Two known recombinant patterns, G^{env}/A^{pol} and A^{env}/D^{pol}/A^{gag}, have been found in patients LE24 and LE9, respectively. However, a potential recombinant is sometimes difficult to recognize, especially between subtypes B and D viruses, which are relatively closely related; specimen LE22 illustrates such a situation. The protease gene sequence, the most outside branch in this clade, grouped weakly with subtype B (Figure 2a). This association, however, was not stable by analysis that used pruning techniques and not supported by bootstrap analysis (only 48% value as opposed to 72% without this sequence). These data suggest that it is unlikely that LE22 *prot* is subtype B, and because both *env* and *gag* sequences clustered into subtype D, we have classified this sample as subtype D.

Epidemiologic Characteristics

To better understand the transmission dynamics and temporal introduction of these HIV variants, we analyzed patients' data (demographics, behavioral risk, modes of transmission, and travel histories) (Table).

Of eight male patients with subtype B HIV-1 infection, six traveled to Europe, the Americas, Asia (Thailand), or different locations in the Middle East. All reported sexual contacts abroad either with commercial sex workers or homosexual or bisexual men. Of the remaining two patients, one traveled selectively to Africa and had sexual contacts in Côte d'Ivoire and Nigeria, whereas the other reported no travel outside Lebanon and had no sexual contacts with commercial sex workers. In contrast, all nine male patients infected with non-subtype B HIV-1, including three mosaic viruses, traveled to Africa or the United Arab Emirates and reported sexual contacts with commercial sex workers.

The behavioral risks of the female patients differed substantially from those of the male patients. All female patients were married, and none reported high-risk activities or contacts with commercial sex workers. Moreover, all female patients (except one [LE12], who had acquired HIV-1 infection from blood transfusion) reported that their spouses were HIV-infected and traveled abroad with them or alone.

To determine if the female patients acquired HIV infection from their spouses, we analyzed available epidemiologic data on the spouses of three female patients LE17, LE19, and LE13. The female patients LE17 and LE19 were spouses of patients LE18 and LE23, respectively. These two couples, LE17/LE18 and LE19/LE23, were infected with HIV-1 subtype A and subtype B viruses, respectively (Figure 2b). In further detailed pairwise analysis of viral DNA isolated from the husband and wife, a small nucleotide divergence of 3.1% within *env* and of 0.6% for both *prot* and *gag* (13,20) showed that in the first couple viral strains were closely related. These data are concordant with epidemiologic findings, which strongly indicate that the husband was the source of infection because of his high-risk sexual activities with commercial sex workers during travels to the United Arab Emirates. His wife did not travel outside Lebanon and did not report activities that would have placed her at risk for HIV-1 infection. In the second couple (LE19/LE23), however, only weak relation between viral strains was observed (nucleotide divergence of 9.8% within *env* and 2% within *prot*). This could be due to many factors including 1) the time that elapsed from infection; 2) transmission of different quasispecies to the spouse; 3) detection of only major quasispecies by direct sequencing, which differed in the couple; and 4) a different source of HIV-1 infection in the sex partner. The latter hypothesis was not supported by epidemiologic data, which indi-

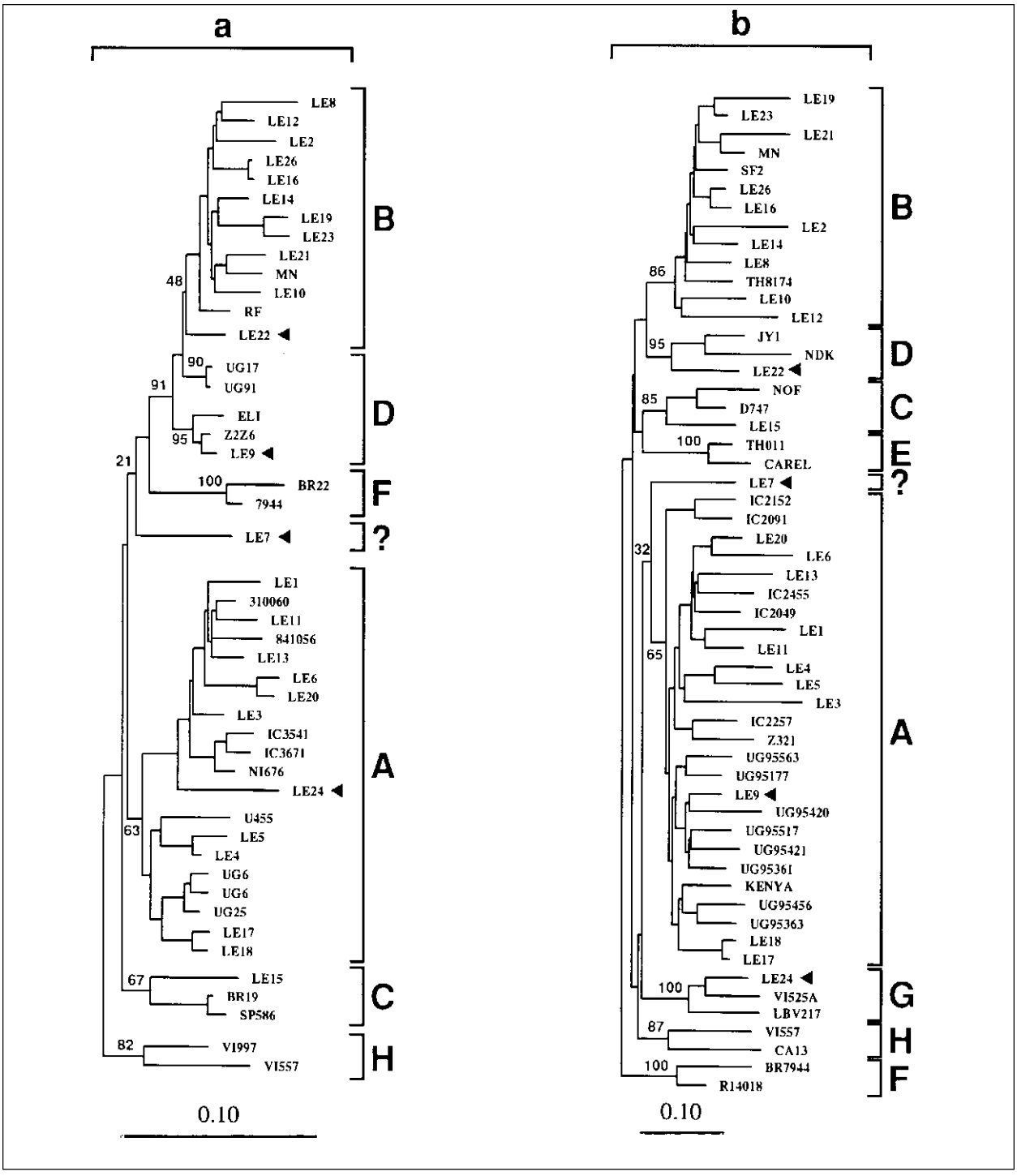

Figure 2. Phylogenetic classification of HIV-1 sequences from Lebanese patients denoted with LE prefix (GenBank accession nos. AF025691-AF025745). The trees were constructed on the basis of the DNA sequences of *prot* (a), *env* (b and c), and *gag* (d) by the maximum likelihood method. Numbers at the branch nodes connected with subtypes indicate bootstrap values. *Env* trees: (b) includes all 25 Lebanese sequences of 325 bp and reference sequences from eight subtypes A through H; and (c) includes only Lebanese LE7 sequence of 216 bp and marker sequences from subtypes A through J.

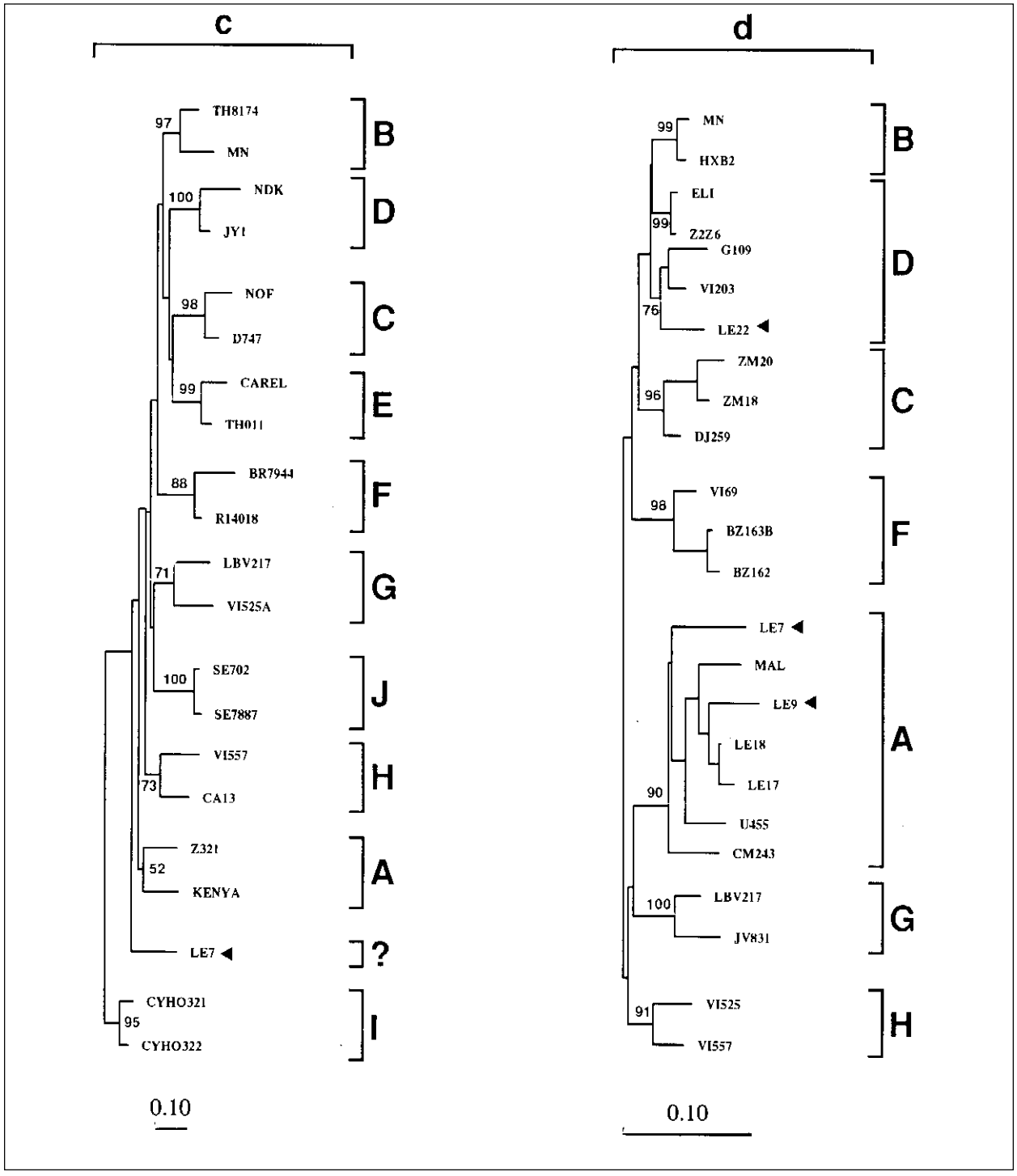

Figure 2. continued. Arrows indicate Lebanese samples that clustered in different lineages depending on which parts of their genome were analyzed. The distinct HIV-1 subtypes are delineated. ? represents the unclassified position of *prot* and *env* sequences of sample LE7. The scale bar indicates an evolutionary distance of 0.10 nucleotides per position in the sequence. Vertical distances are for clarity only.

Table. Demographic, clinical, and behavioral information

Patient no.	Gender[a]	Age (yrs)	Status[b]	CD4/ mm^3	WHO class[c]	Sex partner characteristics: AIDS/ HIV	Sex partner characteristics: Bi/homosexual male	Sex partner characteristics: CSW[d]	Travel/sexual contacts abroad	Subtype
LE1	M	37	M	172	3	No	No	Yes	West Africa (Côte d'Ivoire)[e]	A
LE5	M	42	M	NA	3	NK[f]	No	Yes	West Africa[e]	A
LE6	M	29	M	168	2	Yes	No	Yes	West Africa[e]	A
LE11	M	26	S	44	3	Yes	No	Yes	Africa[e]	A
LE18[g]	M	34	M	400	1	Yes	No	Yes	Middle East (United Arab Emirates)[e]	A
LE22	M	42	S	NA	3	No	No	Yes	West Africa (Côte d'Ivoire)[e]	D
LE9	M	57	M	253	1	No	No	Yes	Middle East (United Arab Emirates)[e], Asia, Europe, North America	A/D/A[h]
LE24	M	54	M	180	1	NK	No	Yes	West Africa (Nigeria)[e]	G/A[i]
LE7	M	54	M	177	3	No	No	Yes	West Africa (Côte d'Ivoire)[e], Middle East	NC/A[j]
LE16	M	32	M	86	2	NK	No	Yes	Europe	B
LE26	M	36	M	86	1	No	No	Yes	Europe (France)[e]	B
LE2	M	27	S	396	3	Yes	Yes	No	Europe (France, Germany, UK)[e], North America (USA)[e], Middle East	B
LE8	M	43	S	98	3	No	Yes	No	Europe[e], South America, Middle East (Bahrain)[e]	B
LE10	M	35	S	56	4	No	Yes	No	Europe (France)[e], Middle East	B
LE14	M	45	D/SP	7	3	No	No	Yes	Europe (Spain, Germany)[e], Asia (Thailand)[e], South America	B
LE23[k]	M	47	M	NA	1	Yes	No	Yes	West Africa (Côte d'Ivoire, Nigeria)[e]	B
LE21	M	31	S	340	1	No	No	No	Lebanon only	B
LE3	F	31	M	378	1	Yes	No	No	Lebanon only	A
LE17[g]	F	32	M	300	3	Yes	No	No	Lebanon only	A
LE20	F	32	M	NA	1	Yes	No	No	Europe	A
LE4	F	36	M	233	1	Yes	No	No	Africa	A
LE13	F	35	M	350	1	Yes	No	No	West Africa	A
LE15	F	34	M	171	1	Yes	No	No	Asia	C
LE12[l]	F	31	M	109	3	NK	NK	NK	Africa	B
LE19[k]	F	42	M	30	4	Yes	No	No	West Africa	B
LE25	F	28	M	200	2	Yes	No	No	West Africa	HIV-2

[a]M=male, F=female.
[b]M=married, S=single, D/SP=divorced/separated.
[c]World Health Organization classification based on opportunistic infections.
[d]Commercial sex worker.
[e]Sexual contact reported in region, country listed in parentheses.
[f]Not known.
[g]Couple no. 1.
[h]Subtype A -*env*, subtype D -*pol*, subtype A -gag.
[i]Subtype G -*env*, subtype A -*pol*.
[j]Unclassified subtype for *env* and *pol*, subtype A - *gag*.
[k]Couple no. 2.
[l]Reported to be infected by blood transfusion.

cated that only the husband was involved in high-risk sexual activities in and outside Lebanon. The third female patient, LE25, was infected with HIV-2 subtype B strain. Her husband, who died of AIDS-related complications, had traveled to Côte d'Ivoire, a region in which this HIV variant is endemic (21). These data suggest that very likely the female patients acquired infections from their HIV-infected spouses.

The molecular and epidemiologic data indicate multiple HIV-1 introductions to Lebanon. First, most male patients were infected abroad with the HIV-1 strains prevalent in the geographic region they visited. Second, the topology of HIV-1 *env* subtype A demonstrated that viral sequences of patients who had sexual contacts with persons from West Africa clustered in the branch with marker sequences from Côte d'Ivoire. In contrast, HIV-1 sequences of patients who reported contacts with persons from the United Arab Emirates (LE9 and LE18) clustered in another branch with Ugandan/Kenyan marker sequences (Figure 2b). Independent branching of HIV-1 *env* sequences of subtype A from West and East Africa had been observed (22). Third, the structures of two mosaic viral genomes and epidemiologic data of patients suggested that they were infected with potential recombinant viruses, which had been identified in two geographic regions. Briefly, patients LE24 and LE9, who reported sexual contacts with commercial sex workers in Nigeria or the United Arab Emirates, respectively, have been infected with mosaic viruses G^{env}/A^{pol} and $A^{env}/D^{pol}/A^{gag}$. Such recombination patterns were found in West-Central Africa and East Africa, respectively (23,24). Finally, analysis of *env* sequences from Lebanese patients revealed a relatively high intrasubtype genetic diversity within subtype B (average 14.9%, range 4.2% to 25.6%) and subtype A (15.5%, range 2.5% to 28.9%). Epidemiologic data and a low prevalence of HIV-1 infection suggest multiple introductions (rather than long existence) of genetically diverse HIV-1 strains to Lebanon.

Conclusions

The results presented in this report provide new information on HIV genetic variation in Lebanon. The identification of HIV-2 and 6 *env* HIV-1 subtypes, including three distinct HIV-1 mosaics, confirms high heterogeneity of HIV variants in Lebanon. Given the limited number of samples tested, finding both HIV-2 and multiple HIV-1 subtypes is remarkable and indicates that the distribution pattern of HIV variants resembled that of Cyprus or the Philippines rather than that of Thailand or India. These data indicate that the spread of complex HIV-subtype distribution patterns involving a large number of distinct HIV-1 and HIV-2 strains to countries without endemic HIV may be more common than previously thought.

The HIV-2 infection detected in this study, the first documented in Lebanon, reiterates the importance of appropriate screening methods for detecting both HIV-1 and HIV-2 to protect the blood supply. Our findings provide preliminary information for monitoring the spread of HIV variants in Lebanon and developing subtype-specific subunit vaccines.

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Dr. Pieniazek is a molecular biologist in the HIV/AIDS and Retroviral Branch, CDC. Her research interests include implications of mixed HIV infections for the evolution of the HIV/AIDS epidemic and for vaccine development; her areas of expertise include developing molecular assays for detection of novel HIV strains and the molecular epidemiology of HIV.

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Sporadic STEC O157 Infection: Secondary Household Transmission in Wales

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We conducted a study to quantify and characterize household transmission of Shiga toxin (Vero cytotoxin)–producing *Escherichia coli* O157 (STEC O157) following sporadic infection in Wales. Through total population surveillance, we identified 83 index case-patients and their household contacts. We screened fecal samples submitted from household contacts for STEC O157 and calculated the household transmission rate for sporadic STEC O157 infection to be 4% to 15%. Household contacts in groups at high risk (particularly children under 5 years of age) present a risk of spreading the infection in the wider community.

Shiga toxin–producing *Escherichia coli* O157 (STEC O157) infection causes severe enteric symptoms (hemorrhagic colitis with diarrhea, severe abdominal pain, and blood in the stools) (1) and hemolytic uremic syndrome (2% to 7% of cases) (2). The bacteria, which are from a predominantly bovine reservoir, are found in food or water (particularly beef products and milk) and are transmitted through zoonotic or environmental spread or person to person by the fecal-oral route (3). Fecal-oral transmission would explain why children with hemolytic uremic syndrome are seven times more likely than otherwise healthy children to have had close contact with a person with diarrhea in the 2 weeks before onset of illness (4).

Outbreaks attributed to person-to-person transmission have been confined mainly to children's day-care facilities and institutions providing care for the elderly or for those with physical or mental disabilities (5-8)—facilities in which hygiene is difficult to maintain—although an outbreak among siblings in a large family and a playmate has been described (9).

Most (90%) of STEC O157 cases reported in England and Wales are sporadic (10). Evidence suggests that the infectious dose is very low, perhaps as few as 10 organisms (11,12); hence the potential for person-to-person transmission among sporadic STEC O157 cases might be expected to be far greater than among sporadic cases of other common gastrointestinal pathogens. Although secondary spread of STEC O157 by sporadic cases has been reported (13), such transmission has not been accurately quantified or characterized on a population basis. This study aimed to quantify and characterize the secondary household transmission of STEC O157 from sporadic cases.

Cases (in persons residing in Wales and the English Borders) in which STEC O157 was isolated from initial fecal specimens and was subsequently confirmed by the Public Health Laboratory Service Laboratory of Enteric Pathogens were included in the study. Only the index case (i.e., the first to be recognized in a household) and sporadic cases (i.e., not part of a general outbreak) were included. Index case-patients were interviewed, face to face, by using a standard structured questionnaire to record age group, gender, onset, and hospitalization details. The names, as well as the age, gender, and occupation, of all household contacts were recorded in a confidential database.

Any person staying in the same household as a person with STEC O157 infection for one or

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more nights during the 7 days before disease onset and up to the interview date was considered a household contact. Diarrheal illness (defined as three or more loose stools in any 24-hour period during the 7 days before the onset of disease in the index patient and up to the date of the interview with the index patient) in the contact and the date of onset of the symptoms were also recorded. All household contacts were requested to provide one fecal specimen, which was examined as described (Table).

To determine whether the household contact cases were secondary or coprimary, the dates of disease onset for index case-patients were compared with those of their symptomatic household contacts. Also, the rates of infection in the households of index case-patients who had been hospitalized were compared with rates in households of patients who remained at home. We constructed matrices to determine the frequency of STEC O157 isolation for each age group of household contacts and index case-patients. The frequency of isolation of the organism from household contacts in the households of male index case-patients was compared with that from households of female index case-patients by calculating relative risks (RR) and Mantel-Haenszel chi squares. To determine any bias in the household contacts screened, we quantified factors affecting an individual contact's likelihood of submitting a fecal specimen (i.e., age, gender, and symptomatic status), as well as age and gender of index case-patient. We examined the delay between disease onset in the index case-patient and date of submission of specimens by contacts and compared the delays for household contacts of different ages. Household contacts infected with STEC O157 who belonged to certain groups (food handlers, health-care workers, children under 5 years of age, older children or adults unable to implement good hygiene) were considered a risk to the community, in accordance with current guidelines (14,15). The number of contacts falling into these groups was recorded.

Eighty-three persons considered index case-patients had 181 household contacts. Of the 181 contacts, 101 (56%) submitted fecal samples. Samples were submitted a median of 12 days after disease onset in the index case-patient (mean = 13.46 days; mode = 12 days; range = 6-40 days); timeliness of sample submission did not differ by age group. Of the 101 contacts examined, 15 had STEC-positive sample**s,** giving a crude secondary household transmission rate of 15%. If it is assumed that contacts who did not submit fecal samples were not excreting STEC O157, the rate is 8% (15 of 181).

Of the 181 household contacts, 32 reported diarrhea: of these, 3 became ill on the same day as the index case-patient (none had confirmed STEC O157), 8 became ill before the index case-patient (2 had confirmed STEC O157), and 21 became ill after the index case-patient (7 had confirmed STEC O157) (Figure). If only the cases of household contacts who became ill after the index case-patient are considered secondary cases, the household transmission rate is 4% (7 of 181 cases).

STEC O157 were isolated from 15 of 109 contacts of case-patients under 14 years of age compared with 0 of 72 contacts of case-patients 15 years of age and older ($p < 0.01$) (Table). STEC O157 was no more likely to be isolated from male than female household contacts (8 of 95 versus 7 of 86, RR = 1.03, confidence interval

Table. Probability of isolating Shiga toxin-producing *Escherichia coli* (STEC) O157 from household contacts, by age group of index case-patient and age group of household contact, for all contacts[a]

Age group of household contact	Age group of index case-patient (%)				
	< 1 yr	1-4 yr	5 -14 yr	15 + yr	Total
< 1 yr	0/0 (0)	0/3 (0)	0/1 (0)	0/0 (0)	0/4 (0)
1-4 yr	1/2 (50)	3/8 (38)	1/3 (33)	0/3 (0)	5/16 (31)
5-14 yr	0/0 (0)	1/11 (9)	0/7 (0)	0/8 (0)	1/26 (4)
15 + yr	2/6 (33)	5/49 (10)	2/19 (10)	0/61 (0)	9/135 (7)
Total	3/8 (40)	9/71 (13)	3/30 (10)	0/72 (0)	15/181 (8)

[a]All initial fecal samples submitted to laboratories serving the resident population of Wales between 1 January 1994 and 31 December 1995 were cultured on sorbitol MacConkey agar (Oxoid) and incubated at 37°C for 18 hours. All types of morphologic appearance of nonsorbitol-fermenting colonies were tested. Five colonies of each type were picked and tested for latex agglutination with O157 antiserum (Oxoid) and biochemically confirmed as *E. coli* by API 20E (bioMerieux sa69280 Marcy L'Etoile, France). *E.coli* O157 isolates were referred to the Public Health Laboratory Service Laboratory of Enteric Pathogens for confirmation and testing for Shiga cytotoxin production.

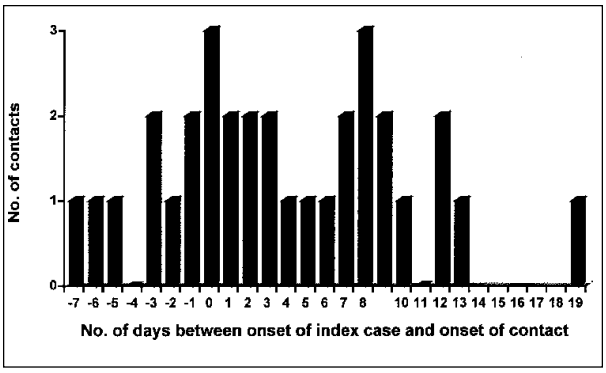

Figure. Time interval between onset of index case and contacts.

[CI] = 0.39-2.73, p = 0.95). The contacts of male index case-patients were no more likely to have STEC-positive samples than those of female index case-patients (11 of 100 versus 4 of 81, RR = 2.23, CI = 0.74-6.73, p = 0.14). Among 53 parents of children with STEC O157, 2 (8%) of 24 fathers and 5 (17%) of 29 mothers had STEC-positive samples. Household contacts were more likely to be STEC positive if the index case-patient had not been hospitalized (12 of 102 versus 3 of 79, RR = 3.10, CI = 0.90-10.61, p = 0.05).

Seven household contacts were food handlers; five submitted samples, and none was STEC positive. Of 12 staff members at healthcare facilities, 11 submitted samples, and none was STEC positive. Of 13 contacts who were children under 5 years of age attending a day-care facility, 11 submitted samples, and 2 were STEC positive. No household contacts were categorized as adults unable to implement good hygiene. Female household contacts were as likely as male to submit fecal samples (RR = 1.13, CI = 0.87-1.46, p = 0.37). Household contacts of female index case-patients were equally as likely as contacts of male patients to submit fecal specimens (RR = 0.81, CI = 0.62-1.06, p = 0.12).

Symptomatic contacts were more likely to submit specimens than asymptomatic contacts (81% [26/32] versus 50% [75/149], RR = 1.61 [1.28-2.03], p = 0.001) and also more likely to excrete STEC O157 than asymptomatic contacts (35% [9/32] versus 4% [6/149], RR = 6.98, CI = 2.67-18.24, p < 0.001 Fisher exact 2-tailed test).

The study showed that 8% to 15% of household contacts of persons with sporadic cases of STEC O157 were STEC positive. However, the household contacts could have had cases coprimary to the index case or even to the primary cases in the household. By excluding contacts who had symptoms before the index case-patient did and those who had no symptoms, we arrived at a household transmission rate of 4%.

The method of identifying secondary cases had certain biases. First, household contacts who had diarrheal symptoms were more likely to submit a stool sample, and their cases were therefore more likely to be recognized. As 40% of the secondary cases were asymptomatic, the true level of asymptomatic and mild infection in household contacts and overall secondary transmission were probably underestimated. This statement, however, does not take into account any role preexisting immunity might play in determining the severity of symptoms and the likelihood of fecal carriage. Persons with higher titers of immunoglobulin (Ig) G antibodies to VT1 in two family outbreaks of STEC have been more likely to be both asymptomatic and not excreting STEC than other family contacts with lower titers (16). Certain household contacts thus might not be able to contract secondary cases. Secondly, the age of the household contact is clearly associated with the likelihood of submitting a fecal sample. Younger contacts were more likely to be screened; therefore, their cases were more likely to be recognized. Nevertheless, the pattern of household transmission demonstrated using as the denominator 101 screened contacts was similar to that using all 181 contacts. The timeliness of specimens did not vary with age.

Overall, household contacts of 1 to 4 years of age and adults of 15 to 34 years of age are most likely to contract STEC O157 from an index case-patient. Children of 1 to 4 years of age explore the domestic environment and frequently put their hands (and other objects) in their mouths, and adults 15 to 34 years of age are likely to be looking after children with diarrhea. When we compared contacts who are parents of an infected child with the remainder of contacts, we observed an elevated risk of contracting STEC O157 in the home (not significant at the 10% level). The pattern of transmission suggests person-to-person spread by the fecal-oral route rather than by food.

Our findings have several implications for the control of STEC O157. Current U.K. guidelines (14,15) advise the prompt investiga-

tion of sporadic cases of STEC and the identification of other persons with symptoms in the household. Since STEC O157 is readily transmitted in the home, contacts of the index case-patient may excrete the organism and present a risk to the wider community through attendance at child day-care centers or through their occupations. Among household contacts, 2 of 13 children under 5 years of age who attended day care had STEC-positive samples. Our data support information from published outbreaks in day-care centers showing that children attending with diarrhea pose a major risk (8) and reinforcing U.K. guidelines (14,15) that, irrespective of symptoms, young siblings of an index case-patient under 5 years of age be excluded from day care until they have two negative fecal specimens at intervals not smaller than 48 hours.

Secondary spread of STEC O157 both during outbreaks and by sporadic cases has been documented (13,17,18). In a large hamburger-associated outbreak in the United States in 1993, an estimated 11% of all cases resulted from secondary spread (19). Whatever the source of the initial infection, secondary transmission, particularly by children, contributes significantly to the incidence of STEC O157 infection. Advice on the risk and on necessary hygienic precautions in the home when a family member has a diarrheal illness should be provided promptly to all affected persons.

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Ehrlichia Infection in Italy

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Immunoglobulin M seroconversion to *Ehrlichia chaffeensis* was documented in U.S. citizens bitten by ticks in Sardinia. Seven cases of suspected ehrlichiosis in local residents were not confirmed by laboratory tests. In Alpine areas antibodies to *E. phagocytophila* were detected in persons at high risk, i.e., foresters (8.6%) and hunters (5.5%), and in controls (l.5%). Of 153 persons bitten by ticks, only one was *Ehrlichia* antibody-positive after 6 months.

A newly recognized rickettsial disease, human ehrlichiosis is emerging in tick-infested areas. First described in the United States (1), the disease, in both monocytic and granulocytic forms, is usually found where Lyme disease is endemic (2,3). Sporadic cases of ehrlichiosis have been reported in southern and northern Europe. Recently, cases were reported in Slovenia, not far from the Italian border (4). Preliminary studies show that 17.1%, 5%, 6.3%, and 11.4% of forestry workers in Switzerland, United Kingdom, Italy, and Sweden, respectively (5-8), have antibodies to *Ehrlichia (E. phagocytophila* and *E. equi).*

Ehrlichiosis in Southern Italy

In 1995 through 1996, four U.S. citizens (two boys and two women) living on a U.S. Navy base in Sardinia (La Maddalena) became ill with acute fever, headache, malaise, and cytopenia after a tick bite. Once rickettsial disease was excluded (Sardinia is a boutonneuse fever–endemic focus), ehrlichia infection was suspected. Coupled serum samples (S1 and S2 a week apart) from each patient were processed by immunofluorescence assay (IFA). All S1 were negative for both species of *Ehrlichia,* while three S2 were positive for immunoglobulin (Ig) M (1:128) to *E. chaffeensis* and negative for IgG (Table), meeting the case definition criteria for ehrlichiosis (Olson, pers. comm.). Subsequently, seven cases of ehrlichiosis-like illness following a tick bite were reported in Sardinian residents. The cases were not confirmed by serologic tests in Rome and Atlanta (E. Lillini, unpub. data).

Table. Immunoglobulin (Ig) M antibodies to *Ehrlichia chaffeensis* in four U.S. citizens bitten by ticks in Sardinia (La Maddalena)

Case	Date of onset	S1	No. weeks	S2
8-yr-old boy[a]	8/95	Neg	1	1:128
12-yr-old boy	7/96	Neg	1	1:64[b]
25-yr-old woman	7/96	Neg	1	1:128
24-yr-old woman	7/96	Neg	1	1:128[c]

[a]Evacuated to U.S. Army Hospital in Landsthul, Germany.
[b]Not diagnostically significant.
[c] Third IgM control 1 month later = 1:256.

Ehrlichiosis in Alpine Areas of Italy

In the last 10 years, tick-borne diseases, especially Lyme disease and tick-borne encephalitis (TBE), have become more widespread in Alpine mountain areas (9). Although new diagnostic tests aid clinical diagnosis, some febrile illnesses in persons bitten by ticks remain unexplained. Human ehrlichiosis must now be considered an emerging problem in tick-infested areas. Pioneering studies in the northern Veneto region (7,10) have recorded *Ehrlichia* antibodies in residents of the Alpine area, where *Ixodes* ticks are present and Lyme disease appears widespread (with more than 500 clinical cases

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reported annually). This study included Cadore, Bellunese (Veneto region), and Trento Districts, neighboring zones located around an area of 80 to 100 miles in the upper northeastern Alpine region (Figure), which pose equal risk of acquiring tick-borne pathogens.

In 1995 through 1996, 544 serum samples were collected from inhabitants of the northeastern Alpine area presumably at risk for ehrlichiosis because they lived in wooded areas and spent time outdoors. A simplified classification system subdivided the participants according to job or primary vocation: healthy residents (controls, n = 193), rangers and forestry workers from Belluno Regional Park and Forestry Services of Trento District (n = 242), and hunters (n = 109) from both areas. Ehrlichia IgG antibodies were detected by IFA using *E. phagocytophila*–infected ovine neutrophils as antigen (courtesy of Dr. K.J. Sumption, Royal School of Veterinary Studies, Edinburgh University, UK). An IFA titer ≥ 1:80 was considered positive (all analyses contained controls of conjugate antibodies and positive and negative sera). Antibodies to *Borrelia burgdorferi* sensu lato were routinely detected by a commercial standard immunoenzyme test kit (Behring, Marburg, Germany). IgG and IgM were considered positive at values (optical density) of >0.6 for IgG and >0.50 for IgM. The TBE antibody (positive titer ≥ 150) was performed by immunozyme and confirmed by immunoblot (Immuno AG, Wien, Austria). This retrospective serologic study of a cohort of persons at high risk showed an IFA positivity (without benefit of immunoblot confirmation) to *E. phagocytophila* (highest titer 1:160) in 21 (8.6%) of 242 foresters, 6 (5.5%) of 109 hunters, and 3 (1.5%) of 193 controls, without significant difference between the three wooded areas studied. Concerning the *Borrelia* positivity, the mean prevalence value among the 541 persons tested was 14.6%, with a lower value (10%) in foresters from Trento District. Seropositivity was most frequent in persons ages 40 to 60 years.

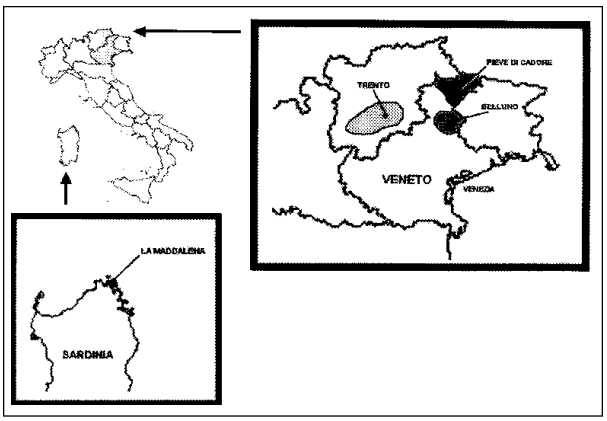

Figure. Areas under study, Sardinia and northeastern Alpine areas.

In summer and autumn of 1997, we followed for 6 months 153 Cadore residents bitten by ticks; we detected *Borrelia, Ehrlichia*, and TBE antibodies immediately after the tick bite and 2 and 6 months later. At the preliminary examination, serologic evidence indicated exposure to multiple tick-borne agents in 26 (16.9%) of 153 patients**,** 15% to *Borrelia*, 0.6% to *E. phagocytophila,* 1.3% to TBE virus. At 2 months, four of the 131 patients tested had IgM antibodies to *Borrelia* (overall incidence 3%); two of these had a clinical pattern of borreliosis. The mean IgG titer to *Borrelia* was 46 (range 7 to >375); the IgM titers were 0.511 to >1.892. Concomitantly, one patient showed typical clinical features of TBE infection, followed by a diagnostic IgM and IgG titer rise (>400); a febrile flulike illness in another patient remained undiagnosed. In the only *E. phagocytophila*–positive person, the titer remained unchanged (1:80). Among the 128 patients tested at 6 months, the four previously recorded as IgM positive to *Borrelia* were confirmed with increased titers (>1,892), and a concomitant IgG positivity (titer >375) was observed in one of these four cases. This study may be one of the largest prospective serologic evaluations for tick-borne pathogens; previous studies have been single-sample serosurveys.

Our data confirm the well-documented high incidence of *Borrelia* infections in upper Alpine areas and show remarkably lower antibody prevalence rates (1%) for TBE and *Ehrlichia*. The different values observed between foresters and healthy controls (8.6% versus 1.5%; p <0.050) from the same zones could be justified by different lifestyles. Foresters spent most of their time in potentially infected areas, changing worksites daily, coming into frequent contact with ticks, and reporting one or more tickbites per year. Controls, on the contrary, had limited outdoor activity, generally restricted to the vicinity of their homes, and less contact with ticks.

Seroconversion to either *Borrelia* or TBE virus were strongly associated with a history of tick attachment; this association and the origin of the persons from a Lyme disease–endemic area support the likelihood that the observed seroconversions were due to a tick-borne agent. The local residents, mainly living in small villages, have close contact with nature but often do not remember tick bites, perhaps because ticks are small and may remain attached for a long time without being noticed. In fact, in approximately 30% of the Lyme disease patients, tick bites are not detectable or reported; consequently, it is difficult to divide these persons into well-defined groups according to tick bite history (9).

Since Lyme disease and ehrlichiosis are zoonoses transmitted by the same tick species *(Ixodes)* and perpetuated in the same reservoir (rodents, deers, sheep), human infections by these pathogens are likely to occur in the same wooded environments. Hunters and rangers, who frequently come in direct contact with animal (deer) carcasses infested by *Ixodes* ticks, or who camp overnight, are at high risk for tick-borne infections (9). Hunters may be infected directly by deer blood (11). *Ehrlichia* seropositivity in persons at risk suggests that mild or subclinical illness due to an infection by *Ehrlichia* species is possible, as recently confirmed (Caruso and Brouqui, pers. data). The low titers observed until now (maximum 1:160) are probably related to a past, mild infection or to the use of an antigen with low specificity.

Conclusions

Our findings indicate the presence of ehrlichiosis in southern Italy (Sardinia) and of subclinical infections in the upper Alpine northeastern areas. In these forested areas, where most patients and physicians are still unaware of the disease, clinical cases of ehrlichiosis had not been documented, although we can presume that some cases of unexplained febrile illness, especially in areas where Lyme disease is endemic, could be related to *Ehrlichia* infection. Further epidemiologic surveillance and controls, now in progress, may better clarify the emergence of this new infection in Italy.

Dr. Nuti is associate professor of tropical and infectious diseases at the La Sapienza University of Rome. His research has focused on leprosy, viral hepatitis, malaria, dengue, Hantaan virus, and tick-borne diseases.

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Salmonella Enteritidis PT6: Another Egg-Associated Salmonellosis?

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Salmonella Enteritidis phage type 6 (PT6) increased dramatically in the United Kingdom during 1997. The sharp rise suggests that PT6 contamination has spread rapidly throughout a basic food commodity; however, the source and food vehicle remain unknown. We present evidence from three outbreaks suggesting a possibile link between PT6 and eggs. Poor documentation of the egg supply network continues to pose problems for public health investigators. Thorough investigation of all future PT6 outbreaks and case-control studies of sporadic infections are needed to confirm the etiology of PT6 infection.

Nearly 10 years ago, evidence from both sides of the Atlantic first implicated eggs in the sharp increase during the 1980s of food poisoning due to *Salmonella* Enteritidis (1,2). In the United Kingdom and Western Europe, the predominant phage type (PT) responsible for eggborne *Salmonella* infection is PT4 (3). In the United States, although PT8 and PT13a are the most common phage types, no single phage type is more likely to be associated with eggborne infection (4). We report the possible emergence of a new egg-associated *Salmonella* phage type in the United Kingdom.

During 1997, we investigated three *Salmonella* Enteritidis PT6 food poisoning outbreaks in Cardiff, Wales. In outbreak 1, five of approximately 200 staff of an education establishment who regularly used a staff canteen reported diarrhea; three were positive for *S.* Enteritidis PT6. All five had eaten fish cakes from a batch of 18 served at the canteen; no illness was reported by other staff, nearly all of whom had eaten the alternative meal. Fish cakes, made with fish and reconstituted dried mashed potatoes, were shaped by hand, coated with raw shell egg, dipped in breadcrumbs, and shallow-fried in oil. When we reproduced the cooking process, as described in a previous outbreak (5), we found that fish cakes were too thick to be properly cooked by the shallow-frying method. The temperature varied greatly both between fish cakes and between parts of the same fish cake. Although the top and bottom of the fish cake were cooked, the egg coating on the sides had insufficient contact with the frying oil to achieve adequate cooking temperature.

Outbreak 2 occurred in a nursing home accommodating 99 residents on four floors. Two residents from different floors and two staff (a cook and a health-care worker) were initially confirmed with *S.* Enteritidis PT6 infection. The only common factor linking the residents was a pureed food diet. When we screened all 10 residents on pureed food diets and a sample of 30 residents on normal diets, we found four other *S.* Enteritidis PT6 cases, all in patients on pureed food diets. The health-care worker was probably infected by secondary spread. The cook's illness began 4 days after the index patient's, which suggested that the cook was infected from a common food source and had not in fact been the cause of the outbreak. Raw shell egg was regularly used in the kitchen; pureed food may have been cross-contaminated from a mixing bowl used for both raw and pureed food.

In outbreak 3, eight customers of the same small Italian restaurant had *S.* Enteritidis PT6

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food poisoning. All had eaten the dessert tiramisu during a 5-day period. Two batches of tiramisu containing raw shell egg were implicated, but neither was available for microbiologic examination. However, a layered cassata ice cream also containing raw egg was tested, and the top layer, made the same day as the first batch of tiramisu, was *S.* Enteritidis PT6–positive. Kitchen inspection indicated that the second batch of tiramisu was probably cross-contaminated when the same whisks and mixing bowls used to make the first batch were reused.

PT6 is a hitherto uncommon phage type in the United Kingdom. In Cardiff, one case not associated with foreign travel was reported in 1994 and in 1995, and two in 1996. During 1997, in addition to the outbreaks described, 33 sporadic cases were reported in Cardiff. In Wales as a whole, reports of *S.* Enteritidis PT6 rose from 23 in 1995 and 43 in 1996 to 279 in 1997, while reports of PT4 remained constant (PHLS Communicable Disease Surveillance Centre [Wales], unpub. data) (Figure). More than 1,000 cases of *S.* Enteritidis PT6 were reported in England and Wales in 1996, almost twice the number in 1995, making it the second most common phage type after PT4 (6). Most cases appear sporadic and acquired within the United Kingdom. Poultry meat and shell eggs were implicated in three of the six outbreaks reported in 1996 (2). The largest outbreak involved 49 persons, of whom 13 had laboratory confirmed

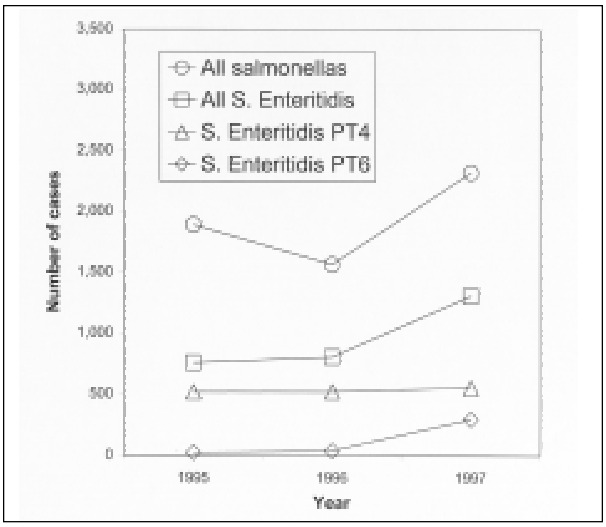

Figure. Salmonella isolates reported in Wales by serotype and phage type, 1995-1997. Source: PHLS Communicable Disease Surveillance Centre (Wales).

S. Enteritidis PT6 (7). Eating egg sandwiches served at a buffet meal was strongly statistically associated with illness, and *S.* Enteritidis PT6 was isolated from several environmental samples from the kitchen where the food had been prepared. However, the investigation did not show whether or not shell eggs used in the sandwiches were the original source or had been cross-contaminated.

The three *S.* Enteritidis PT6 outbreaks described occurred in very different settings: a work canteen, a nursing home, and a public restaurant. Although the evidence is circumstantial, our reports suggest a possible link between *S.* Enteritidis PT6 and eggs. Traceback of eggs was particularly difficult, both because of poor egg labeling and because of the complex network of suppliers and distributors involved. As far as could be ascertained, the eggs associated with the three outbreaks were from different sources, which suggests a general egg contamination problem; if this is the case, the egg contamination signals a complete failure of measures to eradicate salmonellae from poultry-breeding flocks. Alternatively, egg contamination may be restricted to one particular sector of the egg industry. Salmonella-contaminated eggs imported into the United Kingdom have been associated with particular packing stations (8). Traceback investigations of eggs in the United Kingdom continue to pose difficulties for public health investigators because of deficiencies in documentation kept on the egg distribution network. Identifying the source of egg-related outbreaks requires better egg labeling and documentation at all levels of the supply chain—from farmer through distributor to the point of retail sale.

The increase in *S.* Enteritidis PT6 could also be explained by molecular change in the previously dominant PT4. However, PTs 4 and 6 are well established distinct types whose type strains have remained unchanged for many years (5); PT6 is probably not replacing PT4 since no concomitant decrease in PT4 incidence has been observed. The sharp rise in PT6 incidence in the United Kingdom over the past 18 months therefore suggests that this phage type has found a new ecologic niche and that contamination has spread rapidly throughout a basic food commodity. The source and food vehicle remain unknown. All *S.* Enteritidis PT6 outbreaks

should be throughly investigated, and case-control studies are needed to establish the etiology of sporadic PT6 infections.

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Reemergence of Epidemic Malaria in the Highlands of Western Kenya

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Hospital records (1990-1997) of a tea company in the Kericho district, western Kenya, showed malaria epidemics almost annually from May to July, with an annual attack rate of 50%, 857 hospitalizations per 100,000 per year, and 42 deaths per 100,000 per year; 32% of deaths in hospitalized patients were caused by malaria. A questionnaire survey (June 1997) of 244 patients hospitalized for malaria showed that only 8% had traveled to an area with known malaria transmission 30 days before diagnosis. The increasing malaria incidence may be due to drug resistance.

This study investigated reports of epidemic malaria in a highland area of Kenya traditionally thought to be free of malaria to 1) formulate recommendations for reducing disease incidence and preventing future epidemics and 2) determine whether malaria was imported or was due to local transmission.

The factors necessary for malaria transmission are the *Plasmodium* parasite, the *Anopheles* mosquito vector, and the human host. Both parasite and vector are affected by temperature and rainfall. Although temperature extremes kill *Anopheles* mosquitoes, higher temperatures (15°C–30°C) increase their rate of development. The gonotrophic cycle (interval between blood meals) shortens with increasing temperature, and the effect of a small temperature increase is greatest at the cooler ambient temperatures. Thus, a small rise in temperature from 19°C to 21°C shortens the gonotrophic cycle from 4 to 3 days and increases the vectorial capacity of the mosquito (1). Altitude is thought to be a proxy for temperature, so the actual limiting factor for malaria at high altitude is the effect of the lower temperature on the parasite (2). Despite the effect of altitude on ambient temperature, microclimatic factors (e.g. heated houses) can play an important role in facilitating malaria transmission and epidemics at higher elevations. Unlike the parasite, the mosquito vector can commonly be found at altitudes from >1,600 m (3,4) to 3,000 m, demonstrating that the limiting factor for malaria transmission at high altitude is the survival of the *Plasmodium* parasite. The *Anopheles* population is very sensitive to rainfall, which increases the availability of mosquito breeding sites. *A. gambiae,* the primary malaria vector of western Kenya, lays its eggs in small pools and puddles; in this region, rainfall of 150 mm per month leads to rapid expansion of the *A. gambiae* population (3,5) and hence increased risk for malaria transmission. Finally, epidemic malaria requires sufficient numbers of human gametocyte carriers (to infect *Anopheles* mosquitoes) and nonimmune human hosts (to acquire clinical infection).

Malaria did not exist in the western Kenya highlands until the second decade of the 20th century (6). In 1901, completion of a railway from the Kenya coast across the highlands and down to Lake Victoria and increased road transport facilitated the gradual spread of infective mosquitoes into the highlands from the low-lying hyperendemic-disease areas (5). The development of tea estates and agriculture in the highlands, with the concomitant clearing of the forests, provided suitable mosquito breeding grounds. Finally, importation of infected laborers completed the conditions necessary for malaria transmission.

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The first reported epidemic was in 1918 to 1919 when local soldiers returned from Tanzania after World War I (6). Two epidemics were recorded in the 1920s and four in the 1930s. Garnham (2) reported epidemics of malaria in the Londiani area of western Kenya from 1941 to 1944, close to the site of the present study, at an altitude of approximately 2,200 m. After the military camp in the area was disbanded in 1944, the local outbreaks ceased, but highland malaria continued to be a serious public health problem until the late 1950s, when an extensive control program essentially eliminated the disease (7). The highlands were considered free of malaria through the 1960s, but since the 1980s malaria has been increasing (8).

More than 90% of malaria in Kenya is caused by *P. falciparum* (4,5) and transmitted most often by *A. gambiae*, with *A. funestus* as a secondary vector. It has always been assumed that malaria in the highlands of western Kenya is not due to local transmission but is imported from the nearby holoendemic-disease areas around Lake Victoria by the frequent travel of the tea plantation workers and their families.

The Study

Geography

This study was conducted in the Kericho tea-growing area at 0° 22' south and 35° 17' east in the Rift Valley highlands of western Kenya, 80 km from the holoendemic malaria area of the Lake Victoria basin. The altitude of the tea estates is 1,780 m to 2,225 m; the mean monthly temperature is 18.7°C. February is the warmest month of the year (Figure 1), with a mean maximum daily temperature of 28.3°C and mean minimum daily temperature of 10.8°C; July is the coolest month, with a mean maximum daily temperature of 25.4°C and mean minimum daily temperature of 10.2°C. Annual rainfall is 1.79 m with a March-to-June rainy season, and relative humidity is generally slightly below 60% except for May to July. April is the wettest month, with mean rainfall of 252 mm; December is the driest with a mean rainfall of 78 mm.

Demographics

The study data were obtained from the health-care system of a major tea company, which has 18 estates with 22,000 workers and approximately 100,000 persons eligible for health care; 32% of company employees belong to ethnic groups whose traditional home areas have holo/hyperendemic malaria. Given that marriage between ethnic groups is not common, it follows that about 30,000 workers and dependents are from malarious areas; most people travel back to their home regions at least once per year. Approximately 31% of employees originate from the highland areas and the remaining 37% from nonendemic-disease areas around the country. This labor composition has remained stable for at least 20 to 30 years. The employees and families generally live in cement-walled, corrugated metal-roofed duplex style houses, situated close together in large housing areas. The houses usually have 1½ rooms, with a wood or charcoal-burning fireplace, one door, and two unscreened windows. The Central Hospital has 67 beds, two physicians, and two clinical officers, and averages 50 to 80 hospitalized patients per day and 20,000 outpatients per year. Three outlying medical centers, each with

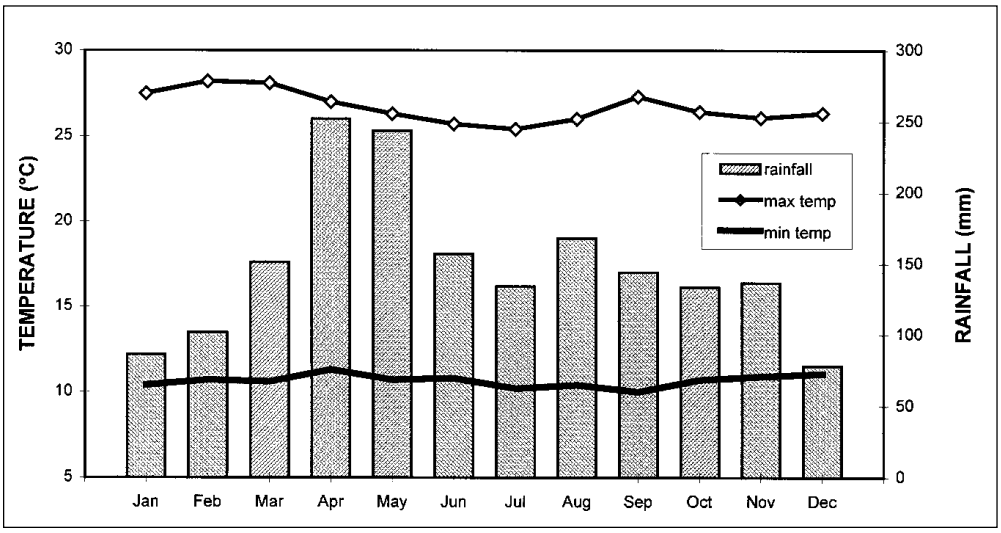

Figure 1. Mean monthly rainfall (1971–97), maximum and minimum temperatures (1984–97), Kericho area.

a clinical officer, see 15,000 to 20,000 patients per year; and 26 dispensaries, each with a nurse provider, see approximately 180,000 to 240,000 outpatients per year.

Data Sources

Retrospective study data were extracted from the Central Hospital monthly reports, which include inpatient and outpatient illness, death, and laboratory data for the whole health-care system. To determine local risk factors for malaria infection, we used daily logbooks on the inpatient wards to identify patients admitted with malaria during June 1997; a simple questionnaire was field tested in consultation with hospital staff and administered to 244 of the 257 identified malaria patients hospitalized during June 1997. The questionnaire asked for demographic and recent travel history and was administered by the primary investigator with a local field worker/translator or by one of the hospital clinical officers.

Findings

Central Hospital illness and death data were available from 1990 through 1997, except for outpatient and death data from October to December 1992. Tea company temperature data were available from 1984 through 1997, and rainfall data from 1971 through 1997 (Figures 1 and 2). The retrospective illness data clearly show the nearly annual occurrence of epidemic malaria (Figures 2 and 3) and the importance of known factors in highland malaria transmission; rainfall >150 mm per month in this area promotes epidemic malaria, and average temperature <18°C appears to extinguish epidemics. In the Kericho area, malaria epidemics occur during May to July, after the long rains and before the temperature drops in July (Figure 3). Figure 4 shows outpatient and inpatient malaria and malaria deaths from 1991 through 1997. For outpatient malaria, the mean annual rate was 48,112 per 100,000 (range 33,914 to 56,040 per 100,000), i.e., each year malaria was diagnosed in approximately 50% of company health-care beneficiaries; for malaria admissions the mean annual rate was 857 per 100,000 (range 361 to 1,058 per 100,000); and for malaria deaths the mean annual rate was 42 per 100,000 (range 14 to 64 per 100,000). In hospitalized patients malaria diagnosis was laboratory confirmed, but in most outpatients malaria was diagnosed purely on a clinical basis. Of the 764 inpatient deaths documented from 1991 through 1997, 254 (32%) were caused by malaria, the leading cause of death in this highland population. Of the 254 malaria deaths, 37% were recorded as being due to severe anemia, 31% cerebral malaria, and 32% malaria with gastroenteritis or pneumonia.

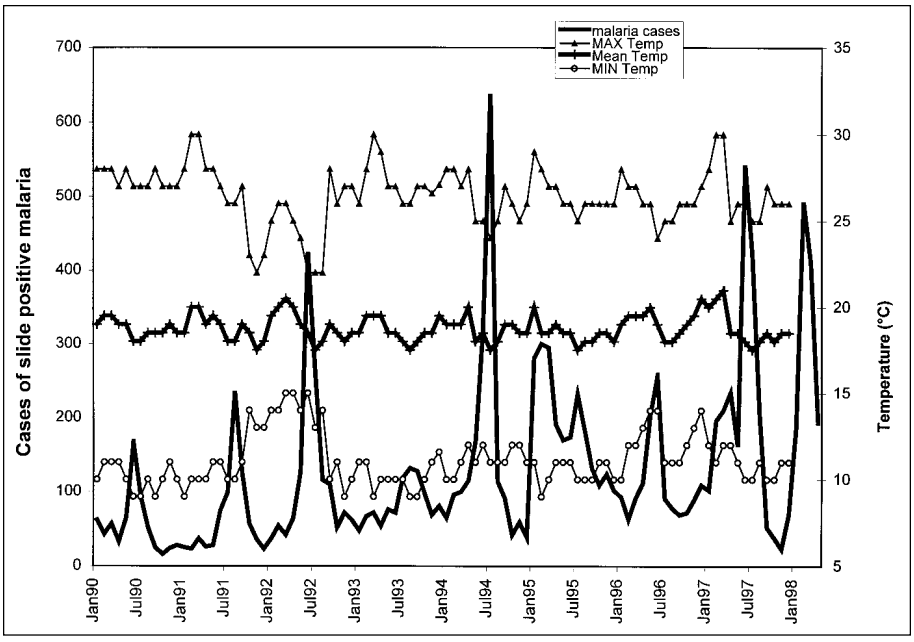

Figure 2. Monthly cases of slide-positive malaria versus maximum, minimum, and mean temperature.

Questionnaire results were available for 244 (95%) of the 257 patients admitted for malaria in June 1997. The remaining 13 patients were either discharged before interview and could not be traced to their houses on the estates, died before interview and the family was not available for questioning, or were minors whose parents were not available for interview. These malaria

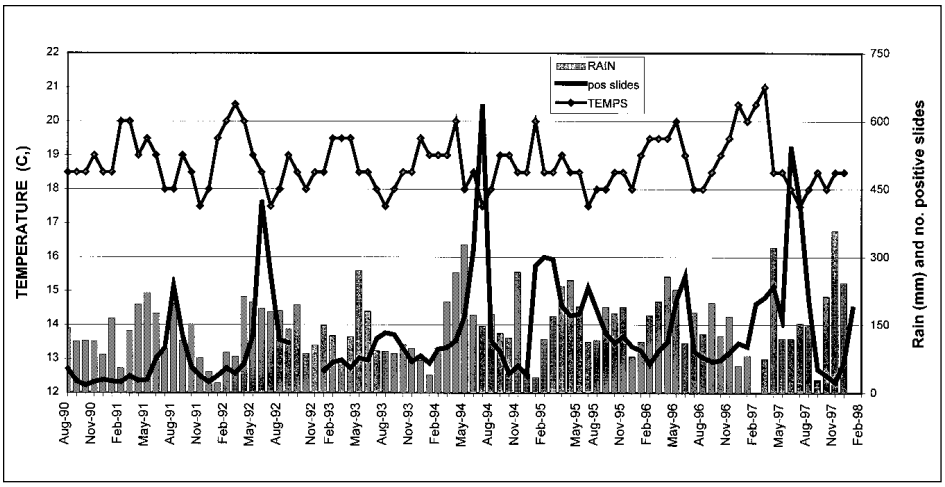

Figure 3. Positive slides, rainfall, and average temperature, per month.

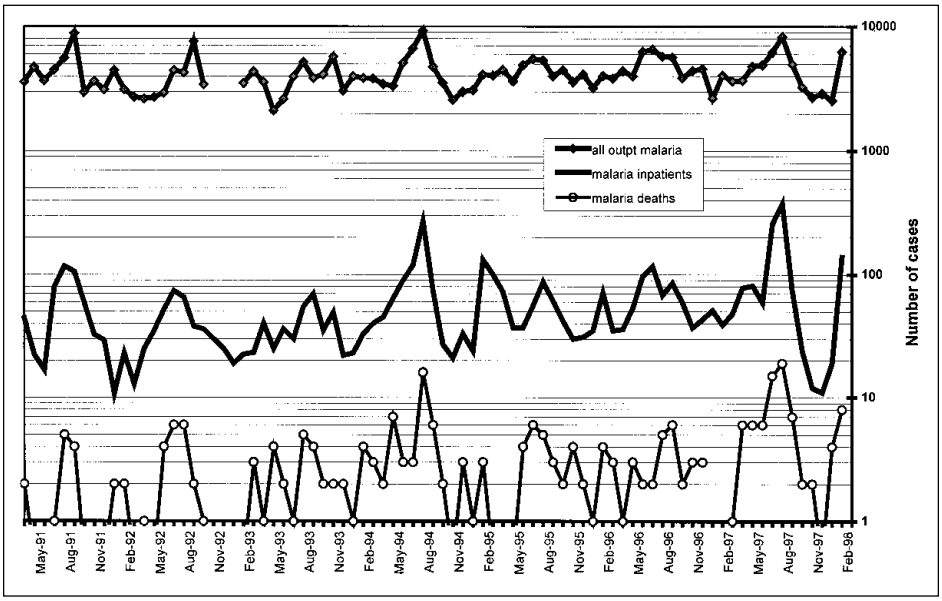

Figure 4. Malaria outpatients, inpatients, and deaths, versus time.

hospitalized patients were 48% female, 52% male, with mean age 13.2 years (range 1 month to 58 years); 20% were tea company employees; 80% were dependents. Only 16% of malaria hospitalized patients belonged to ethnic groups originating from areas with holo/hyperendemic malaria, versus 32% of all company employees (relative risk = 0.4 [95% confidence interval 0.29 to 0.57] for hospitalization for malaria if ethnic origin was from a holoendemic-disease area versus a nonendemic disease area). Forty-three (18%) of the patients hospitalized with malaria had traveled away from the Kericho area in the 30 days before admission, with an average of 19 days away (range 1 to 90 days). Of the 43 hospitalized patients who had traveled, 19 had been to malarious areas; therefore, only 8% of the 244 malaria hospitalized patients had recently traveled to an area with known malaria transmission.

Conclusions

These data document the reemergence of recurrent epidemic malaria due to local transmission in the highlands of western Kenya. Although malaria in the highlands was a serious problem after World War II, because of improved transportation and increased immunity, recurrent epidemic malaria had not been seen in a generation. Several pertinent factors have remained stable and do not appear to explain the increase in malaria in this area. Firstly, climate data from the Kericho area show no obvious change in average temperature or rainfall over the last 10 to 20 years that would explain the present almost annual epidemics in the highlands. Although the climate data used in this study may not be sensitive enough to detect small changes in environmental temperature, global warming would also not explain the epidemics seen in the 1940s to 1950s. Deforestation may be relevant, providing more

breeding sites for vector mosquitoes in sunlit pools and perhaps leading to localized changes in weather patterns and increased microenvironmental temperatures (1).

Secondly, travel of infected persons from adjacent malaria-endemic areas is not new. The main road from the malaria-holoendemic Lake Victoria basin to this highland area was built in the 1950s; in fact, people had been making the same journey using ox wagons decades before the road was paved (5,6). According to the tea company's central office, the tribal/ethnic composition of the employees has remained stable at least since the 1970s, as has the total number of health-care system beneficiaries. Therefore, the increase in malaria does not seem to be explained by an increase in the number of nonimmune relative to semiimmune/immune persons nor simply by an increase in the total population.

A third factor that could lead to an impression of increasing malaria would be the degradation of the health-care infrastructure: if the system became overwhelmed by other diseases and lack of funding, it would be unable to cope with the same number of malaria cases as before, leading to increased hospitalizations and deaths. Indeed, the public health sector in much of sub-Saharan Africa has been overwhelmed by the AIDS epidemic, lack of funding, and other institutional problems; however, the tea company in this study continues to provide excellent care.

One hypothesis for the increase in highland malaria is the failure of the drugs used to cure malaria infections. The period during which epidemic malaria was absent from the highlands corresponds to the time when pyrimethamine and chloroquine were still effective malaria treatments. In 1953, pyrimethamine was used successfully as the first step of a large malaria control campaign in the Kenya highlands, but by 1954, rapid development of resistance was reported in several areas (9,10). Nevertheless, widespread use of chloroquine as well as incorrect use of other malaria medications continue. Clinical control of symptoms (but not cure) creates a large number of semiimmune gametocyte carriers, who can infect mosquitoes when transmission is favored at midyear. In this way, the failure to cure falciparum malaria infections leads to an increase in the human gametocyte-carrier pool, resulting in the rapid spread of epidemic malaria among the largely nonimmune highland population. Studies are under way to further investigate this hypothesis. While increased drug resistance may be the major factor in the documented increase of epidemic malaria, the causes are undoubtedly multifactorial and include vector, host, and environmental components.

Several possible interventions to stop the cycle of epidemic malaria and the associated high costs may be worth exploring. Spraying of houses on the tea estates with residual insecticide could be effective against the endophilic and endophagic *A. gambiae* (11), which appears to be the primary vector in these highlands (5,7). Indoor spraying campaigns in the past in western Kenya with DDT (12) and Dieldrin (7) have effectively combated malaria epidemics. Even now, worker acceptability would likely not be an issue as houses are regularly sprayed for other public health reasons. Insecticide-impregnated bed nets may also be effective with proper education and follow-up. Finally, improved treatment regimens to cure malaria infections, gametocytocidal drugs to prevent transmission, and chemoprophylaxis of workers during travel to malaria-holoendemic areas should be evaluated. Protection of a well-defined, economically important, and less immune population may make effective certain strategies of drug use, such as intermittent chemoprophylaxis, which would not normally be considered in areas with higher levels of malaria endemicity.

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Murine Typhus in Travelers Returning from Indonesia

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We report the first three documented cases of murine typhus imported into Europe from Indonesia, discuss clues for the diagnosis of the disease, and urge that murine fever be considered in the diagnosis of febrile disease in travelers.

One of the oldest recognized, most common, but least reported arthropod-transmitted zoonoses (1,2), murine virus is caused by *Rickettsia typhi*, formerly named *R. mooseri*, a gram-negative obligate intracellular bacterium (1,2). The main vector of murine typhus is the rat flea *Xenopsylla cheopis,* which maintains *R. typhi* in rodents. Flea bites and contamination of excoriated skin or the respiratory tract with infected flea feces are the major sources of human infection throughout the world (2). The classic reservoir and vector are the rat and rat flea, respectively (2); however, the role of cat fleas and opossums in maintaining this microorganism has been suggested in some areas of the United States where rats and rat fleas are absent (2,3). Although murine typhus has a worldwide distribution, it is often unrecognized, and documented cases are rarely reported, particularly in tropical countries. Three recent cases of murine typhus in 2 months in patients returning to Europe from Indonesia indicate that murine typhus should be considered a possible cause of imported fever from Indonesia.

Case 1

In October 1997, a 29-year-old man living in France was hospitalized with a 2-day history of fever. On admission, his temperature was 40°C and his pulse, at 70/min, was dissociated. No signs of eruption or other abnormalities were observed. The patient had returned from a 3-week trip to Bali, Indonesia, 2 weeks before his hospitalization but reported no arthropod bites during his trip. Increases of alanine aminotransferase (110 U/L), aspartate aminotransferase (120 U/L), lactate dehydrogenase (1000 U/L) and reactive protein C (130 mg/L) associated with a low white blood cell count were noted. Repeated blood smears did not disclose any malaria parasites. The patient received a 21-day treatment with doxycycline plus rifampin and became afebrile on day 3. He was discharged from the hospital and remained well. An indirect immunofluorescence assay (IFA) for antibodies reactive with *R. typhi* antigens (4) showed raised levels of immunoglobulin (Ig) M (1:512) and IgG (1:256).

Case 2

In November 1997, a 28-year-old man living in France was hospitalized with a 6-day history of fever. On admission, he had a low-grade fever (38°C). Physical examination found no abnormalities except for a few skin ulcerations of the inguinal area. The patient had returned from Bali 3 weeks before his hospitalization but reported no arthropod bites during his trip. Biologic findings were unremarkable. Blood smears did not disclose malaria parasites. The blood cell count, hemoglobin level, and blood chemistry values were within normal limits. Serologic testing for antibodies reactive with *R. typhi* antigens showed raised levels of IgM (1:512) and IgG (1:256).

Case 3

The third patient, a 28-year-old man from Belgium, became acutely ill during a trip to Indonesia (Bali, Lava, Lombok), with a fever

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(40°C), shivers, diffuse myalgia, but no rash. He was treated in Indonesia with amoxicillin, followed by cefuroxim-axetil (500 mg twice a day for 10 days), without success. The patient recalled multiple insect bites during his trip. Clinical examination on arrival in Belgium showed severe disease with bilateral conjunctivitis and splenomegaly, but no adenopathies. Biologic findings were unremarkable except raised levels of alanine aminotransferase (681 U/L), aspartate aminotransferase (659 U/L), and reactive protein C (17 mg/L). Analysis of the cerebrospinal fluid showed 20 leukocytes/ml with 75% polymorphonuclear cells. Repeated blood smears did not disclose any parasites. The patient received empirical treatment with quinine, doxycycline, and a quinolone to treat suspected malaria or salmonellosis. The fever rapidly resolved, and the patient recovered. Serologic testing with IFA was positive for *R. typhi* (IgG, 512; IgM, 1024). Antibodies to *R. typhi* were also detected in the cerebrospinal fluid (IgM, 2).

All patients exhibited serologic cross-reactions (low titers) with *R. prowazekii*, the agent of epidemic typhus, and *R. conorii*, the agent of Mediterranean spotted fever (Table;1,4). Serologic findings were completed by cross-absorption. When absorption was performed with *R. typhi*, homologous and heterologous antibodies disappeared, but when absorption was performed with *R. prowazekii*, only homologous antibodies disappeared; therefore, murine typhus was diagnosed as the cause of illness in the three patients (4).

Murine typhus is a mild disease with nonspecific signs (1). The incubation period is 7 to 14 days. The classic triad of fever, headache, and skin rash is observed in fewer than 15% of cases (5). While fever and headache are frequent, a rash occurs in only half of the patients and is often transient or unimpressive. This is illustrated in these three cases, where no rash was noted. Arthralgia, myalgia, or respiratory and gastrointestinal symptoms are frequent (5). Leukocytosis and mild leukopenia, anemia, and thrombopenia occur frequently; hyponatremia, hypoalbuminemia, hepatic abnormalities, or renal dysfunction may occur (5). Fewer than half of the patients report exposure to fleas or flea hosts. Of the cases reported here, only one involved insect bites. Untreated patients may have fever and symptoms for 7 to 14 days and then rapidly convalesce. The treatment of choice is antibiotic therapy with tetracyclines. A single 200-mg dose of oral doxycycline usually leads to defervescence during the first 72 hours (6).

Like our patients, most murine typhus patients may have isolated fever and nonspecific biologic abnormalities. Diagnosis may be missed because the rash, the hallmark of rickettsial diseases, is absent (1). For example, in a recent review in the United States, 22 different diagnoses were proposed for 80 patients with murine typhus (5). Consequently, the diagnosis of the disease is based on serologic testing. The reference method is immunofluorescence; however, a latex test, a dot blot enzyme-linked immunosorbent assay, and an immunoperoxydase assay have also been described (4). The fact that travel-associated cases of murine typhus have been identified and may represent a source of imported fever indicates that serologic testing should be considered in febrile patients returning from disease-endemic areas (Figure).

Table. Immunofluorescence testing for antibodies to *Rickettsia typhi* (Wilmington), *R. prowazekii* (Breinl), *R. conorii* (Moroccan), and *Orientia tsutsugamushi* (Kato, Gilliam, Karp, Kawazaki) in febrile patients returning from Indonesia

	Titer (IgG/IgM)			
Antigens	*R. typhi*	*R. prowazekii*	*R. conorii*	*O. tsutsugamushi*
Patient 1				
Acute serum	512/256	128/128	<16/128	<16/<16
Convalescent serum	512/64	128/32	<16/<16	<16/<16
Patient 2				
Acute serum	256/512	128/512	<16/128	<16/<16
Convalescent serum	256/512	<16/<16	<16/64	<16/<16
Patient 3				
Acute serum	512/1024	256/2048	<16/64	<16/<16
Convalescent serum	1024/1024	512/1024	<16/32	<16/<16

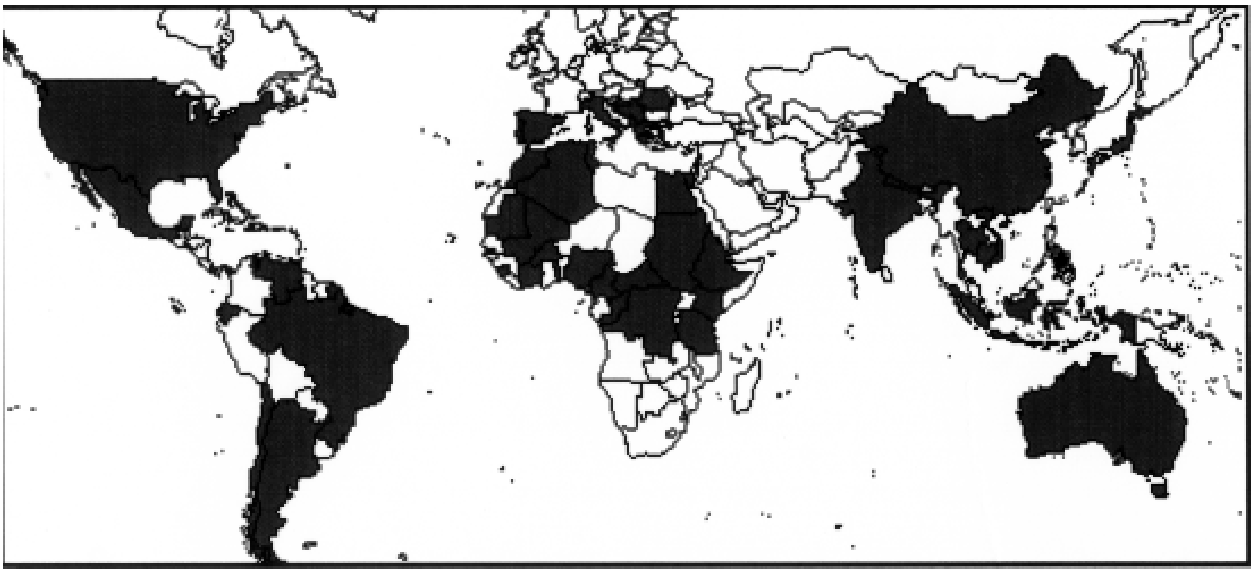

Figure. Areas in which murine typhus poses a risk according to seroepidemiologic studies, case series, or imported cases in travelers (1,2,6-13).

Tissot-Dupont et al. recently reported that the prevalence of antibodies against *R. typhi* in humans in Africa was higher in coastal areas where rats are prevalent (Figure; 7). Other seroepidemiologic studies demonstrated substantial seroprevalence in Asia (Thailand, Vietnam, Japan, Indonesia, China), Europe (Croatia, Greece), and Australia (6,8). Cases have recently been reported in Greece, Spain, Portugal, Israel, and Thailand (6). The disease is known to be endemic in the United States (particularly California, Hawaii, and Texas) and Mexico (6). Some authors have reported the disease in Rumania, Yugoslavia, Albania, Italy, areas around the Caspian Sea, in Kuwait, Latin America (Venezuela, Argentina, Brazil, Chile, Ecuador, Panama, Guatemala, Puerto Rico), and French Guyana (9). However, serologic cross-reactivity may occur between *R. typhi* and *R. prowazekii*, the agent of epidemic typhus (1,4). Epidemic typhus, transmitted by body lice, occurs more frequently in cool areas where clothes are infrequently changed and particularly during human conflicts (1). The disease is prevalent in countries at high altitudes in Central America or Africa (10,14); interpretation of serologic results in these countries has at times been difficult (7,10). However, cross-absorption is a useful technique for identifying the infecting rickettsia to species level as reported in the present cases (4).

Cases of murine typhus have been reported in travelers returning from India, Nepal, Morocco, Canary Islands, Spain, and Africa (6,11-13). Although a case of murine typhus was reported in a patient after a trip to Malaysia and Indonesia (11), the cases reported here are the first serologically documented cases clearly imported from Indonesia. In Malang, East Java, humans are highly exposed to *R. typhi,* and murine typhus appears to be endemic in the region (8).

Fever is a common problem in patients returning from travel abroad. Although malaria is often suspected, rickettsial diseases (including murine typhus) are generally not. Our cases indicate that murine typhus should be considered in the diagnosis of febrile illness in travelers returning from disease-endemic areas, particularly if fever is present and blood smears show no malaria parasites. Rickettsial diseases were recently cited as the third most frequent cause of imported fever in Switzerland (12). On the basis of this study and two previous reports from France (13) and the United States (11), murine typhus is a frequently imported rickettsial disease. Increased development of tourism and travel to tropical countries should lead to an increase in the documentation of cases.

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Differentiating Human from Animal Isolates of *Cryptosporidium parvum*

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We analyzed 92 *Cryptosporidium parvum* isolates from humans and animals by a polymerase chain reaction/restriction fragment length polymorphism method based on the thrombospondin-related anonymous protein 2 gene sequence. Used as a molecular marker, this method can differentiate between the two genotypes of *C. parvum* and elucidate the transmission of infection to humans.

Cryptosporidium parasites cause infection in humans and other vertebrates, including mammals, birds, reptiles, and fish. More than 20 species of *Cryptosporidium* have been reported, of which six are considered valid species on the basis of oocyst morphologic features and site of infection (1,2). *Cryptosporidium parvum,* the species that infects humans and most mammals, has a monoxenous life cycle in which all stages of asexual and sexual development occur within one host. The parasite generates large numbers of viable oocysts in feces. Cross-infection studies in various mammalian systems have indicated zoonotic transmission to humans (1,3). *C. parvum* has caused waterborne outbreaks of cryptosporidiosis and (in AIDS patients) life-threatening diarrhea for which no effective treatment exists (4). A waterborne outbreak of cryptosporidiosis in Milwaukee, Wisconsin, in 1993 affected more than 400,000 people (5).

Molecular characterization techniques used to detect intraspecific variations in *C. parvum* include isozyme profiles (6); random amplified polymorphic DNA (RAPD) analyses (7); nucleotide sequence studies of the 18S rRNA (8,9) and DHFR gene (10); and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the undefined repetitive sequence (11), polythreonine motifs, and oocyst wall protein (12,13). Two distinct genotypes of *C. parvum* parasites have been detected in humans.

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In a previous article, we identified several mutations in the gene thrombospondin-related anonymous protein 2 of *C. parvum* (TRAP-C2) that differentiate between anthroponotic and zoonotic infection in humans (14). Our objective in the present study was to develop a simple, rapid protocol that can be used as a diagnostic tool to differentiate between the two genotypes of *C. parvum* and elucidate the transmission of infection in humans. We analyzed 92 *C. parvum* isolates from humans, calves, deer, dogs, and monkeys and found that this new PCR/RFLP method based on the TRAP-C2 gene sequence can be used as a molecular marker to differentiate between the two genotypes of *C. parvum*.

Analytic Approach

Isolates

We summarized data for 92 isolates of *C. parvum,* 50 from human and 42 from animal sources (Tables 1, 2). Twenty-one of the 50 human isolates were from AIDS patients; the rest were primarily from cryptosporidiosis outbreak case-patients. Seven of the human isolates came from a previous TRAP-C2 sequencing study (14), but because of the lack of DNA, other isolates we used in the previous study were not used in this study. Fecal samples were stored at 4° C in 2.5% potassium dichromate before oocysts were isolated. Oocysts were purified from fecal samples by first using the discontinuous density sucrose gradient centrifugation and then the Percoll gradient centrifugation (15,16).

Table 1. *Cryptosporidium parvum* human isolates, restriction pattern, and sequence type

Source	Isolate	Host	Restriction pattern	Sequence type
1993 Milwaukee	HM3	Hum[a]	Hum	Hum[b]
1993 Milwaukee	HM5	Hum	Hum	Hum
1993 Milwaukee	HM7	Hum	Hum	ND[c]
1995 Florida	HFL1	Hum	Hum	Hum[b]
1995 Florida	HFL5	Hum	Hum	Hum[b]
1995 Florida	HFL6	Hum	Hum	ND
1995 Atlanta	HGA1	Hum	Hum	Hum[b]
1995 Atlanta	HGA4	Hum	Hum	Hum
1995 Atlanta	HGA5	Hum	Hum	ND
1995 Atlanta	HGA6	Hum	Hum	ND
1996 Canada	HCAN9	Hum	Bov[d]	Bov[b]
1995 Canada	NC30	Hum	Hum	ND
1994 Nevada	HCNV2	Hum	Hum	ND
1994 Nevada	HCNV4	Hum	Hum	ND
1997 Pennsylvania	PA41	Hum	Bov	Bov[b]
1997 Pennsylvania	PA46	Hum	Bov	Bov[b]
1997 HIV-Guatemala	HGMO7	Hum	Hum	Hum
1997 HIV-Guatemala	HGMO8	Hum	Hum	ND
1997 HIV-Guatemala	HGMO9	Hum	Hum	Hum
1997 HIV-Guatemala	HGMO10	Hum	Hum	Hum
1997 Minnesota	HMOB1	Hum	Bov	Hum
1997 Minnesota	HMOB3	Hum	Bov	Bov
1997 Minnesota	HMOB4	Hum	Bov	Bov
1997 Minnesota	HMOB5	Hum	Bov	Bov
1997 HIV-New Orleans	HNO2	Hum	Hum	Hum
1997 HIV-New Orleans	HNO3	Hum	Hum	Hum
1997 HIV-New Orleans	HNO4	Hum	Hum	ND
1997 HIV-New Orleans	HNO5	Hum	Bov	Bov
1997 HIV-New Orleans	HNO6	Hum	Hum	Hum
1997 HIV-New Orleans	HNO7	Hum	Hum	Hum
1997 HIV-New Orleans	HNO8	Hum	Hum	Hum
1997 HIV-New Orleans	HNO10	Hum	Hum	Hum
1997 HIV-New Orleans	HNO11	Hum	Bov	ND
1997 HIV-New Orleans	HNO12	Hum	Hum	Hum
1997 HIV-New Orleans	HNO13	Hum	Hum	Hum
1997 HIV-New Orleans	HNO14	Hum	Hum	Hum
1997 HIV-New Orleans	HNO15	Hum	Hum	Hum
1997 HIV-New Orleans	HNO16	Hum	Hum	Hum
1997 HIV-New Orleans	HNO17	Hum	Hum	Hum
1997 HIV-New Orleans	HNO18	Hum	Hum	Hum
1997 HIV-New Orleans	HNO19	Hum	Hum	ND
1997 India	HIND4	Hum	Hum	Hum
1997 India	HIND5	Hum	Hum	ND
1998 Washington State	HWA1	Hum	Hum	Hum
1998 Washington State	HWA2	Hum	Hum	ND
1998 Washington State	HWA3	Hum	Hum	ND
1998 Washington State	HWA4	Hum	Hum	ND
1998 Washington State	HWA5	Hum	Hum	Hum
1998 Washington State	HWA6	Hum	Hum	Hum
1998 Washington State	HWA7	Hum	Hum	Hum

[a]Hum=human
[b]Sequencing data reported earlier (14)
[c]ND= Not done.
[d]Bov=bovine.

Table 2. *Cryptosporidium parvum* bovine isolates, restriction pattern, and sequence type

Source	Isolate	Host	Restriction pattern	Sequence type
1996 Alabama	AAL35	Calf	Bov[a]	Bov
1996 Georgia	AGA43	Calf	Bov	Bov
1996 Georgia	AGA44	Mon[b]	Bov	Bov
1997 Georgia	AGA75	Calf	Bov	Bov
1996 Idaho	AID21	Calf	Bov	Bov
1996 Kansas	AKA19	Calf	Bov	Bov
1996 Maryland	AMD36	Calf	Bov	Bov
1996 Maryland	AMD38	Deer	Bov	Bov
1996 Massachusetts	AMA61-GCH1	Calf	Bov	Bov
1997 Iowa	AIO62	Calf	Bov	Bov
1996 Ohio	AOH6	Calf	Bov	Bov
1996 Ohio	AOH7	Calf	Bov	Bov
1996 Ohio	AOH8	Calf	Bov	Bov
1996 Ohio	AOH9	Calf	Bov	Bov
1996 Ohio	AOH10	Calf	Bov	Bov
1996 Ohio	AOH11	Calf	Bov	Bov
1996 Ohio	AOH12	Calf	Bov	Bov
1996 Ohio	AOH13	Calf	Bov	Bov
1996 Ohio	AOH14	Calf	Bov	Bov
1996 Ohio	AOH15	Calf	Bov	Bov
1996 Ohio	AOH16	Calf	Bov	Bov
1996 Ohio	AOH17	Calf	Bov	Bov
1997 Ohio	AOH45	Calf	Bov	Bov
1997 Ohio	AOH47	Calf	Bov	Bov
1997 Ohio	AOH48	Calf	Bov	Bov
1997 Ohio	AOH49	Calf	Bov	Bov
1997 Ohio	AOH50	Calf	Bov	Bov
1997 Ohio	AOH52	Calf	Bov	Bov
1997 Ohio	AOH53	Calf	Bov	Bov
1997 Ohio	AOH54	Calf	Bov	Bov
1997 Ohio	AOH55	Calf	Bov	Bov
1997 Ohio	AOH56	Calf	Bov	Bov
1997 Ohio	AOH57	Calf	Bov	Bov
1997 Ohio	AOH58	Calf	Bov	Bov
1997 Ohio	AOH59	Calf	Bov	Bov
1997 Ohio	AOH107	Dog	Bov	Bov
1996 Oklahoma	AOK3	Beef cattle	Bov	Bov
1996 Oklahoma	AOK29	Calf	Bov	Bov
1997 Pennsylvania	APE89	Calf	Bov	Bov
1996 Utah	AUT37	Calf	Bov	Bov
1996 Washington	AWA5	Beef cattle	Bov	Bov
1997 West Virginia	AWV65	Calf	Bov	Bov

[a]Bov=bovine.
[b]Mon=monkey.

Extraction of Genomic DNA and PCR Amplification

We followed the protocol of Kim et al. in isolating the total genomic DNA from the purified oocyst (17). A 369 base pair (bp) fragment of the TRAP-C2 gene of *C. parvum* was amplified by using a forward (cua cua cua cua CAT ATT CCC TGT CCC TTG AG) and a reverse (cau cau cau cau TGG ACA ACC CAA ATG CAG

AC) primer (lower case represents nucleotide used for cloning); these primers correspond to positions 848-867 (positive strand) and 1,180-1,199 (negative strand) of the GenBank sequence X77586, respectively. The PCR reaction consisted of 50 ng genomic DNA, 200 µM of each dNTP (Perkin Elmer, Foster City, CA), 40 ng of primer, 1X PCR buffer, and 0.5 units of Taq polymerase (GIBCO BRL, Frederick, MD) in a total volume of 100 µl. DNA amplification was carried out for 35 cycles, each consisting of denaturing (94° C, 45 sec), annealing (48° C, 45 sec), and elongating (72° C, 60 sec), with an initial hot start at 94° C for 5 min in a Perkin Elmer Gene Amp PCR 9600 thermocycler. An additional cycle of 7 min at 72° C was done for final extension. Each experiment used three negative controls (reaction mixtures without Taq polymerase, primers, or template DNA) and a positive control.

DNA Sequencing and Analysis

PCR products were purified by the Wizard PCR Preps DNA purification system (Promega, Madison, WI) and cloned by the CLONEAMP pAMP1 System for Rapid Cloning of Amplification Products (GIBCO BRL, Frederick, MD) according to the manufacturer's protocol. DNA sequencing of recombinant clones that had the correct size insert was carried out on an ABI 377 Automated Sequencer by the dRhodomine Terminator Cycle Sequencing Kit (Perkin Elmer-Applied Biosystems).

RFLP

To develop an RFLP technique for differentiating between the two genotypes of *C. parvum*, the TRAP-C2 sequences were aligned and mapped for restriction enzyme sites by the Genetics Computer Group program (18). Enzymes with predicted exclusive cutting in each genotype were used in RFLP development and analysis. For RFLP analysis, 10 µl of amplification products was digested in a 30-µl reaction mix consisting of 10 units of *Bfa*I (New England BioLabs, Beverly, MA), *Bse*tEI (Boehringer Mannheim, Indianapolis, IN), *Eco*571 (MBI Fermentas, Gariciuno, Vilnius, Lithuania), *Hae*III (New England BioLabs), *Hph*I (New England BioLabs), *Mae*III (Boehringer Mannheim, Germany), *Nru*I (New England BioLabs), *Pac*I (New England BioLabs), or *Tsp*45I (New England BioLabs), and 3 µl of respective restriction buffer for 1 hr, under conditions recommended by the supplier. The digested products were fractionated on 2.0% agarose gel and visualized by ethidium bromide staining.

Findings

Sequence Analysis of Human and Bovine Isolates

Two genotypes of *C. parvum* exist in humans, as shown by the primary sequence of the TRAP-C2 gene (14). Nucleotide sequences differed at five positions between most human and bovine isolates. To confirm and extend this observation, we sequenced additional human and bovine isolates, as well as isolates from dogs, deer, and monkeys. We obtained 42 additional sequences of the TRAP-C2 gene from animal sources and 27 additional sequences of the TRAP-C2 gene from human sources; results of DNA sequencing confirmed that *C. parvum* is highly conserved at the TRAP-C2 locus. All animal isolates, including those from nonbovine animals, showed bovine genotype characteristics (Table 2). Differences between the two genotypes are shown in Table 3. Of the additional 23 human isolates showing human genotype pattern, four isolates (HGMO7, HGMO9, HGMO10, and HNO18) showed "C" at the fifth place, whereas the rest showed "T".

Table 3. Human and bovine *Cryptosporidium parvum* isolates based on multiple alignment[a]

Position (nt)	Human genotype	Bovine genotype
51	G	A
78	C	T
100	T	G
147	C	T
280	T or C	C

[a]Representative sequences have been deposited in the GenBank, with accession numbers AF082521 to AF082524.

PCR-RFLP Method To Discriminate between Human and Bovine Genotype Isolates

To avoid expensive and lengthy DNA sequencing when determining the genotype of *C. parvum* isolates, we developed a simpler, quicker method—PCR amplification of the TRAP-C2 gene followed by RFLP. Restriction

enzyme mapping on the aligned sequences of both genotypes showed five human-genotype–specific (*Hae*I, *Hae*III, *Nru*I, *Pac*I, and *Tha*I) and six bovine-genotype–specific (*Bfa*I, *Bse*tEI, *Eco*571, *Hph*I, *Mae*III, and *Tsp*45I) restriction enzymes. All human-genotype– and bovine-genotype–specific restriction enzymes except *Hae*I and *Tha*I were tested for the TRAPC-2 PCR-amplified products of genomic DNA of *C. parvum*. After restriction and gel electrophoresis, the resulting bands were the size predicted by the mapping analysis (Figure). Digestion of PCR products with these enzymes resulted in a distinct band pattern for the human genotype and bovine genotype isolates. In all cases, the DNA sequencing and PCR-RFLP mapping data matched.

Using PCR-RFLP in Outbreak Investigations

We validated the PCR-RFLP technique by using isolates from outbreaks and sporadic cases of human cryptosporidiosis. Human genotype characteristics were evident in all samples from HIV-infected patients from Guatemala and most patients with sporadic clinical cases, as well as samples from the following outbreaks: Milwaukee (1993), Florida (1995), Atlanta (1995), Canada (1995), Nevada (1994), and Washington (1998). Of the 17 samples from HIV-infected patients in New Orleans, two demonstrated bovine-genotype pattern, while the rest were similar to human genotype. However, bovine-genotype characteristics were evident in the human isolates from outbreaks in British Columbia, Canada (1996), Minnesota (1997), and Pennsylvania (1997).

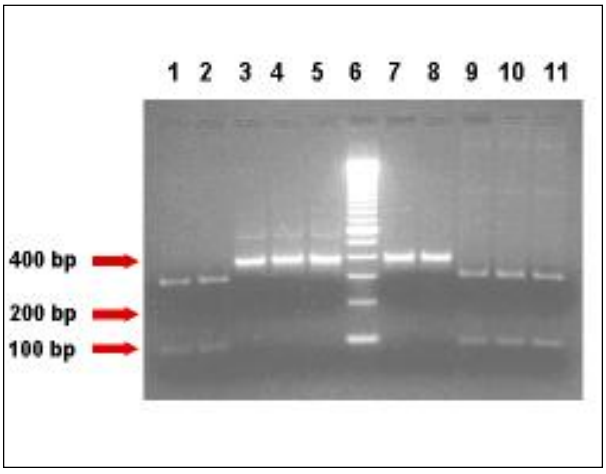

Figure. Human- and bovine-specific restriction enzymes showed distinct banding pattern for genotypes of *Cryptosporidium parvum* isolates. The different lanes represent the TRAP-C2 PCR-amplified products belonging to AGA43, AMD36, AOH6, HM3, and HM5 isolates of *C. parvum*, respectively, after digestion with *Hae*III (Lanes 1-5, human-specific marker) and *Bst*E II (Lanes 7-11, bovine-specific marker) restriction enzymes and agarose gel electrophoresis. Lane 6 is the 100 bp marker. Samples AGA43, AMD36 and AOH6 are bovine (bovine genotype) and samples HM3 and HM5 are human (human genotype). Human- or bovine-specific restriction enzyme markers can cut only the TRAP-C2 amplified product of the respective genotype of *C. parvum* isolates.

Conclusions

We examined a large number of *C. parvum* isolates (92) from human and animal sources from patients in outbreak and nonoutbreak settings to determine the two transmission routes of the parasite in humans. Molecular markers were generated by restriction digestion of PCR-amplified TRAP-C2 products with one of the 12 enzymes to differentiate the two genotypes of *C. parvum*. The results based on TRAP-C2 gene PCR-RFLP showed that this method could also be used in future cryptosporidiosis outbreak investigations.

Results of our characterization of outbreak and nonoutbreak cases of human cryptosporidiosis indicate that anthroponotic organisms account for most cases. We find a large number of human genotype parasites in sporadic cases and in HIV-infected patients. Most cryptosporidiosis outbreaks examined are caused by anthroponotic (human genotype) parasites. Our results suggest similar epidemiologic features of cryptosporidiosis in HIV-infected persons from New Orleans and Guatemala because both were infected with human genotype parasites.

The results of this study confirm the polymorphic nature of *C. parvum*. As we showed in a previous study, two alleles of the TRAP-C2 gene exist, each representing a distinct genotype of *C. parvum* with different transmission cycles in humans. The simple PCR-RFLP technique we developed can effectively differentiate between these two genotypes and transmission cycles and can be used as a tool in outbreak investigations of cryptosporidiosis. Information generated from these investigations will be useful not only in

identifying the sources of contamination but also in controlling the disease.

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Dr. Sulaiman is a postdoctoral research associate in the Molecular Vaccine Section, Division of Parasitic Diseases, National Center for Infectious Diseases, CDC. For the last 7 years, he has focused on the genetic polymorphism of various organisms. He is now conducting molecular typing of *Cryptosporidium* to understand the transmission routes of the parasite.

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An Outbreak of Hantavirus Pulmonary Syndrome, Chile, 1997

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An outbreak of 25 cases of Andes virus-associated hantavirus pulmonary syndrome (HPS) was recognized in southern Chile from July 1997 through January 1998. In addition to the HPS patients, three persons with mild hantaviral disease and one person with asymptomatic acute infection were identified. Epidemiologic studies suggested person-to-person transmission in two of three family clusters. Ecologic studies showed very high densities of several species of sigmodontine rodents in the area.

Hantavirus pulmonary syndrome (HPS), first recognized in 1993 after a cluster of acute respiratory distress syndrome deaths in the southwestern United States, is now considered a pan-American zoonosis (1,2). HPS is characterized by fever, myalgia, gastrointestinal symptoms, and headache, with subsequent characteristic cardiopulmonary dysfunction and a 40% to 60% case-fatality rate (3). Although the etiologic agent, Sin Nombre virus (SNV), causes clinical symptoms that appear different from those that Eurasian hantaviruses cause in hemorrhagic fever with renal syndrome, SNV shares many features with these Old World hantaviruses (4,5), including an association with a single primary rodent host *(Peromyscus maniculatus* [deer mouse] in the case of SNV), which acts as the natural reservoir (6).

After SNV was identified, numerous other New World hantaviruses were rapidly identified throughout the Americas by reverse transcription-polymerase chain reaction (RT-PCR) amplification of viral RNA from captured antibody-positive rodents and, occasionally, from infected patients (2). HPS was first identified in Chile in 1995 in the Cochamo, Los Lagos region. Genetic sequencing of RT-PCR products from the autopsy tissues of another patient presumptively infected in this region in 1996 confirmed infection with Andes virus (7), which had previously been identified in southern Argentina as a cause of HPS, with *Oligoryzomys longicaudatus* postulated as the reservoir host (8). Sporadic cases were reported in Chile until an outbreak that included two family clusters was recognized in the Coyhaique Health District, Aysen region, in August 1997; a third cluster was reported in January 1998. This outbreak prompted a joint investigation by the National Administration of Laboratories and Institutes of Health (Argentina), Pan American Health Organization, Centers for Disease Control and Prevention (CDC, USA), in collaboration with the Ministry of Health of Chile, to define the epidemiology and ecology of the syndrome in Chile.

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The Investigation

Epidemiologic Surveillance and Case Identification

National and local surveillance for HPS was reinforced with explicit case definitions for HPS, asymptomatic hantavirus infection, and mild hantaviral disease. HPS was defined as an acute febrile illness (temperature >38.3°C) characterized by unexplained acute respiratory distress syndrome or bilateral interstitial pulmonary infiltrates, with respiratory compromise requiring supplemental oxygen, or an unexplained illness resulting in death, with autopsy results showing noncardiogenic pulmonary edema without an identifiable specific cause of death. In addition to a compatible clinical illness, the following laboratory evidence of infection was required: 1) hantavirus-specific immunoglobulin M (IgM) or a fourfold rise in IgG titer, or 2) positive RT-PCR results for hantavirus RNA, or 3) positive immunohistochemical results for hantavirus antigen.

Asymptomatic hantavirus infection was defined as laboratory evidence of acute hantavirus infection (presence of IgM antibodies) in persons with no documented concurrent illness. Mild hantaviral disease was defined as IgM-positive results and a febrile illness without objective pulmonary dysfunction (i.e., hypoxemia and radiographic abnormalities). All other persons with isolated detectable hantavirus-specific IgG antibodies were classified as "other seropositives."

All available clinical charts and case reports from the Aysen region were reviewed in conjunction with interviews of family members from the two described clusters and survivors. A third cluster was identified in January 1998 and was also reviewed.

Ecologic Studies

Rodents were trapped at two principal sites near the residence sites of the first two reported family clusters, as well as in several areas of less disturbed native vegetation in Lago Atravesado. Traps were placed at each of the two case-households, as well as at two neighboring controls for each case-household (one within 500 m and the other 500 m to 1,000 m). Each evening, 150 to 200 live-capture traps were placed within and around residences, outbuildings, gardens, and woodpiles, along fence lines, and within remnant patches of native vegetation. Traps were checked in the early morning, and samples of blood and organ tissues were collected according to standardized protocols (9). For comparison, we used similar trapping techniques at additional sites near Valdivia (700 km north of the outbreak area) and the capital city of Santiago (1,400 km north). Rodent taxonomy is as described by Musser and Carleton, 1993 (10).

Laboratory Testing

Human serum specimens were tested for IgG antibodies reactive with SNV and Andes antigens by an enzyme-linked immunosorbent assay (ELISA) (11). Rodent whole-blood specimens were similarly tested for IgG antibodies to SNV. An IgM-capture ELISA was performed on human sera by using inactivated Laguna Negra virus at CDC and an IgM-capture ELISA with recombinant Andes nucleocapsid antigen at the National Institute for Infectious Disease, Buenos Aires, Argentina (P. Padula, unpub. data). Immunohistochemical analysis for hantaviral antigens was performed by using a cross-reactive monoclonal antibody directed against conserved hantaviral nucleocapsid epitopes (12). Specimens from HPS patients were also examined for viral genetic materials (M and S segment) by nested and heminested RT-PCR from either autopsied tissues or blood clot samples (13,14). Genetic sequencing of RT-PCR products derived from patient samples was also performed.

The Outbreak

From October 1995 when HPS was first recognized in Chile through June 1997, seven additional cases were identified (Figure 1). Between July 1, 1997, and January 22, 1998, 25

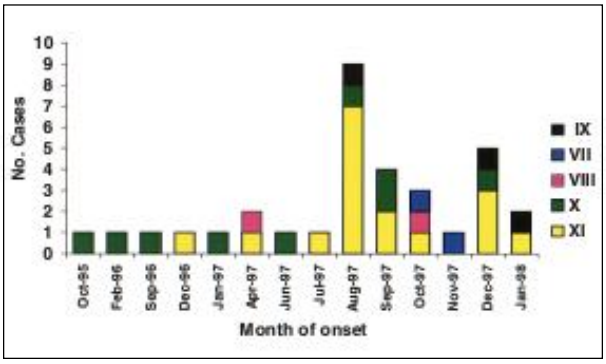

Figure 1. Temporal distribution of hantavirus pulmonary syndrome cases, Chile, 1995-1998.

HPS patients and three family clusters were identified in Chile. By RT-PCR we confirmed that 16 of these patients had been infected with Andes virus. Except for six cases, the recent outbreak was centered in Regions X (Llanchipal) and XI (Aysen) (combined population = 1.1 million) (Figure 2). The areas where these patients lived are sparsely populated, with numerous lakes, in a *Nothofagus* (southern beech) forest ecosystem. From October 4, 1995, to January 22, 1998, a total of 33 cases were identified in Chile, with a case-fatality rate of 54%. The mean age was 31.4 years (range: 1 year 11 months to 60 years); five (15.2%) of the patients were children under 17 years of age; and 76% of the patients were male. No cases were reported among health-care workers.

No significant difference in mean age, case-fatality rate, or proportion of male patients was evident between the July to January outbreak and previous cases; however, all the pediatric cases occurred during the July to January outbreak.

Family Clusters

The first family cluster (Cluster 1) of HPS cases was reported from Cisne Medio, Lago Verde community, Aysen region. The 39-year-old male head of the family became ill with an acute febrile illness on July 15 and died in transit to the regional hospital on July 21; HPS was subsequently confirmed by immunohistochemistry and RT-PCR (M and S segment), with a genetic sequence consistent with Andes virus. The family moved to the village of Villa Amengual, 7 km away, on July 22 and remained there except for a brief visit to Cisne Medio on July 27 to retrieve personal belongings. The wife of the index patient became ill with HPS on August 2 and died on August 8. Her 2-year-old and 12-year-old sons became ill 1 week and 16 days, respectively, after she became ill. HPS was confirmed in both children by serologic testing; both children survived. The brother-in-law of the index patient, who continued to reside intermittently in the original household, became ill 33 days after his sister became ill and died (Figure 3). Thus, the immediate family members became ill 12, 19, and 28 days after leaving the family homestead, and the intervals between the onset of the index and later cases were 18, 25, and 34 days.

In contrast, the second family cluster (Cluster 2) included all four members of a household in Lago Atravesado, Coyhaique community, Aysen region, who became ill within 5 days of each other; one of them, a 2-year-old child with no respiratory symptoms, was classified as having a mild hantaviral infection. The third family cluster (Cluster 3) included a husband, who worked in a rural area, and his wife, who remained in the family home in urban Coyhaique, a city of 60,000 inhabitants. The husband became ill with symptoms suggestive of HPS 12 days after returning to his family home. He was hospitalized and died on December 19.

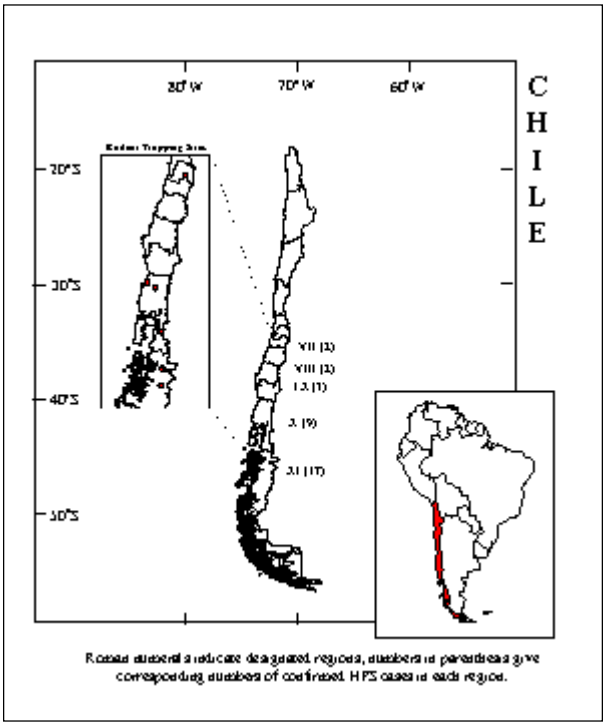

Figure 2. Geographic distribution of hantavirus pulmonary syndrome cases, Chile, 1995-1998.

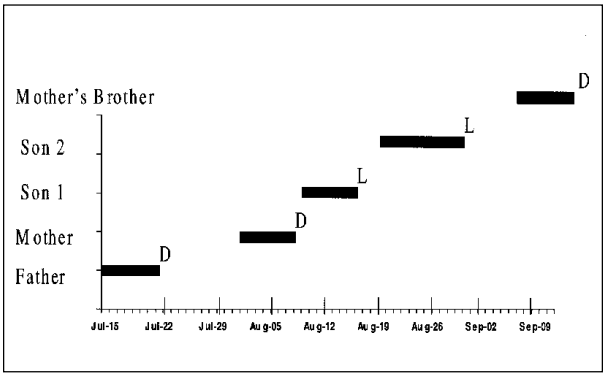

Figure 3. Time-line listing for hantavirus pulmonary syndrome cases in Cluster 1, Chile, 1997.

His wife became ill 22 days after the onset of the husband's symptoms. She had not traveled outside the town of Coyhaique during the previous 12 months and reported no exposure to rodents or their excreta. The only known exposures for the wife were washing her husband's clothing and caring for him while he was ill. She survived and was discharged from the hospital on January 28.

Genetic sequencing of viral RNA from cases of immediate family members from Clusters 1 and 2 demonstrated 3.6% divergence in a 167-nucleotide G2 fragment between the clusters. However, the genetic sequence was identical within each of the two clusters. Genetic sequencing of viral RNA from an additional patient from Cisne Medio not related to the family members in Cluster 1, and the brother-in-law in Cluster 1, demonstrated a distinct G2 sequence, with 1%-4% sequence divergence between them (P. Padula, unpub. data).

Clinical and Histopathologic Features

In general, the clinical description of HPS cases in Aysen was similar to that of the cases in North America. However, included in the Aysen cases were three children with petechiae; one of them died rapidly with hemorrhagic pulmonary secretions and bleeding from puncture sites. Six of the seven patients on whom urinanalysis was performed had microscopic hematuria and casts; three had proteinuria of >100 mg/dl. In addition, one child in the Llanchipal region had concurrent acute rubella infection. One of the most recent cases occurred in Maule, Region VII, in a 23-week pregnant woman who had typical HPS symptoms and fetal death. Subsequently, disseminated intravascular coagulopathy developed and the woman died of multisystem failure.

The histopathologic features in the lung of HPS case-patients included an interstitial mononuclear infiltrate and intraalveolar edema. Immunohistochemical staining showed hantaviral antigens in the microvascular endothelial cells of the lung and other tissues—typical SNV-associated HPS cases. Prominent immunostaining of pulmonary macrophages was also noted in some cases. A unique feature in the series was fine granular immunostaining in the hepatic cells in some cases, a pattern not observed in a large series of North American HPS cases previously examined (Figure 4) (12).

Laboratory Test Results

Serologic testing was conducted for all patients and contacts of patients with confirmed HPS cases in Aysen. Among 53 contacts of 14 patients, two (3.8%) had serologic evidence of an acute infection (one had no illness, and another [described previously in cluster 2] had a mild febrile illness without pulmonary disease that did not meet the HPS case definition), and one was IgG positive. In addition, two hospitalized patients had mild hantaviral disease (M. Tapia, unpub. data). Test results of specimens (SNV and Andes antigens were used) were 100% concordant. Nested and heminested RT-PCR assays were conducted for 20 (71.4%) of 28 HPS cases; all 20 were positive. Sixteen (57.1%) were sequenced and characterized as Andes virus.

Ecologic Studies

A total of 253 rodents were captured during 3 nights of trapping (574 trap nights) in the vicinity of Coyhaique. Overall trap success (captures per 100 trap nights) at case and control home sites was 37% at Cisne Medio and 50% at Lago Atravesado. Trap success at Cisne Medio within and adjacent to the case-household was 66%, compared with only 18% at control homes; this difference may reflect the use of rodenticides in and around the control households. Trap success at distances greater than 100 m from the households was more similar between case and controls (63% versus 43%). The most frequently captured rodent species was *O. longicaudatus*, which comprised 47% of the captured rodents in the area; 13 (12.7%) of 102 tested were hantavirus-antibody–positive. *Akodon olivaceus* comprised a further 33% of the captures (6 [7.5%] of 80 were antibody-positive), and 16% were *Akodon longipilis* (1 [2.7%] of 36 antibody-positive). Eight of 10 rodents captured inside the case-home at Cisne Medio were *O. longicaudatus,* and one was hantavirus-antibody–positive. High trap success in a forested area near Lago Atravesado (22 captures in 40 trap nights, or 55%) indicated that high rodent population densities were not restricted to peridomestic areas.

Trap success was moderate near Valdivia (52 captures from 660 trap nights, or 8% trap success) and very low near Santiago (8 captures from 453 trap nights, or <2%). The species composition was also very different from that encountered in the Coyhaique area: despite

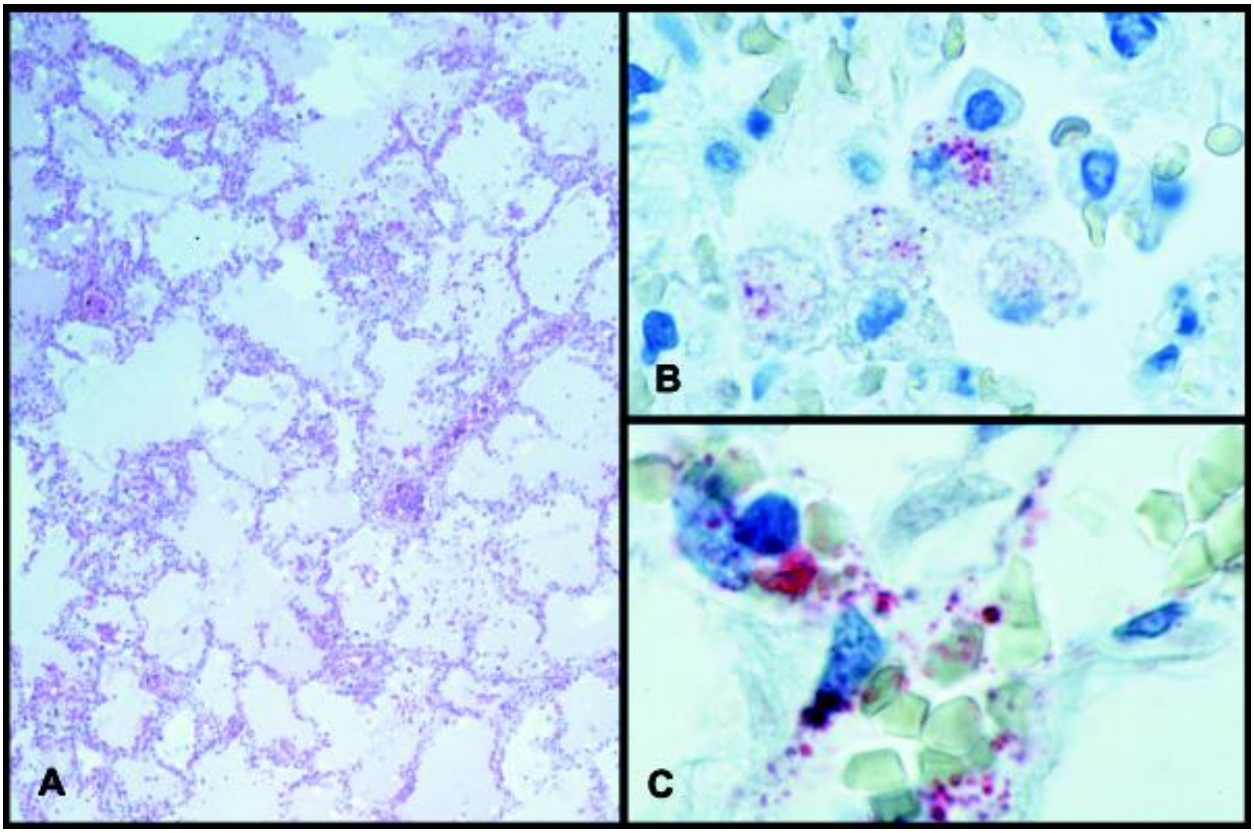

Figure 4. A) Low-power photomicrograph of lung showing interstitial pneumonitis and intraalveolar edema. B) Andes virus antigen–positive intraalveolar macrophages. C) Fine granular immunostaining of hantaviral antigens in endothelial cells of pulmonary microvasculature. (A, Hematoxylin and eosin; B,C, napthol-fast red with hematoxylin counterstain; original magnifications A x 50, B and C x 250.)

trapping in rural areas, 31% of rodents captured near Valdivia and Santiago were the murine species, *Rattus rattus, R. norvegicus,* and *Mus musculus*. No hantavirus-antibody–positive rodents were found from Valdivia or Santiago.

Conclusions

This outbreak of HPS and the presence of endemic disease in Chile support the assumption that HPS will continue to occur throughout the Americas (range of the sigmodontine rodents that host the genetic group of HPS-causing hantaviruses). In Latin America, endemic HPS and epidemics have been reported in Argentina, Brazil, and Paraguay (15-19). Sporadic cases and SNV-like viral sequences have been reported in these countries and in Bolivia and Uruguay (P. Padula, pers. comm.,7,20). Retrospective studies have verified that HPS cases occurred in the United States as early as 1959 (21). The factors responsible for the outbreaks in the Americas have not been well defined and may not be the same in all regions. However, periodic climatic and ecologic events cause dramatic local increases in rodent reservoir populations, known as "irruptions" or "ratadas" (22) and may lead to an increase in the number of rodent-human contacts.

Small-mammal trap success in the Coyhaique area was 5 to 30 times higher than in rural areas 700 km to 1,400 km further north. These extremely high trap success values indicate a rodent irruption in southern Chile whose causes may be related to the flowering of a species of bamboo and a relatively benign preceding winter (22). Regardless of causes, however, the extremely high rodent densities and evidence of hantavirus infection in the two most common species strongly implicate contact with rodents as the primary mode of disease transmission to humans. The rodent species with the highest

antibody prevalence, *O. longicaudatus*, is the reservoir for Andes virus in Argentina and Chile (8,23). No hantavirus has been associated with *A. olivaceus*. RT-PCR conducted on tissue samples from seropositive animals yielded amplifiable viral RNA from seven of seven *O. longicaudatus*, two of four *A. olivaceus*, and one of one *A. longipilis*. Sequencing showed that all PCR products represented Andes virus, and sequences were nearly identical among all three species (S. Morzunov, unpub. data). The near identity between sequences from all three species and the fact that two of four samples from *A. olivaceus* were PCR negative suggest that hantavirus infection in *A. olivaceus* and *A. longipilis* represents spillover of virus from the primary reservoir, *O. longicaudatus*. The absence of viral RNA in two of the four samples from *A. olivaceus* also suggests that the infection is of shorter duration in this presumably nonhost species.

A number of features may eventually differentiate HPS caused by Andes virus from that caused by other New World viruses. Several mild and asymptomatic hantavirus infections have been reported in Argentina and Chile, whereas in North America the case-to-infection ratio is approximately 1:1 (24,25). In this investigation, four (11%) of the patients with serologic evidence of acute infection did not have HPS. More mild and asymptomatic disease in some South American hantavirus infections, in addition to infection with nonpathogenic hantaviruses, may be among the factors explaining antibody prevalence as high as 40% among indigenous persons of Argentina (C. Johnson, unpub. data). Similar case-to-infection ratios may explain the high prevalence (35% to 57%) of hantavirus antibodies in some regions of Paraguay (16,26).

Disease in Chile has also been characterized by an increased propensity toward bleeding, with petechiae in 50% of the pediatric cases and increased renal involvement with microscopic hematuria and cellular casts in the three patients whose urinanalysis results were available. Flushing of the head and upper thorax is indicative of vascular dysregulation and is a well-described sign in hemorrhagic fever with renal syndrome. It has also been described in Andes virus-infected patients from the adjacent areas of Argentina (17,27), but not in Chile.

The relatively large proportion of children with HPS reported in Chile is unexplained. Infection in children is relatively rare in the United States, where only 8 (4.5%) of 179 reported cases were in children 16 years old or younger. Moreover, this dearth of pediatric cases has also been reported for hemorrhagic fever with renal syndrome (28). It is unclear if this greater incidence of pediatric cases is Andes virus- or outbreak-specific. However, Argentina has also seen a larger proportion of pediatric HPS cases than the United States, caused by three different genotypes: Andes, Oran, and Lechiguanas.

Immunostaining showed hantaviral antigens in the microvascular endothelial cells of the lung and other tissues with prominent staining of pulmonary macrophages in some cases. A unique feature not previously described in SNV-associated HPS cases was fine, granular immunostaining in the hepatic cells of three patients. The specificity of this hepatic staining is being investigated. In Argentina, person-to-person transmission has been demonstrated in at least one epidemic of Andes virus infection (17). Although the mechanism of that transmission is unknown, the increased staining of intraalveolar pulmonary macrophages suggests the presence of virus within the alveolar space with the potential for small-particle aerosolization. A comparison of disease variants that may be hantavirus-strain–specific will be required to address this hypothesis.

No discrepancies were observed in serologic results from testing with SNV or Andes antigens, which underlines the current recommendation that diagnosis be based on serologic testing, especially given the difficulties in handling and transporting fresh tissues for RT-PCR and the possibility of cross-contamination. Formalin-fixed tissues from case-patients who died should be used for immunohistochemical tests, particularly if no serum or blood was obtained. However, even in fatal cases, an attempt should be made to obtain heart blood for serologic tests.

Person-to-person transmission cannot be excluded in the first or third family cluster. We did not trap rodents within the town limits at Villa Amengual or in the city of Coyhaique. However, although large numbers of rodents were seen in rural areas, rodent infestation in Villa Amengual or Coyhaique had not been reported. Further, since *O. longicaudatus* is a sylvatic species not generally associated with urban habitats, subsequent exposure to infected

O. longicaudatus was likely not responsible for the wife's illness in family cluster 3. However, as in Cluster 2, Andes and other associated hantaviruses can cause clustering attributable to a single point–source contact with infected rodents.

Person-to-person transmission of Andes virus-associated HPS has been documented (17,29), although this type of transmission has not been observed with SNV-associated HPS (24,30). The lack of cases among health-care workers suggests that current protective methods are adequate; however, standard precautions should be reinforced. Until the propensity of Andes virus to be transmitted from person to person is clarified, patients with suspect cases should be isolated in separate rooms, contact and droplet precautions should be used, and respiratory precautions should be considered.

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Host Genetics of Infectious Diseases: Old and New Approaches Converge

The increasing interest in infectious disease genetics over the last 5 years reflects several trends in the biomedical sciences, not least the explosion of knowledge in human genomics. However, infectious disease genetics is not a new field; some of the largest twin studies were performed more than 50 years ago, and the first malaria resistance gene was identified soon afterwards. The current excitement reflects a recent increase in power in several of the approaches being used to map and identify the genes responsible for variable susceptibility to many major infectious diseases. Four distinct approaches have been used to identify infectious disease susceptibility and resistance genes; these approaches may now be converging.

Approaches

Candidate Genes

The most popular type of study in the host genetics of infectious disease compares the allele or genotype frequencies of so-called "candidate genes" in clinical cases with matched controls. Candidate genes are selected on the basis of their function and likely relevance to the disease of interest and on the possession of one or more genetic variants (1). This approach has been very successful. Starting with the globin genes and glucose-6-phosphate deficiency, several candidate genes were found in case-control studies to be associated with malaria resistance. A different approach associated the Duffy blood group with *Plasmodium vivax* malaria resistance many years before the blood group was known to be a chemokine receptor. In many infectious diseases, HLA variation has been studied, and several associations have been established. The early associations between HLA-DR2 and susceptibility to leprosy and tuberculosis (TB) in India have been reconfirmed in recent years. Most recently, chemokine receptors and a deletion variant of CCR5 have been found to affect both risk for HIV-1 infection and rate of progression to AIDS.

Despite these successes, problems remain with the candidate gene approach. Most sample sizes have been adequate to detect only very strong associations; however, less marked associations may be more common. Matching controls to cases may be problematic and has led to the recent trend for family-based association studies in other types of polygenic disease. Frequent geographic heterogeneity in HLA associations has complicated the interpretation of studies of these important genes. Finally, the limited effects observed in most studies suggest that this approach might be missing some other genes with more major effects. Nonetheless, with more promising candidate polymorphisms appearing each month, the popularity of this approach will likely increase.

Mouse Genetics

A quite different approach to finding human infectious disease resistance and susceptibility genes has been identifying relevant murine genes. The relative ease of mapping susceptibility genes in inbred strains of mice has led to the mapping of numerous named loci in mice by linkage analysis. In some cases, such as with the murine Mx influenza resistance locus, the gene has actually been identified (2). Far more genes have been mapped in this way than have been identified. A well-studied exception is the murine *Nramp1* gene that was positionally cloned on chromosome 1 (3) after this locus was shown to influence susceptibility to certain leishmanial, mycobacterial and salmonella infections in mice. Another important recent approach has been the use of gene knockout mice to determine whether particular genes affect susceptibility to various infectious diseases.

A difficulty with the mouse genetics approach is that a gene identified in mouse studies may turn out to lack polymorphism in humans. This difficulty is particularly relevant for artificially generated knockout mice; inactivating mutations of key genes may be too deleterious to humans. Such mutations, like mutations of the interferon-gamma receptor gene (4), may, however, turn up rarely in immunodeficient children. However, even mutations that are polymorphic in inbred mouse strains, such as the *Nramp1* variant, may be nonpolymorphic in wild mouse populations. Given the differences in selection pressure that mice and humans have been exposed to over tens of millions of years, the major susceptibility genes in the two species are unlikely to be the same. Another difficulty is, of course, the extent to which a murine model may mimic human infectious disease. The human malarias, for

example, cannot be studied in mice.

Complex Segregation Analysis

Complex segregation analysis models the transmission of the disease in multicase families and compares the likelihood of different ways of inheriting the disease. Several parameters may be optimized, including the contribution of a potential single major gene, polygenic and environmental components, allele frequencies, and dominance. The remarkable result is the regularity with which a single major gene is reported. For example, a single major gene has thus been implicated in leprosy, schistosomiasis, TB, and even malaria (regulating parasite densities) (5). Even allowing for some publication bias, these remarkable results are surprising in view of the highly polygenic nature of infectious disease susceptibility suggested by candidate gene analysis. A major gene, as proposed in these models, would need both a high frequency and very high odds ratio to account for a substantial proportion of the overall genetic effect. One explanation might be that such single major genes remain to be identified in these and perhaps many other infectious diseases.

The usefulness of complex segregation analysis is difficult to assess because in no case has the single major gene proposal been confirmed by mapping and identifying such a gene. However, this difficulty may be ending as genome scanning technology is increasingly applied to infectious diseases.

Human Genomewide Analysis

The most recent approach applied to human infectious diseases is mapping and subsequently identifying major genes affecting susceptibility or resistance through genomewide scans. This approach entails an initial linkage analysis in large numbers of multicase families, which is followed by association study analysis for gene identification. Typically, a few hundred microsatellite markers search for evidence of increased sharing of parental alleles identical by descent in affected sibling pairs. This nonparametric approach does not require information on how the disease was inherited, which is almost always unknown. However, in schistosomiasis the results of complex segregation analysis have been used in a parametric, or model-based, analysis to map a susceptibility gene to chromosome 5 in a small number of Brazilian families (6), as reviewed by Abel and Dessein in this issue. The degree to which this genomewide approach can be used to map major susceptibility genes in reasonable numbers of families remains uncertain. However, the genes with the largest effects are those that can be mapped with most power and may be of greatest interest.

The main limitation of a genomewide linkage scan in polygenic infectious diseases is that its power is lower than that of association studies. Recruiting the required large numbers of clinically well-defined multicase families may require multicenter collaborations. Technical advances may make genomewide association studies feasible, which would remove the need for the use of multicase families, although the requirement for large numbers of cases would remain.

Approach Convergence

Until recently, these various genetic approaches were typically pursued by different research groups with relatively little synergistic interaction. The major candidate genes studied in humans were different from those mapped in mice, and none of the associations found appeared strong enough to fit the single major genes being proposed by complex segregation analysis. However, researchers working on the *Nramp1* gene in mice suggested that the human homologue, *NRAMP1*, could correspond to a major mycobacterial susceptibility gene suggested by segregation analysis in humans. This theory has now become testable with the identification of numerous polymorphisms in *NRAMP1*. A clear association emerges between variation in this gene and TB susceptibility, at least in a West African population (7). Thus a gene identified through whole genome analysis in mice, potentially the major gene suggested by complex segregation analysis, is associated with susceptibility when investigated as a candidate gene in a human case-control study. The separate approaches have converged, we hope for the first of many meetings.

However, little in complex disease genetics is simple. The effect observed in Gambia is different from BCG susceptibility in mice in that in human TB, susceptibility appears dominant (7), while in mice it is recessive. Also, the effect in humans, though highly statistically significant, is relatively modest, perhaps a few percentage points of the overall genetic component of

susceptibility. Although not the elusive major gene, this effect is well worth investigating for the light it may throw on mechanisms of disease susceptibility. As other candidate genes are identified from mouse studies, they may add to the long list of candidate genes available for human association studies. Meanwhile, the hunt for elusive major susceptibility genes continues. Ongoing genome scans should soon help determine whether these genes really exist in particular infectious diseases; if they do, their mechanisms of action as well as the forces underlying their evolutionary maintenance should be of great interest.

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Does Restricted Distribution Limit Access and Coverage of Yellow Fever Vaccine in the United States?

Yellow fever, the original "viral hemorrhagic fever," is a mosquito-borne infection characterized by hepatic, renal, and myocardial injury, bleeding, and a high case-fatality rate. The disease occurs in tropical South America and sub-Saharan Africa. Maintained by zoonotic transmission between sylvan mosquitoes and nonhuman primates, the virus cannot be eradicated, and prevention of human infection requires high vaccination coverage. Recently the incidence of yellow fever has increased dramatically (1), with 23,543 cases and 6,421 deaths officially reported to the World Health Organization (WHO) between 1985 and 1996. The true incidence is believed to far exceed the reported incidence. Yellow fever remains a public health problem because of failure to implement effective immunization, particularly in Africa.

The recent upsurge in yellow fever activity has been associated with an increased risk for infection among unimmunized travelers. In 1996, two tourists infected in the Amazon region of Brazil died after returning to the United States (2) and Switzerland (3). Fatal and other cases in tourists were also reported in previous years (4-8). Other cases were likely missed, misdiagnosed as viral hepatitis, treated abroad, or not reported.

Since viremic humans are the source of infection for the epidemic mosquito vector *Aedes aegypti*, unimmunized travelers may import yellow fever to *Ae. aegypti*-infested regions, including the United States, West Indies, Central America, Mediterranean region, and Asia. The expansion of international travel, including adventure travel to remote and tropical areas, has increased exposure to yellow fever and other exotic infections (9). Each year eight to nine million tourists arrive in yellow fever-endemic countries in South America and Africa (10,11); the number of tourists visiting regions within countries in which yellow fever transmission occurs is unknown but probably exceeds three million. In the United States, 150,000 to 300,000 civilian travelers are immunized annually (12)—likely only a fraction of the population requiring the vaccine. However, few data exist on vaccine coverage in travelers to yellow fever–endemic regions. One survey in the United States found that 30% of travelers to developing countries between 1980 and 1986 received yellow fever vaccine (13). Yellow fever vaccine coverage of European travelers varied from 0% to 90% (14,15).

WHO and the Centers for Disease Control and Prevention strongly recommend vaccination for travel outside urban areas in the yellow fever–endemic zone (16,17). Yellow fever 17D, a live, attenuated, vaccine developed in 1936, has been used in more than 300 million persons and has a remarkable record of safety, tolerance, and efficacy (18). Immunity develops rapidly after a single dose and is extraordinarily durable, probably lifelong. Yellow fever vaccine can be administered simultaneously with most other vaccines or immune serum globulin recommended for international travel. Cost of the vaccine is approximately $48 for a single-dose vial in the United States. Thus, the attributes of the vaccine itself do not constitute a barrier to the immunization of travelers.

Yellow fever is the only human vaccine subjected to regulations regarding distribution. The International Health Regulations (19) require that yellow fever vaccine be given in approved vaccinating centers, listed in a 1991 WHO publication (20) and updated in the WHO Weekly Epidemiological Record. The restriction was instituted to ensure that the relatively thermolabile vaccine is properly stored and delivered within the specified time after reconstitution and that a signed vaccination certificate is properly completed (specifying the date of immunization and the vaccine lot) and bears an official stamp. Only authorized medical facilities may order vaccine directly from the manufacturer, and the vaccine is not available through the normal distribution channels for drugs and biologic products.

In the United States, registration and approval of yellow fever vaccinating centers has been delegated by the U.S. Public Health Service to the state health departments. With the expansion of international travel in recent years and a growing number of travel clinics, this system has become unwieldy, and its listing of active centers is inaccurate.

The Problem, the Risks, a Solution

The United States currently has 800 to 1,000 yellow fever vaccinating centers. Many are county health departments or clinics specializing

in travel medicine. However, most travelers first seek pretravel medical advice from their primary-care providers. Immunizations and prophylactic measures against many travel-related threats, such as hepatitis A, typhoid, diphtheria, and measles, are commonly given by primary-care providers; even more specialized travel vaccines, such as rabies, meningococcal, and Japanese encephalitis vaccines, are readily available to general practitioners. Specific recommendations for the use of travel vaccines are in publications (19,20) and on the Internet.

Yellow fever is the only travel vaccine for which the primary-care physician must refer the patient to another provider. Compliance with recommendations for immmunization is affected by multiple factors (21), including inconvenient access to a vaccine. A Tennessee resident who died of yellow fever in 1996 was not vaccinated before going to Brazil because the official vaccinating center nearest his home was 25 miles away (2). In contrast, he had received other medical advice, hepatitis A vaccine, and a prescription for malaria chemoprophylaxis from his primary-care physician. Since requests for yellow fever vaccine may be few, most physicians do not initiate the complicated process of obtaining authorization as a designated vaccinating center. In the United Kingdom, however, where general practitioners have become proactive in providing travel advice to their patients, the number of physicians who obtained yellow fever vaccinating center status rose from 181 to 1,231 between 1990 and 1992 (22).

The disadvantages of general practitioners' engaging in travel medicine, including the immunization of travelers against exotic infections and prescription of malaria chemoprophylaxis, have been examined in several studies, which suggest that improvements are needed in the quality of advice provided to patients (23,24). Among Swiss and German general practitioners, only 11% and 1%, respectively, provided correct recommendations to travelers to Thailand and Kenya regarding malaria prophylaxis and vaccinations (25). County health departments in the United States responsible for yellow fever vaccination have limited expertise in travel medicine. While extending the distribution of yellow fever vaccine to primary health-care providers would increase access to the vaccine and improve coverage, additional steps are required to improve the quality of medical advice by giving practitioners accurate and simple information to guide use of the vaccine. Public health department nurses and inspectors in Ontario, Canada, provided correct recommendations for yellow fever vaccination 96% of the time (26), reflecting the value of education in this field.

Travelers requiring yellow fever and other live virus vaccines face special problems. The current recommendation is for either simultaneous administration or separation of the vaccines by 4 weeks to avoid potential interference (16). Compliance with this recommendation is difficult, and the recommendation is probably ignored when the patient receives some vaccines from the primary-care physician and yellow fever vaccine at a designated center. The recommendation provides a further incentive for a single health professional to administer a schedule of immunizations appropriate for the individual traveler.

We sampled 15 states, which contained a very high proportion of the U.S. population (44%) and were representative with respect to geography, urban-rural distribution, and race. Although we could not estimate whether these states were different from the United States as a whole with respect to international travel, we believe that it is reasonable to extrapolate the findings with respect to access to vaccinating centers to the general population. We found that the current system of designated yellow fever vaccinating centers in the United States provides reasonable access to approximately 95% of the population, where "reasonable access" is defined as residence within a 25-mile radius of the vaccinating center (Table). Although this would appear sufficient, in fact there may be substantial obstacles—from the perspective of the patient—to utilization of centers within geographic proximity (the 25-mile catchment area), including extra costs, inconvenience of access to centers within congested urban areas, and unfamiliarity with the provider. Approximately 5% to 6% of the total U.S. population (12 to 14 million) resides in rural areas that are relatively inaccessible to vaccinating centers (Table). In some states, such as Montana, Wyoming, Oklahoma, and Vermont, approximately 25% of the population resides outside 25-mile catchment areas, and very long distances must be traveled to reach a center (Figure), with the result that only highly motivated patients are vaccinated. Although it is possible that urban

Table. Populations (1990 census[a]) inside and outside 25-mile radius 'catchment areas' of intrastate authorized yellow fever vaccinating centers

		Population x10^3		
Region	State	Total	Inside catchment area (%)	Outside catchment area (%)
East	New York	17,991	17,679 (98.3)	312 (1 .7)
	Massachusetts	6,016	5,965 (99.2)	51 (0.8)
	Connecticut	3,287	3,287 (100)	0 (0)
	Vermont	563	418 (74.2)	145 (25.8)
South	Florida	12,938	12,410 (95.9)	528 (4.1)
	Louisiana	4,220	3,767 (89.3)	453 (10.7)
Central	Illinois	11,431	10,125 (88.6)	1,306 (11.4)
	Wisconsin	4,892	4,331 (88.5)	561 (11.5)
	Nebraska	1,578	1,293 (81.9)	285 (18.1)
	Oklahoma	3,146	2,317 (73.6)	829 (26.4)
Mountain	Montana	799	595 (74.5)	204 (25.5)
	Wyoming	454	350 (77.1)	104 (22.9)
Pacific	California	29,758	29,138 (97.9)	620 (2.1)
	Oregon	2,842	2,676 (94.2)	166 (5.8)
	Hawaii	1,108	983 (88.7)	125 (11.3)
15 states		101,023	95,334 (94.4)	5,689 (5.6)

[a]U.S. Bureau of the Census, 1990 Decennial Census.

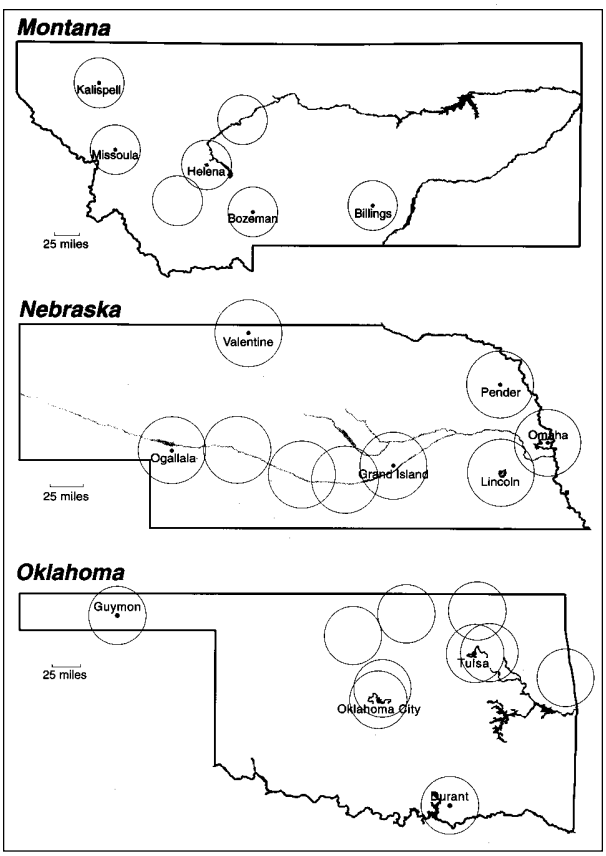

Figure. Location of yellow fever vaccinating centers in three western states. The circles indicate the area within a 25-mile radius from each center (catchment areas). Only names of selected locations are shown.

residents with better access to vaccinating centers are more likely to internationally travel than rural residents, our analysis clearly demonstrated that restricted access to yellow fever vaccine by populations in rural or sparsely populated areas.

In the past, a principal concern underlying the restricted distribution of yellow fever vaccine was the thermolability of the vaccine and the need to ensure its proper storage and handling. However, other vaccines in wide use have similar or even more stringent storage requirements. Tetanus toxoid must be stored refrigerated away from the freezer compartment, measles-mumps-rubella must be stored refrigerated and protected from light, and varicella vaccine must be stored frozen. Yellow fever vaccine manufacturers now include stabilizers in the product that render the lyophilized vaccines quite resistant to degradation (27-29). Yellow fever vaccine is stored in the refrigerator at the point of use, as are most other vaccines. Yellow fever vaccine is discarded 1 hour after reconstitution; in contrast, varicella vaccine must be discarded within 30 minutes. Thus, the storage and handling of yellow fever vaccine do not pose special difficulties.

The International Health Regulations, adopted nearly 30 years ago (19), require a valid certificate of vaccination only for yellow fever (17,19). This regulation recognizes the risk for importation and spread of yellow fever across international boundaries by viremic travelers. However, enforcement is problematic and lax. For example, in the Calcutta airport, closure of the health inspection desk in 1986 virtually eliminated verification of vaccination status of disembarking passengers (30). A number of yellow fever–receptive (*Ae. aegypti*-infested) countries, including the United States, do not require vaccination certificates (16,17), and many countries require but do not enforce the inspection of certificates. The United States abandoned inspection of vaccination certificates (because of the high cost and questionable public health value of yellow fever quarantine regulations) in favor of a system of surveillance and optimization of health information to the

traveling public. The difficulty in controlling yellow fever vaccination through designated centers and the lack of a clear justification on the basis of thermostability of the vaccine led a WHO working group on revisions to the International Health Regulations to recommend reconsideration of designation procedures of vaccination centers (31). We think the link between the requirement for certification of yellow fever vaccination imposed by some national health authorities and the distribution of vaccine through designated centers should be the focus of future discussions between parties to the International Health Regulations. Certification of vaccination could be provided by any licensed physician, as was the case for smallpox and cholera vaccines at the time that these were required for international travel. The blank certificate (available from the Government Printing Office) could, for example, be shipped together with the vaccine from the manufacturer or distributor to the physician. A change would be required in the International Health Regulations to allow validation of the certificate by physician signature alone without application of the uniform stamp currently used on the certificate.

The risk of acquiring yellow fever during travel is related to the level of virus transmission at the time and in the location visited. During yellow fever epidemics, the risk to an unimmunized person is very high, even during a short visit to an endemic-disease area. In recent outbreaks affecting indigenous populations in West Africa, the incidence of infection during the 3-month epidemic period was 20% to 30% (32,33). The risk of developing severe disease with jaundice if infected has been estimated at 1:3.8 to 1:7.4 and the risk of dying if ill with jaundice is 20% to 60% (18). In areas where the indigenous population is protected by vaccination, yellow fever virus may circulate silently between monkeys and mosquitoes without any reported human cases as a guide to risk. Moreover, the southern United States is infested with *Ae. aegypti*, and importation of yellow fever poses a considerable threat (34,35). The potential for importation by persons infected with yellow fever has increased with the establishment of direct links between the United States and disease-endemic areas, such as Manaus, Brazil, and Iquitos, Peru (9). These factors provide a strong rationale for immunizing all persons who visit disease-endemic zones.

The current system of designated yellow fever vaccinating centers and relative inaccessibility to yellow fever vaccine are impediments to full vaccination coverage of travelers. Extension of vaccinating privileges to primary-care providers, together with clear and concise information regarding recommendations for use of the vaccine, may improve protection of travelers at risk and enhance the barrier to importation of the disease.

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Aedes aegypti in Tucson, Arizona

To the Editor: The highly domestic mosquito species *Aedes aegypti,* a tropical, nonnative vector of dengue and yellow fever, has been identified in several desert communities, including the city of Tucson and the border towns of Douglas, Naco, and Nogales (1). *Ae. aegypti* has now been found in the southern Arizona communities of Benson and Sahuarita Heights, which indicates that its distribution is probably expanding. Moreover, mosquito surveillance indicating that *Ae. aegypti* populations in Tucson are established throughout the city supports local concerns that the mosquito poses a public health risk in Arizona's second largest metropolitan area.

Since it was first detected in 1994 on Tucson's west side, the mosquito has been found in the central downtown and university districts, northern foothills, and the east and south sides (both in residential areas, including schools and parks, and in business districts). Between 1995 and 1997, almost 200 adult *Ae. aegypti* (female and male) were recovered in CDC CO_2 traps from 26 sites in Tucson by vector biologists from the Arizona Department of Health Services, Pima County, and the University of Arizona Veterinary Science Department.

These trapping events occurred between May and October of each year and were associated with either routine arbovirus surveillance or nonsystematic *Ae. aegypti* "spot-checks." Many of the sites were sampled more than once and 30% contained *Ae. aegypti* on multiple occasions. Catches from individual sites contained 1 to 40 adult mosquitoes (mode = 1).

A larval survey of the city has not yet been conducted because of limited staff and problems associated with oviposition trapping, an important part of *Ae. aegypti* larval surveillance (2). Initial attempts at ovitrapping by state, county (1), and later university personnel were not successful because the hay infusion water in ovitraps evaporated rapidly in Tucson's arid climate. However, the few larvae recovered from household containers suggest that Tucson's urban environment is providing a breeding habitat. Since the local climate requires full-time vigilance of ovitraps, this method of surveillance appears too labor intensive for the present.

In 1997, the University of Arizona Entomology Department initiated a mosquito survey of the city. This multiyear project is funded by the Entomology Department, the city of Tucson, and the Pima County Health Department. Neighborhood associations in Tucson were surveyed for their perceptions of the magnitude of the mosquito problem in their areas. On the basis of survey results, the city was divided into regions: north, east, south, west, and central. Four trapping stations were established in each region for a total of 20 sites spanning the metropolitan Tucson and outlying areas. The five regions were surveyed for mosquitoes through use of CO_2 traps approximately every 10 days starting July 1, 1997; traps were set in the late afternoon and collected in the late morning. Daytime CO_2 trappings were not effective.

The Entomology Department's 1997 surveillance data suggest that the central part of the city is the most heavily infested. Of 95 adult *Ae. aegypti* trapped, 49.5% were from the central region of Tucson, 18.9% from the west side, 17.9% from the east side, 10.5% from the north side, and 3.2% from the south side. The mosquito populations appeared to fluctuate with the weather, increasing in size after rainfall. Long-term trapping and future larval surveys should shed more light on this association.

Pima County and the University of Arizona Veterinary Science Department trapping activities in 1997 also produced evidence of *Ae. aegypti* in two communities near Tucson. Six adult mosquitoes were recovered in the town of Benson, 30 miles southeast of Tucson, and seven were trapped in Sahuarita Heights, 15 miles south of Tucson. The presence of the mosquitoes in these communities, as well as in Douglas, Naco, and Nogales, demonstrates that Arizona's smaller desert communities are also susceptible to *Ae. aegypti* infestations. The humidity emitted by home evaporative coolers may be crucial for the survival of tropical mosquitoes, such as *Ae. aegypti,* in Arizona's arid climate (N. Monteny, pers. comm.).

Genetic analysis of the *Ae. aegypti* collected from southeastern Arizona, Texas, and Mexico is under way at the University of Arizona Ecology and Evolutionary Biology Department to determine the structure, history, and origin(s) of the reemergent mosquito populations. Preliminary findings from mitochondrial DNA sequences suggest that *Ae. aegypti* in Arizona represent a single (panmictic) population, which

indicates frequent local migration. More extensive sampling is necessary to confirm these results and determine a point of origin.

A community outreach program has been developed to inform the public about *Ae. aegypti* breeding and control in Tucson. Public involvement will be a key factor in the control of these urban breeders. Major emphasis will also be placed on programs for children and teachers as both groups can be instrumental in maintaining long-term interest in this problem. As these programs are developed, they can be expanded and amended to meet the needs of other infested communities in southern Arizona. A mosquito control abatement district is under consideration in a central part of Tucson. The primary purpose of this district would be to provide approximately 10,000 homeowners with information on controlling *Ae. aegypti* breeding on their property.

Just how long the *Ae. aegypti* infestation will last is difficult to assess. Records of the city's earlier infestation indicate the mosquito was present for at least a 15-year period (1931 to 1946) (1,3,4). Since their identification in early 1998 summer mosquito samples from Tucson, adult *Ae. aegypti* have been part of the city's local environment for at least 5 consecutive years (1994 to 1998). Their continued presence and the abundant breeding habitat provided by the expansion of Tucson's urban landscape suggest that *Ae. aegypti* could survive for an extended period.

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Can the Military Contribute to Global Surveillance and Control of Infectious Diseases?

To the Editor: Numerous networks—both formal (e.g., Ministries of Health and WHO Collaborating Centers and collaborating laboratories) and informal (e.g., nongovernmental and humanitarian organizations, the media, and electronic discussion groups)—contribute to WHO's network of networks for the global surveillance of infectious diseases (1).

A potential source of additional information on infectious diseases is the network of military health facilities and laboratories throughout the world. In addition to health facilities serving populations at high risk for infectious diseases, the military also has laboratories, often among the better-equipped, in developing countries. To evaluate the feasibility and potential usefulness of including military laboratories in the WHO global surveillance network, we conducted three surveys.

The first survey identified military laboratories willing to participate in global surveillance activities and obtained information about their infectious diseases reporting systems. Of the 107 countries surveyed, 76 replied. Among them, 53 (70%) reported having a central military laboratory that coordinates laboratory activities throughout the military, and 62 (82%) reported that military clinical facilities had a reporting system for infectious diseases.

The second survey quantified laboratory capabilities in the 53 laboratories identified in the first survey and obtained details about the 62 reporting systems. Among the 39 (74%) laboratories that replied, all can perform at least one of the following activities: isolating and identifying bacterial, viral, or parasitic agents. Twenty-nine (55%) have the capacity for specialized immunologic or molecular study. In addition, one of these laboratories has a biosafety level 4 facility, six have a biosafety level 3 facility, and 10 have a biosafety level 2 facility. Twenty-seven (51%) of the laboratories perform compulsory screening of new recruits for HIV, 17 (33%) for hepatitis B, 7 (13%) for hepatitis C, 39 (74%) for tuberculosis, 35 (67%) for syphilis, 18 (34%) for intestinal parasites, 13 (25%) for schistosomiasis, 12 (23%) for malaria, and 2 (4%) for Chagas disease.

Among the 54 reporting systems for which further information was obtained, clinical diagnoses (in some countries laboratory confirmed) are reported through the hierarchical chain, normally by mail or facsimile, but in two countries by electronic links. Almost all military reporting systems are parallel to civilian systems. Thirty-four (63%) of 54 systems feed into the civilian system, with a built-in mechanism to avoid duplicate reporting; 16 (30%) systems feeding into the civilian system have no such mechanism in place; and four have no link with the civilian system.

The third survey addressed vaccination policies. Among 52 countries that replied, 47 (90%) have a compulsory military vaccination schedule: 45 (87%) for tetanus, 30 (58%) for diphtheria, 23 (44%) for typhoid, 16 (31%) for bacillus Calmette-Guérin and polio, 12 (23%) for meningococcal meningitis, and 10 (19%) for measles, mumps, and rubella.

These surveys show that military populations are protected against many infectious diseases and that a wealth of information is obtained by military laboratories and health-care facilities on populations at high risk for infectious diseases. While most of the information collected from the health-care facilities is reported through civilian systems as well, incorporating the military network of laboratories into the WHO global surveillance network could ensure broader coverage.

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Dual Infection with *Ehrlichia chaffeensis* and a Spotted Fever Group Rickettsia: A Case Report

To the Editor: In their article, Daniel J. Sexton et al. state, "Well- documented cases of simultaneous human infections with more than one tick-borne pathogen are rare" (1) and mention only two reports of such cases. However, another report should be mentioned because of its historical interest and the lessons it may teach.

In 1900 to 1905, in the Bitter Root Valley, a tick-borne disease emerged, which became known as Rocky Mountain spotted fever. Although Ricketts et al. later published a report (2), which identified the causative agent, in 1904 L.B. Chowning and W.M. Wilson published Studies on *Pyroplasma hominis* (3). They reported finding *Pyroplasma* (since changed to *Babesia)* in the blood of approximately 20 patients with spotted fever. They studied this organism in detail and even found the reservoir for it in the local rodent species. Wilson et al. thought that the organism was the causative agent of spotted fever. On the basis of their excellent plates and descriptions, it is clear that the organism they were describing was what we later came to know as *Babesia microti.*

The work of Wilson and Chowning was ignored and forgotten for many years. They had incorrectly concluded that spotted fever was caused by a parasite. For many years it was "well known" that *Babesia* infections became apparent in human patients only on removal or inactivation of the spleen. That persons with functional spleens were subject to infection with *B. microti* was finally established by the so-called Nantucket outbreak (4) and subsequent publications.

Therefore, Wilson and Chowning's work reports several cases of simultaneous infections of humans by two tickborne pathogens; i.e., patients had spotted fever and *B. microti* in the blood. More poignant was that an "emerging" disease of humans was missed and not discovered again for some 70 years.

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Dual Infection with *Ehrlichia chaffeensis* and a Spotted Fever Group Rickettsia: A Case Report—Reply to Dr. Sulzer

To the Editor: Several investigators have suggested that some of Wilson and Chowning's patients may have had coinfection with *Babesia* and *Rickettsia rickettsii* (1-4). Furthermore, the organisms that Wilson and Chowning observed in red cells of 20% of the local Columbian ground squirrels are consistent with later reports of various species of *Babesia* in the erythrocytes of other species of squirrels (4). However, most rickettsiologists who have commented on Wilson and Chowning's paper have concluded that intraerythrocytic organisms observed in blood samples did not contribute substantially to the illnesses of the 23 patients described. Although Stiles, Wenyon, and Brumpt concluded that the organisms in human blood samples observed by Wilson and Chowning were artifacts or malarial parasites (5-7), contemporary experts who have reviewed the colored plates that accompanied Wilson and Chowning's 1904 paper believe that there is "little" or "no doubt" that Wilson and Chowning actually described organisms of the genus *Babesia* (1,2,8).

In a commentary that followed the republication of Wilson and Chowning's landmark paper in 1979 (9), Richard Ormsbee reviewed the sequence of events that followed the publication of Wilson and Chowning's report in 1904 (10). After more than 200 hours of careful microscopy, C.W. Stiles could find no evidence of *Pyroplasma* in the blood of 12 patients with Rocky Mountain spotted fever (RMSF). He refuted Wilson and Chowning's findings (5) and challenged Chowning, who was also in the Bitter Root Valley, to demonstrate the presence of organisms in the blood of a person with a typical case of RMSF. Chowning was unable to find *Pyroplasma* in blood smears from these patients (10). Ricketts did not arrive in the Bitter Root Valley to begin his studies of RMSF until 1906 (11); thus he could not have published his classic paper on the etiology of RMSF in volume 1 of the Journal of Infectious Diseases.

To our knowledge, ecologic studies done in the Bitter Root Valley have not demonstrated endemic foci of babesial infection. A serologic survey of 246 Bitter Root Valley residents in 1978 showed no antibabesial antibodies (12). Although it is possible that 4 of the 23 patients with RMSF described by Wilson and Chowning had incidental preexisting latent babesial infection, the clinical and autopsy data they presented suggest that the patients had typical *R. rickettsii* infection. There is no proof that any of the patients described by Wilson and Chowning had simultaneous acute babesial and rickettsial infection, and we agree with Ormsbee that the significance of the "*Pyroplasma hominis*" described in the blood smears of several of Wilson and Chowning's patients is "... a mystery that persists to this day" (10).

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Reemergence of *Plasmodium vivax* Malaria in the Republic of Korea

To the Editor: In "Reemergence of *Plasmodium vivax* malaria in the Republic of Korea" (1), the term eradication was, in my judgment, inappropriately used. In 1981, Yekutiel proposed that eradication is "The purposeful reduction of specific disease prevalence to the point of continued absence of transmission within a specified area by means of a time limited campaign" (2). In 1984, Hinman proposed an important addition that eradication must have followed a "deliberate effort" (3). At the Dahlem Workshop in 1997 (4), a more comprehensive definition was proposed. This definition states that eradication is "Permanent reduction to zero of the worldwide incidence of infection caused by a specific agent as a result of deliberate efforts; intervention measures are no longer needed" (4). At the same conference, two other terms were also defined. Elimination of disease: "Reduction to zero of the incidence of a specified disease in a defined geographic area as a result of deliberate efforts; continued intervention measures are required." Elimination of infection: "Reduction to zero of the incidence of infection caused by a specific agent in a defined geographic area as a result of deliberate efforts; continued measures to prevent reestablishment of transmission are required."

These definitions promote unanimity in using the term eradication and avoid misconceptions over accomplishments.

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Paratyphoid Fever

To the Editor: The letter on paratyphoid fever by Kapil et al. (1) stated that an outbreak of enteric fever due to *Salmonella paratyphi* A has never been reported. A large (227 cases) outbreak of enteric fever secondary to *S. paratyphi* A occurred in the Arabian Gulf nation of Bahrain in 1987. The clinical and epidemiologic details of the outbreak were reported in a local medical society journal (2). Like the outbreak described by Kapil et al., the Bahraini outbreak was associated with sewage leaking into the water supply.

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Hospitalizations After the Persian Gulf War

To the Editor: Knoke et al., Naval Health Research Center, San Diego, California, published two articles on military hospitalizations in Persian Gulf War veterans, the most recent in Emerging Infectious Diseases (1,2).

Although the titles of both articles indicated general hospitalizations, Knoke et al. studied just military hospitalizations among selected, mostly healthy, active-duty Persian Gulf War veterans enlisted as of 1994. They compared military hospitalizations of active-duty Gulf War veterans (cases) with military hospitalizations of active-duty era veterans not in the Persian Gulf between 1990 and 1991 (controls). "Healthy warrior" effects would have predicted low military hospitalization rates for both cases and control populations (3), but both were high.

The studies were "restricted to active-duty personnel" hospitalized in military facilities because active-duty personnel were "rarely hospitalized outside of DoD facilities" (1). However, of 150 surgical procedures, mostly

intestinal and skin biopsies, performed on 85 sick active-duty and reservist Persian Gulf veterans from Pennsylvania between 1991 and 1995, more than one third, 58 (39%), were performed in private facilities (4). Most of the federal procedures were done in Veterans Administration (VA), not military, hospitals. Many active-duty, sick Persian Gulf veterans in Pennsylvania, Texas, and California deliberately avoided military, and some VA, hospitals between 1991 and 1997 because of concerns about competence, convenience, confidentiality, and career opportunities during this era of downsizing and closing of military bases (3,5).

In addition, Knoke et al. excluded at least five groups of sick veterans from their limited case studies: 1) those treated in VA and private hospitals, 2) those from the Reserves and National Guard, 3) those who retired early largely because of illness, 4) those who consented to long military hospitalizations within the DoD Comprehensive Clinical Evaluation Program (CCEP) for Gulf War Veterans, and 5) those who had obstetric complications after returning home from the Gulf War. Thus, many sick veterans were excluded from the case studies.

If we hypothesize that one or more new infectious agents like *Leishmania tropica, Brucella* species, *Bacillus anthracis, Mycoplasma fermentens (incognitus), Coxiella burnetti,* or obscure fungi or molds might be involved, comprehensive research studies in the future would do better to include all workers from the Arabian desert, reservists as well as active-duty personnel.

Few Gulf veterans with Gulf-related illnesses were welcomed by military hospitals and about half of 452 Persian Gulf veterans surveyed by the U.S. General Accounting Office sought health care outside the VA for health problems they believed were related to service in the Persian Gulf (5). An alternative interpretation of Knoke's hospitalization study might be that admitting officers in military facilities prevented sick Persian Gulf War veterans from obtaining medical care within their facilities.

Not only were the case populations studied unusual; recent workers and travelers to the Middle East were not excluded from the control population. "Nondeployed" controls included recently deployed Persian Gulf military personnel as long as nondeployed personnel worked in the Gulf after 1991. Some of those late-deployed Persian Gulf workers also fell ill with the same illnesses as veterans deployed between August 1990 and 1991. Illnesses from late Persian Gulf deployments might explain excess hospitalizations seen in nondeployed controls. All late-deployed personnel from the Middle East should also have been excluded from the nondeployed control population.

Finally, medical ICD-9 diagnoses, while interesting, were incomplete and nonspecific. Medical diagnoses common to Gulf veterans should have been listed in addition to unexplained illnesses. Knoke's condensed diagnostic list, like patient charts we have seen from DoD hospitalizations, may have failed to capture common clinical and laboratory abnormalities seen in many sick Gulf veterans, including (but are not limited to) ulcerative colitis, Crohn colitis, inflammatory bowel disease, intestinal bleeding due to inflammatory colonic polyps, skin acne, nodules, plaques, psoriasiform skin rashes, nose ulcers, nose bleeds, leukocytosis, neutropenia, elevated alanine transaminase (SGPT/ALT) liver enzymes, hepatosplenomegaly, thrombocytopenia, nephrolithiasis (kidney stones), and fevers of unknown origin (4,6). In addition, more than one unexplained illness category should have been tabulated per patient, because "Gulf War Syndrome" is a multisystem illness (4,6-9).

More research is needed on hospitalizations in addition to deaths and new diseases found in Persian Gulf War veterans (3). Civilian scientists and physicians must collaborate closely with other diverse federal and nonprofit organizations to study Gulf War illnesses objectively (5,9). The health problems seen in Gulf War veterans may be part of a new complex of emerging desert-associated illnesses (9-14).

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Hospitalizations after the Gulf War—Reply to K.M. Leisure et al.

To the Editor: We studied all active-duty Persian Gulf War–era veterans who remained on active duty at the conclusion of deployment (July 31, 1991), not as Leisure et al. stated in their letter "selected, mostly healthy, active-duty Persian Gulf War veterans enlisted as of 1994."

Our study was restricted to hospitalizations of active-duty service members because these were the only service members whose records were available on computerized files. No one was excluded from the defined target population. However, there are "sick Gulf War veterans" and healthy Gulf War veterans not in the target population. The difficulty is in studying either a random sample or the entire population of Gulf War veterans. The only published study we know of the entire population is the mortality report of Kang and Bullman (1).

The suggestion that we should have excluded from the control group service members who had ever been in the Gulf War area would have been appropriate for a report of exposure to the Persian Gulf region; ours was a report of exposure to the Persian Gulf War. That we should have studied a different collection of ICD-9 diagnoses also suggests a different report.

While our study may have limitations, we have not seen objective data that support the anecdotal observations of Leisure et al.

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HIV Nursing and Symptom Management. ME Ropka, AB Williams, Editors. Jones and Bartlett Publishers, Sudbury, Massachusetts, 1998, 806 pages.

The need for updated texts on nursing care of patients with HIV is critical for many reasons: the rapidly expanding availability of new medications and need for adherence to complex therapeutic regimens, the requirements of opportunistic infection prophylaxis, the broad constellation of symptoms caused by many opportunistic diseases from early HIV infection through long-term end-stage care, and the vulnerable populations involved. This volume edited by nurses from the University of Virginia School of Medicine and the Yale University School of Nursing gives a cogent overview of these topics, broken into four overarching units.

Unit one provides introductory information on HIV infection including disease pathogenesis and epidemiology, related opportunistic infections and malignancies, and pharmacologic therapy. The second unit, the strongest of this volume, reviews the nursing management of common clinical problems such as neurologic manifestations, nutrition-related changes, fecal incontinence, respiratory changes, psychosocial problems, and pain management. The third unit, HIV Special Treatment Considerations, addresses adherence to therapy, continuity of care, and ethical and legal issues. Unit four gives guidance for approaching HIV care in some of the diverse populations particularly affected by the HIV epidemic: women, children, substance abusers, Hispanics, and African-Americans.

This volume, which contains more than 100 well-organized explanatory tables and figures, provides a good overview of essential topics and allows easy retrieval of information on nursing care as well as basic epidemiology and pathogenesis of frequently encountered HIV-related conditions. HIV Nursing and Symptom Management provides comprehensive background information and practical clinical guidance for nurses in HIV patient care or research.

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Meeting Summary

Overseas Medical Laboratory Conference

Military Infectious Disease Research Program, U.S. Army Medical Research and Development Command, Ft. Detrick, Maryland, USA

The Military Infectious Disease Research Program, U.S. Army Medical Research and Materiel Command, and the Walter Reed Army Institute of Research (WRAIR) hosted a workshop on overseas activities in infectious disease research in December 1997, at WRAIR, Washington, D.C. Approximately 150 Army and Navy medical research scientists met to discuss progress toward specific goals designed to produce products and information applicable to protection of military personnel in the field.

The Department of Defense has developed a network of six overseas laboratories that perform research on infectious diseases of public health and military importance (Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand; the Naval Medical Research Units No. 2 in Jakarta, Indonesia, and No. 3 in Cairo, Egypt; the U.S. Army Medical Research Units in Nairobi, Kenya, and Rio de Janeiro, Brazil; and the Naval Medical Research Institute Detachment in Lima, Peru). The combination of laboratories in the United States (WRAIR, U.S. Army Institute for Infectious Diseases, and the Naval Medical Research Institute) and overseas forms an effective international infectious diseases research program, in which domestic laboratories are positioned to maintain contact with academia and other branches of the government and overseas laboratories detect new trends in naturally occurring endemic and epidemic infectious diseases of potential military relevance (through their own surveillance or close contact with foreign health authorities and conducting risk assessment). The overseas laboratories also conduct trials of new products for disease prevention and control.

For more information on the workshop, contact LTC Daniel Strickman, U.S. Army Medical Research and Development Command, (Attn: MCMR-PLA), 504 Scott Street, Fort Detrick, MD 21702-5012, USA; tel: 301-619-7567; fax: 301-619-2416; e-mail: COL_Charles_Hoke@ftdetrck-ccmail.army.mil.