EMERGING INFECTIOUS DISEASES

Tracking trends and analyzing new and reemerging infectious disease issues around the world

A peer-reviewed journal published by the National Center for Infectious Diseases Vol. 4 No. 2, April–June 1998

Gulf War Illness New Paramyxovirus New Vectors in Africa Update on Malaysia

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service

CDC
CENTERS FOR DISEASE CONTROL AND PREVENTION

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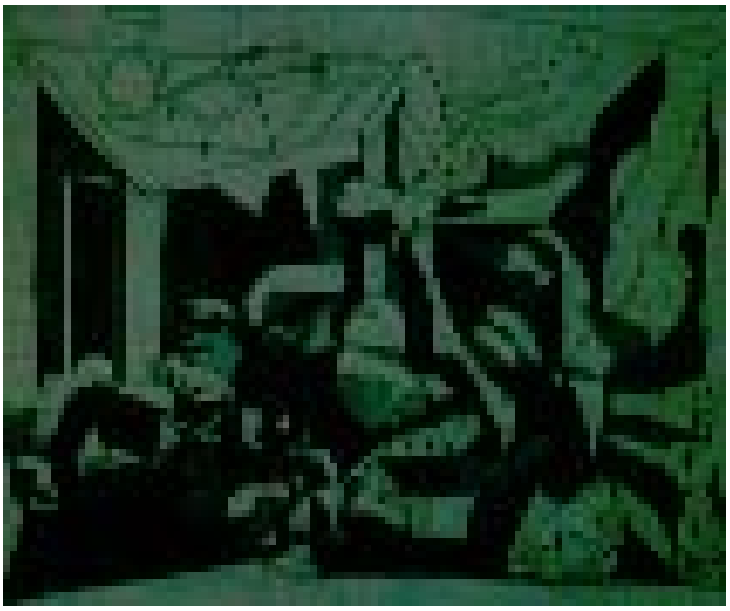

Left: Pablo Picasso. The Charnel House. Paris (1944–45; dated 1945). Oil on charcoal on canvas, 6' 6 5/8" x 8' 2 1/2" (199.8 x 250.1 cm). The Museum of Modern Art, New York. Mrs. Sam A. Lewisohn Bequest (by exchange) and Mrs. Marya Bernard Fund in memory of her husband Dr. Bernard Bernard and anonymous funds. Photograph © 1998 The Museum of Modern Art, New York.

Cover: Batik painting, "Freedom of Childhood," by Malaysian Chinese artist Kuan Kee Peng. Mr. Kuan works as a medical laboratory technologist at the University of Malaya, Kuala Lumpur, Malaysia. The painting was used in the 1976 UNICEF Engagement Calendar.

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International Editors

Emerging Infectious Diseases—Southeast Asia

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The recent emergence of a new strain (H5N1) of influenza A virus, the so-called avian or bird flu, in Hong Kong underlines the importance of the Southeast Asia region as an epicenter not only for influenza A viruses but also for other microbial agents. In late 1992, *Vibrio cholerae* O139 appeared on the Indian subcontinent; and within a few months, it had spread to China, Nepal, Pakistan, Malaysia, and as far as Russia.

The World Health Organization (WHO) has reported that infectious diseases account for more than 17 million deaths per year worldwide and that at least 30 new infectious diseases have emerged within the last 2 decades. Up to half of the 5.8 billion people on earth are at risk for many endemic diseases, with the most overpopulated and economically depressed countries in Southeast Asia at highest risk. Although vaccines and antibiotics are available for many diseases, in 1995 alone, respiratory infections such as pneumonia killed 4.4 million people, 4 million of them children. Diarrheal diseases, including cholera, typhoid, and dysentery killed 3.1 million, most of them children. Tuberculosis (TB) killed almost 3.1 million, malaria 2.1 million, hepatitis B more than 1.1 million, and measles more than 1 million.

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Faced with the reality of infectious diseases on a daily basis, Southeast Asian nations have not been able to give emerging disease surveillance the priority status it deserves. Much of the surveillance in the region is centered in a few well-established laboratories where there is adequate expertise, sufficient funding, and personal interest. Because of influenza A, it is widely believed that Asia is the epicenter of new influenza strains; the emergence of the avian influenza strain in Hong Kong supports this contention. As a result of the activities of the Pacific Basin Respiratory Virus Research Group, an excellent surveillance program for respiratory viral infections is in place. If this small cluster of avian flu had occurred elsewhere, it would most likely have been missed. All the 110 WHO Influenza Centers, particularly those in Southeast Asia, have been alerted and are frequently updated about the Hong Kong outbreak. Diagnostic reagents for the identification of the H5N1 strain will be made available to these centers as part of the surveillance program.

Enteroviruses

Enteroviruses are frequently associated with the hot and humid climate of tropical countries in Southeast Asia. The viruses are responsible mainly for inapparent and mild infection, but occasionally they can give rise to infection of the central nervous system, resulting in aseptic meningitis and (rarely) paralysis; polioviruses are a good example. Hand, foot, and mouth disease, caused by enteroviruses, such as coxsackie A16 and enterovirus 71, is common in many countries in the region. In a recent outbreak of hand, foot, and mouth disease in Malaysia, a new clinical entity of encephalomyelitis emerged, which resulted in several deaths among children under 5 years of age. Four such deaths were investigated thoroughly in the University Hospital in Kuala Lumpur. All four children were seen in the Emergency Department in a state of respiratory and cardiovascular instability with a history of fever (3 to 5 days) associated with reduced oral intake. One child had flaccid paralysis with hyporeflexia of the lower limbs. A constant feature in these patients was the very rapid vascular changes, followed rapidly by cardiac decompensation. Pulmonary

edema followed, suggesting a concomitant increase in pulmonary vascular permeability. Consent for postmortem was obtained with some difficulty because of religious and cultural reasons. The midbrain, pons, medulla, and spinal cord were extensively damaged, with the worst damage at the level of the medulla. The cerebrum and myocardium were relatively normal. Enterovirus 71 was isolated from the medulla and spinal cord of three children and from the cerebrum of the other child, where no brain stem material was available. Other tissue sites including the cerebrospinal fluid did not yield virus. The need for postmortem examinations in investigating fatal emerging diseases must be addressed in areas where such examinations are not possible for cultural reasons.

Arboviruses

Arthropod-borne viruses or arboviruses are yet another group of viruses synonymous with countries in the region and cause considerable sickness and death. Some of these viruses, e.g., Murray Valley encephalitis virus in Australia, have restricted geographic distribution but may have the ability to transcend geographical barriers, as in the emergence of Japanese encephalitis virus in the Torres Strait of northern Australia and in Papua New Guinea.

Dengue

Dengue is by far the most important arbovirus infection in Southeast Asia. According to WHO, dengue has been reported in over 100 countries worldwide and poses a threat to approximately 2 billion people. At the 46th World Health Assembly held in Geneva in May 1993, a resolution was passed to make the prevention and control of dengue a priority.

Dengue is an acute viral infection characterized by abrupt onset of fever, severe headache, pain behind the eyes, muscle and joint pains, and rash. The hemorrhagic form of dengue fever, dengue hemorrhagic fever (DHF), was recognized as a new disease in the Philippines in 1953 and has been seen in India, Malaysia, Singapore, Indonesia, Vietnam, Cambodia, and Sri Lanka. During 1956 to 1992, 1,335,049 cases of DHF (13,723 deaths) were recorded in Vietnam alone. The rise of dengue in tropical and subtropical areas of the world is explained by factors such as rapid population growth, expanding urbanization, inadequate municipal water supplies, and difficulties in refuse disposal. These lead to an abundance of new breeding sites for the mosquito vectors, while human migration patterns disperse vectors and viruses into new areas.

The resurgence of DHF in several countries where only dengue fever had been reported has become worrisome. Sri Lanka reported its first outbreak in 1989, and India and China also faced the same dilemma. DHF/dengue shock syndrome has also been reported for the first time in New Caledonia and Tahiti. The large outbreak in Cuba in 1981 showed how the disease has spread to the Americas and other countries such as Venezuela and Brazil. In Malaysia, where the disease is endemic, encephalitis cases associated with dengue viruses have been documented, and vertical transmission has also been reported in the last two years. Other unusual manifestations observed rarely include acute renal failure and hemolytic uremic syndrome. The emergence of such unusual clinical manifestations should also be monitored and documented in other dengue-endemic countries. Despite intensive efforts by the countries in the region to control the vectors, the disease is still on the rise. A tetravalent live attenuated vaccine developed at Mahidol University, Thailand, is being field-tested, and the preliminary results are promising.

Disease Spread

Rapid transportation has been blamed for the spread of diseases, and this is especially so in countries in the region. Tourism is an important industry in Southeast Asia, and a series of conferences on travel medicine has highlighted the problems, particularly in infectious diseases. Another vast movement of people between countries in the region is that of migrant workers. In Malaysia, an estimated 1.7 million workers are migrant workers, of which only 1.2 million came in legally. Most of the workers are from Indonesia, Myanmar, Philippines, and Pakistan, and they work in the agricultural sector as well as in construction and domestic services. Those who come in legally undergo a medical examination that includes screening for some infectious diseases. Diseases associated with migrant workers are chloramphenicol-resistant typhoid, multidrug-resistant TB, leprosy, malaria, HIV/AIDS, and filariasis. In 1993, the first case of kala-azar caused by *Leishmania donovani* was reported in a Bangladeshi migrant worker in Malaysia, and since then, four other cases have been identified, all

of them in Bangladeshi workers. This disease had not been present in Malaysia.

In 1997, the theme "Emerging Infectious Diseases: Global Alert—Global Response" was chosen by WHO to celebrate World Health Day. This year, as WHO celebrates its 50th anniversary, the region is still plagued with major public health problems threatening the lives of millions of people in the region. These problems underline the need to strengthen epidemiologic surveillance and concentrate public health activities in Southeast Asia, the epicenter of many emerging diseases.

Wild Primate Populations in Emerging Infectious Disease Research: The Missing Link?

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Wild primate populations, an unexplored source of information regarding emerging infectious disease, may hold valuable clues to the origins and evolution of some important pathogens. Primates can act as reservoirs for human pathogens. As members of biologically diverse habitats, they serve as sentinels for surveillance of emerging pathogens and provide models for basic research on natural transmission dynamics. Since emerging infectious diseases also pose serious threats to endangered and threatened primate species, studies of these diseases in primate populations can benefit conservation efforts and may provide the missing link between laboratory studies and the well-recognized needs of early disease detection, identification, and surveillance.

Infectious diseases respect no species or geographic boundaries. For a parasite, closely related hosts offer new environments in which infection, maintenance, replication, and transmission remain possible. The anthropoid primates (which include humans) and to a lesser degree simian primates share broadly similar physiologic and genetic characteristics and thus susceptibility to many viruses, bacteria, fungi, protozoa, helminths, and ectoparasites (1) that have the potential to cross primate-species boundaries (2).

Similarities in pathogen susceptibility have made nonhuman primates ideal laboratory models. During the 20th century, laboratory research on captive primates has elucidated the life cycle and pathogenesis of many infectious agents and facilitated drug and vaccine development. Nevertheless, the ecology of infectious agents found in wild populations of primates has only recently been addressed. Just as captive primates have proved invaluable for research at the level of the organism, wild populations can provide the opportunity to study infectious disease phenomena at the population and ecosystem levels. Research at these levels addresses such pressing questions as the origin(s) of pathogens, determinants of pathogen emergence, and factors influencing maintenance of pathogens in animal reservoirs.

During the past two decades unknown human diseases, including AIDS, Ebola fever, hantavirus infection, and dengue hemorrhagic fever, have emerged from enzootic foci. The emergence of these and other diseases has been linked to the interface of tropical forest communities with high levels of biodiversity and agricultural communities with relative genetic homogeneity and high population densities of humans, domestic animals, and crops. This interface poses a high risk for the emergence of novel disease (3-5).

Since most nonhuman primates live in tropical forest habitats, most interactions between humans and wild nonhuman primates occur in this high-risk interface, which has recently increased because of expanded ecotourism and forest encroachment. These interactions can lead to pathogen exchange through various

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routes of transmission (Table). Arthropod vectors, shared water, and hunting of wild animals have facilitated pathogen exchange and may have played an important role in pathogen transfers since ancient times. In the recent past, laboratory research has led to accidental human exposure to such agents as primate malaria parasites (12) and a simian immunodeficiency virus (SIV) (16). The potential for exchange through xenotransplantation has been discussed (17), and infection from vaccine contaminated with SV40, a primate papovavirus, led to the exposure of millions of persons in the 1950s (15). Conversely, pathogen transmission from humans to nonhuman primates places both captive and wild animals at serious risk for diseases such as measles and tuberculosis (TB), which are deadly in many nonhuman primate species. In general, as levels of interaction increase, so does pathogen exchange, resulting in further risks to both humans and nonhuman primates.

The need for improved surveillance and basic research on emerging infectious disease is well documented (3,18-20). Wild primates can serve as sentinels by signaling which pathogens pose a risk for humans in the immediate area (21) as well as in distant countries (5). Here, we describe potential benefits of incorporating wild populations into emerging infectious disease research.

Pathogen Origins

Nonhuman primates are infected with the closest relatives of important human pathogens. The construction of molecular phylogenies has played an important role in studying the evolution and classification of many pathogens. While trees that result from these analyses must be interpreted with care, they can provide valuable information on the history of pathogens. Furthermore, the selection of genes that are evolving at the appropriate rate should allow phylogenetic analyses to assess both ancient and more recent epidemic origins (22).

Human herpes simplex virus infection is common in nonhuman primates and may reflect contact with humans. A study of viral infection in nonhuman primates found that chimpanzees and gorillas were seropositive to human herpes simplex virus-1 and -2 strains, but orangutans and gibbons were not (23).

Research on SIV phylogeny (24,25) has shown that HIV-1 and HIV-2 are each more closely related to primate pathogens than they are to one another. HIV-1 is in a group with SIV_{CPZ} (26), a chimpanzee (*Pan troglodytes*) virus, while HIV-2 falls within a clade consisting of West African primate viruses (Figure 1; 24). In fact, Mindell (27) has argued that HIVs and SIVs should be referred to as 'primate immunodeficiency viruses' to more accurately reflect their heritage. Preliminary evidence suggests that the origins of global T-cell lymphotropic virus-1 subtype diversity may be analogous, leading Liu et al. (28) to propose that HTLV-I subtypes emerged from three separate nonhuman primate reservoirs.

This pattern is not unique to viruses, as demonstrated through decades of research on primate malarias. More than 26 species of *Plasmodia* infect primates (12,29). Both morphologic and molecular analyses show human and

Table. Routes of pathogen exchange between human and nonhuman primates

Route of exchange	Pathogen	Direction of exchange	Evidence[a]	Reference
Animal bite	Herpes B	Nonhuman primate to human	E	6[b]
	Monkeypox	Nonhuman primate to human	E	7
Fecal-oral	Poliovirus	Human to nonhuman primate	L	2[b]
	Poliovirus	Chimpanzee to chimpanzee	E	8
Hunting, food prep & eating	Ebola	Nonhuman primate to human	E	9
Nasal secretions	*Mycobacterium leprae*	Among primates	P, L	10[b]
Respiratory droplet	Tuberculosis	Human to nonhuman primate	L	11[b]
Vector-borne	Malaria	Both directions	L,E	12[b]
	Filaria	Both directions	L,E	8[b]
Water-mediated	Dracunculiasis	Human to nonhuman primate	L	13
	Schistosomiasis	Nonhuman primate to human	E	14
Xenotransplantation	SV40	Nonhuman primate to human	E[c]	15[b]

[a]L = laboratory; E = epidemiologic ; P = evidence that parasites live naturally in multiple primate hosts.
[b]Evidence reviewed.
[c]The only current evidence for xenotransplantation includes SV40 spread through vaccine production.

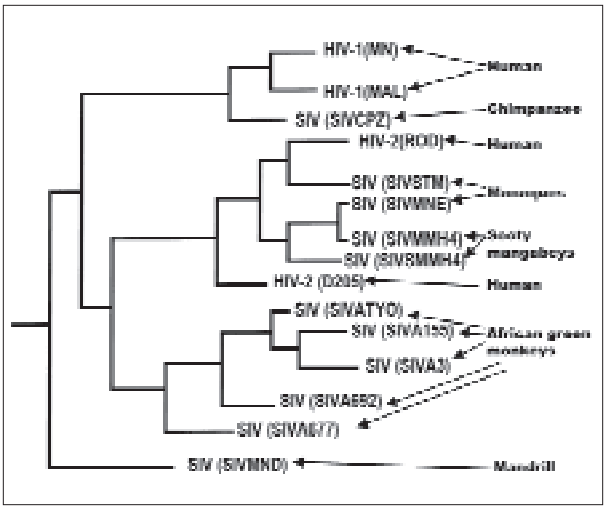

Figure 1. Relationships among primate and human lentiviruses: Phylogeny of primate lentiviruses based on the gag gene obtained by (25). The names of the strains are indicated in parentheses. Hosts are indicated on trees. The description of the lentivirus strains is provided in (25).

nonhuman primate malarias interdigitating on phylogenetic trees (Figure 2; 30,33). Preliminary evidence suggests that this group of parasites has a range of coevolutionary scenarios, including the speciation of *P. vivax* and related parasites in Asian primates, the recent exchange of parasites between humans and New World monkeys (30), and perhaps an ancient exchange of a *falciparum*-like parasite from a bird or lizard to an African hominoid (31,34,35). Further analyses are likely to lead to surprises. For example, recent research has shown that the diversity of human *P. vivax* (previously considered to be a single species) also includes a *P. vivax*-like parasite, a widespread pathogen, which is most closely related to *P. simiovale*, a primate malaria of Asian macaques (*Macaca* sp.) (30,36).

A major limitation of these studies is that they rely on very small numbers of nonhuman primate isolates—only two SIV_{cpz} isolates for the HIV-1 clade, a single *P. simiovale* isolate for malaria phylogeny. In addition, very few data are available on the distribution of these pathogens in wild populations; more data on the distribution and extent of shared pathogens should provide clues to origins (37), thereby pointing to general conditions that may contribute to disease emergence.

Molecular phylogenies can also play a direct role in the control of pathogens, for example, in charting their antigenic diversity, a task necessary for vaccine design (38). Because nonhuman primate pathogens are often evolutionary outgroups, vaccines that are targeted at antigens shared by human and nonhuman pathogens should provide more universal coverage. Molecular phylogenies can also assist in determining the rate of evolution of vaccine candidate antigens. Because low rates of mutation often indicate selective constraint, this technique may point to candidate antigens that cannot easily mutate to evade vaccine-induced antibodies. Similar analyses identifying highly constrained gene products or metabolic pathways as targets for drug development may contribute to slowing the emergence of drug resistance.

Reservoirs

The study of pathogen transmission has been encumbered by the use of inappropriate

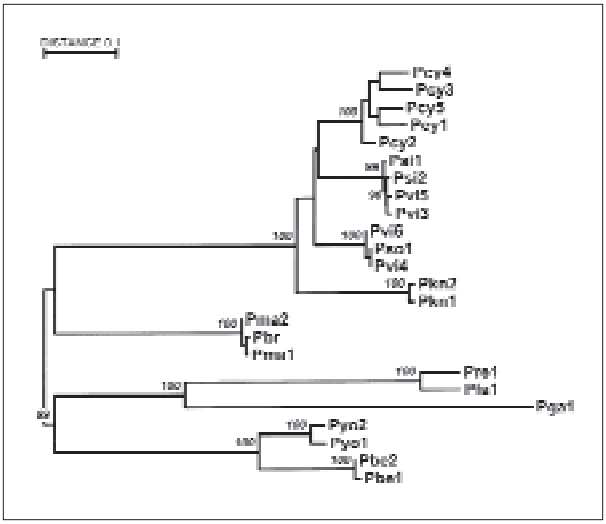

Figure 2. Relationships between primate and human parasites: Malaria phylogeny based on the circumsporozoite protein gene. The alignment does not include the central repeat region. *P. falciparum* (Pfa), *P. vivax* (Pvi), and *P. malariae* (Pma) are from humans; *P. cynomolgi* (Pcy), *P. simiovale* (Pso), and *P. knowlesi* (Pkn) are from macaques; *P. simium* (Psi) and *P. brasilianum* (Pbr) are from New World monkeys; *P. reichenowi* (Pre) is from chimpanzees; *P. gallinacium* (Pga) is from birds; and *P. berghei* (Pbe) and *P. yoelii* (Pyo) are from rodents. The numbers in the names indicate different isolates as described in (30). The sequence of *P. gallinacium* was reported by (31). The numbers on the branches are bootstrap % based on 500 pseudoreplications. The tree was estimated by the neighbor-joining method with the Tajima and Nei distance (32).

terminology. Such terms as "primary hosts," "reservoir hosts," "carriers," "arthroponoses," "xenonoses," and "zoonoses" pose premature assumptions and belie aspects of the origins and natural transmission cycles of pathogens. The factors that determine pathogen viability within a vertebrate host may correspond only roughly, or not at all, to species boundaries. Primate pathogens do not adhere to the faithfully maintained sanctity of the distinction between humans and nonhumans.

The misconception of an evolutionary trend toward increasing host specificity (39) has contributed to the belief that pathogen exchange should be rare. An apparently restricted host range, however, may be the product of specific ecologic conditions and not of an intrinsic characteristic of the pathogen. In many cases, ecologic changes can broaden the host range of a pathogen. For example, the filarid worm *Loa loa* remains outside human populations, primarily because of vector behavior (40); changes in ecology, such as the availability of a novel host, can change vector behavior and expand the pathogen's host range. Similar phenomena may have played a role in the emergence of a range of flaviviruses (e.g., yellow fever, dengue, and Japanese encephalitis) from primarily forest-dwelling nonhuman primate cycles. For these flaviviruses, primate populations may also continue to play a role by introducing novel genetic variants, which, at least in the case of dengue, may be involved in pathogenesis.

Another misconception is that primate populations are too sparse to maintain human pathogens. A number of variables influence whether or not a pathogen is maintained in a given population. The capacity for latency, for example, may decrease the host population size necessary to maintain pathogens. This characteristic has evolved independently in pathogens as diverse as herpesviruses and *Plasmodium* and may help explain sustained transmission in hosts with low population density. Orangutans (*Pongo pygmaeus*), for example, are thought to act as hosts to two distinct *Plasmodium* species (41), despite an estimated population size of two per km^2. While molecular phylogenies have not yet been used to verify that these parasites are unique, the presence of a dormant hypnozoite stage might allow for sustained transmission even at this extreme. Where human and nonhuman hosts overlap, both must be factored into epidemiologic models. Small populations of nonimmune humans alone may not be capable of maintaining a pathogen, but when nearby nonhuman hosts are considered, a critical population size may be reached.

Surveys to assess pathogen prevalence among nonhumans can play an important role in control strategies. Eradication programs must consider animal reservoirs; even if complete human coverage is achieved, long-term reemergence from animal reservoirs can undo the best eradication efforts. For example, it is not known if poliovirus infections can be maintained in nonhuman primate populations. Seroprevalence surveys conducted before and after eradication may prove invaluable.

Accidental exposure to infected laboratory workers has led to poliovirus infections of chimpanzees and gorillas since the 1940s (1). Poliovirus can infect not only our closest living relatives, chimpanzees, gorillas (*Gorilla gorilla*), and orangutans (*Pongo pygmaeus*) (2), but also more distantly related anthropoids like the colobus monkeys (e.g., *Colobus abyssinicus kikuyuensis* [=*guereza*]) (42). Antibodies and shed virus have also been found in recently imported animals (8), and some chimpanzees may act as symptomless carriers (2). Long-term research by Jane Goodall on wild Tanzanian chimpanzees documented the potential for transmission of poliovirus (or a similar virus) in free-ranging chimpanzee populations (43). Since no samples were collected, it is impossible to determine if the epidemic described by Goodall was part of a natural chimpanzee cycle or the result of introduction from local human populations or researchers. As poliovirus eradication efforts intensify, it may be useful to monitor virus prevalence in humans living near primate habitats.

Control efforts that rely on antimicrobial drugs must also take into account the potential for nonhuman primate reservoirs. Despite demonstrations that mass administration of diethylcarbamazine citrate successfully controlled *Brugia malayi* (a filarid worm), Mak et al. found a high prevalence of *B. malayi* after a large-scale administration of chemotherapy (44). Research showed that even though periodic prevalence of *B. malayi* decreased, subperiodic prevalence remained high. The maintenance of subperiodic *B. malayi* was eventually attributed to mosquitoes infected by leaf monkeys (*Presbytis obscura*). In this

particular free-ranging primate host, approximately 83% of the monkeys were infected (44).

Nonhuman "reservoirs" may also confer potential benefits. It is at least theoretically possible that nonhuman primate populations may provide a barrier to the spread of drug-resistant pathogens; while these pathogens benefit from resistance in environments where drug pressure exists, drug resistance can be costly, and resistant pathogens may not compete effectively against susceptible 'wild-type' pathogens in the absence of drug pressure. As drug-free populations, reservoirs may provide havens for susceptible pathogens, thereby decreasing the rate at which drug-resistant genes spread and increasing the rate at which susceptibility may return after drug pressure ends. This hypothesis may help explain why the rates of drug-resistant gram-negative enteric bacteria of wild baboons (*Papio cynocephalus*) living with limited human contact are significantly lower than those of baboons living with human contact (45).

Sentinel Surveillance

In tropical lowland forests, which contain the greatest biodiversity of terrestrial habitats (46), exist rarely seen or unknown pathogens with the potential to enter human populations. These pathogens may affect residents of and visitors to forested regions (21) and act as the source of introduction of infectious agents to distant susceptible populations (47). Increasing human contact with forested systems almost certainly leads to a corresponding increase in the emergence of infections in the human population. Nevertheless, predicting which pathogens humans may encounter and be susceptible to remains a methodologic challenge.

Surveillance methods for predicting emerging pathogens include surveillance of vectors or forest-dwelling human populations and wildlife epidemiology (epidemiologic study of infections in wild populations) (48). These approaches have limitations. While vector sampling may prove the easiest method for widespread surveillance, the pathogens identified from vectors may be difficult or impossible to culture. Even when successful, vector sampling is likely to identify a range of pathogens, only some of which may infect humans. Studies of human populations, while providing valuable information, are limited to regions in which forest-dwelling human populations exist.

Epidemiologic research among free-ranging primate populations has the potential to predict which pathogens might enter human populations as contact with forested regions increases. In addition to their physiologic similarities to humans, primates have other characteristics that contribute to their accumulation of infectious agents. Primates live primarily in forested environments (49); in general, they have large bodies and live in large groups—characteristics that may attract vectors (50). Furthermore, dependence on fruit, a characteristic of most primates, requires mobility (both terrestrially and arboreally), a trait that may increase exposure to pathogens (51,52).

Despite the lack of organized attempts to document the distribution of pathogens in wild populations, recognized "die-offs" in wild primate populations have played an important role in identifying novel pathogens. In 1956, for example, a novel flavivirus was identified through the investigation of large-scale deaths of bonnet macaques (*Macaca radiata*) and hanuman langurs (*Presbytis entellus*) in the Kyanasur Forest of India (Seymour, 1981, cited in [2]) caused by Kyanasur Forest virus. More recently, in 1995, deaths in a chimpanzee population studied by Christoph Boesch in the Täi Forest, Côte d'Ivoire, and a single human case following a necropsy led to the identification of a novel strain of Ebola virus (9,53). The single human case in the Swiss researcher foreshadowed the localized mini-outbreak of Ebola hemorrhagic fever in Mayibout, a village in the northeast of Gabon in January 1996. The Gabon epidemic was linked to the handling, preparation, and consumption of a chimpanzee that had been found dead; 29 of 37 identified cases involved exposure to the dead chimpanzee (54). Close monitoring of such populations, as is being conducted in the Täi Forest, has the potential to identify emergence-linked behavior, such as the consumption of specific plants or insects, which may lead to the still elusive reservoir of Ebola virus. Considering the exceptionally small percentage of wild primate populations under long-term study, these examples represent only the tip of the iceberg. More systematic monitoring of wild primate populations will likely provide a substantial payoff in our understanding, identification, and possible control of novel pathogens, both for humans and endangered primates.

Surveillance for certain types of human-nonhuman primate contact may be particularly useful. Hunting, which involves tracking, capturing, handling, transporting, preparing, and consuming meat, may play a particularly important role in pathogen exchange. In addition to the recent evidence of hunting-mediated Ebola transmission, the hunting of a red colobus (*Colobus pennanti oustaleti* [=*badius*]) has been implicated in a localized epidemic of monkeypox, an orthopoxvirus similar to smallpox, which continued for four generations of human-to-human contact (55). Another example is the increased risk for feline plague among cats that hunt rodents (7). Necropsies share many characteristics with hunting and are appropriately considered a high-risk activity.

Bites from wild primates may also play a role in the transmission of certain pathogens. For example, chimpanzee-to-human transmission of monkeypox occurred when a wild chimpanzee bit a 2-year-old girl (6). Further sampling demonstrated high prevalence in forest squirrel populations (up to 49% among *Funisciurus lemniscatus*) (56), underlining the need for comprehensive studies before determining the ultimate source and reservoir of pathogens. Bites and scratches have transmitted pathogens to laboratory workers. The transmission of B-virus, a herpesvirus infecting rhesus macaques (*Macaca mulatta*), has caused death in 18 of 24 known human cases (57). Surveillance and education in human populations that hunt nonhuman primates, as well as follow-up of reported primate bites in nonlaboratory settings, may be indicated.

Ecologic and Social Factors Involved in Natural Transmission

The potential for disease emergence and reemergence depends on the interaction of complex social, ecologic, and genetic factors at the host, vector, and pathogen levels (58). Because wild populations of primates display diverse social behavior and live in a range of ecologic environments, they exemplify natural transmission. Furthermore, monitoring pathogens in wild primate populations does not involve the treatment or behavior change interventions that monitoring pathogens in humans requires. Exceptions include the increasingly common need for wildlife medicine to maintain the health of endangered species and decrease the impact of pathogens from humans and domestic animals on free-ranging animals (59). Nevertheless, for many pathogens, basic epidemiologic research, and not treatment, remains the primary goal of wildlife medicine (59).

Studies of wild populations can highlight factors associated with the pathogen exchange across species boundaries. Some parasites, for example, the chewing lice–pocket gopher system, complete their entire life cycle on a single host. This high level of host specificity may contribute to the close cospeciation between pocket gophers and their respective lice; the phylogenetic trees of host and parasite are nearly mirror images (60). Nevertheless, such host-specific parasites may be the exception rather than the rule. Many intestinal parasites, for example, seem to be generalists. One epidemiologic study of wild primates in the Kibale Forest National Park, Uganda, evaluated the role of primate distribution on the distribution of intestinal amoebas (61); the park contains a number of spatially separate primate groups, each of which consists of multiple primate species. Most variation in amoeba prevalence was explained by group membership, and little was explained by species, which suggests that these parasites treated the primate groups as biologic islands (62), spreading easily among diverse members of the same island but rarely spreading to new islands.

Long-term behavioral research combined with occasional pathogen sampling may provide valuable data. Long-term research on the SIVs of East African primates, for example, has provided evidence for a recent cross-species transmission of SIV between baboons and African grivet monkeys (*Cercopithecus aethiops aethiops*) (62). This research has documented the incidence of SIV_{agm} among Ethiopian grivet monkeys for more than 20 years (63). By examining prevalence in age groups over time, this research demonstrated the minimal impact of SIV_{agm} on the survival of grivets, the predominance of sexual transmission, and the lack of maternal transmission.

An ecologic approach to pathogen transmission can benefit our understanding of emerging diseases. By determining how humans become a part of the life cycle of pathogens rather than how pathogens enter human populations, we can better understand the factors associated with emergence and improve the quality of public health responses. How humans become part of the life cycle of pathogens depends on human migrations, environmental changes, and cultural

and social factors, in the context of evolutionary history of the pathogens, vectors, and hosts, which make up an infectious system. Ecologic and evolutionary studies of wild animals in general, and primate populations in particular, can address questions arising from these complex interactions.

The Future

Traditionally, the study of wild populations of primates has been the domain of primatologists and wildlife veterinarians, who have worked to overcome logistic difficulties in the field, develop methods, and address ethical issues. Opportunities exist for collaborative work on infectious disease; the benefits of such efforts are considerable. The translocation of animals from vulnerable forest fragments to forest reserves, an increasingly common conservation effort, is an opportunity for pathogen sampling. Other possibilities are provided by collaboration with long-term behavioral research on free-ranging animals. While behavioral research sites may only provide fecal and urine samples, the study of intestinal parasites is often possible; recent advances in urinalysis demonstrate that urine may be a source of both antigen and antibody from systemic infections, such as malaria (64). The distribution of appropriate necropsy protocols and sample collection kits would improve data collection and decrease risks associated with necropsies. Urgently needed are strategies for noninvasive remote diagnosis. For example, combined with sensitive molecular diagnostics, a remote tissue-biopsy dart system (65) may have potential for obtaining epidemiologic samples.

Efforts to preserve endangered primates and monitor disease emergence have some common objectives. Pathogen exchange is a two-way street, and exposure to human pathogens poses a serious threat to endangered animals (11). While the main focus of this article has been risk to humans, nonhuman primates are frequently more threatened by microorganisms indigenous to humans than vice versa (2). TB, which is often fatal to nonhuman primates, represents a serious threat to laboratory primate communities, which commonly are infected by humans and can occasionally reinfect laboratory workers (66). TB is prevalent in wild populations, as demonstrated by the presence in wild olive baboons (*Papio cynocephalus*) of *Mycobacterium bovis* infection, an infection that most likely originated from cattle (67).

Both hunting and forest encroachment threaten endangered primates and increase the possibility of human and nonhuman infectious disease emergence. Research on the pathogens of primates and humans on forest boundaries helps assess risks to wild primates and to humans. In addition to their value in the study of infectious disease and human evolution, many primates are valuable natural resources in their home countries. The veterinary expertise and wildlife management skills of conservation organizations can both supplement the basic pathogen research and control work of the public health community and benefit from it.

The examination of pathogen exchange in regions of host overlap may identify social factors that influence pathogen emergence. Data on forest use by human communities surrounding forest reserves and levels of crop-raiding by nonhuman primates have been collected as part of ongoing conservation projects. A comparison of these data with human and nonhuman primate pathogen prevalence may provide a measure of forest-oriented behavioral risks. Further research comparing infection rates among hunters and nonhunters could confirm the findings and determine the role of behavioral control measures to decrease risk.

Much remains to be done. Recent evidence has suggested that some wild primates self-medicate with plants in their environment (68). An understanding of the underlying wildlife epidemiology, therefore, combined with long-term dietary data and analysis of plant chemistry, may lead to new chemotherapeutic drugs (69). In addition, research on the dynamics of primate pathogens in their natural hosts may elucidate novel host resistance mechanisms. In particular, evidence of impaired host survival and reproduction, as well as long-term host-parasite association, may point to hosts that are likely to have evolved genetically mediated resistance. Such resistance mechanisms in humans (70) have begun to play an increasingly important role in vaccine and drug development (71,72). While the findings from captive primate studies have played an important role in medicine in the 20th century, this period has also been marked by a notable absence of research on the basic ecology of disease systems. Perhaps by learning from primates in their natural environments we may better prepare ourselves for the

disease threats to humans and wildlife populations in the coming century.

Acknowledgments

We thank Adria Tassy Prosser and Daniel G. Colley for valuable suggestions and Mary Bartlett for editorial assistance. Nathan Wolfe is supported by a Taplin Graduate Fellowship and a National Science Foundation-Predoctoral Fellowship. Ananias A. Escalante is supported by the ASM/NCID Postdoctoral Research Associates Program.

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Accommodating Error Analysis in Comparison and Clustering of Molecular Fingerprints

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Molecular epidemiologic studies of infectious diseases rely on pathogen genotype comparisons, which usually yield patterns comprising sets of DNA fragments (DNA fingerprints). We use a highly developed genotyping system, IS*6110*-based restriction fragment length polymorphism analysis of *Mycobacterium tuberculosis*, to develop a computational method that automates comparison of large numbers of fingerprints. Because error in fragment length measurements is proportional to fragment length and is positively correlated for fragments within a lane, an align-and-count method that compensates for relative scaling of lanes reliably counts matching fragments between lanes. Results of a two-step method we developed to cluster identical fingerprints agree closely with 5 years of computer-assisted visual matching among 1,335 *M. tuberculosis* fingerprints. Fully documented and validated methods of automated comparison and clustering will greatly expand the scope of molecular epidemiology.

The combination of conventional epidemiologic investigations with molecular techniques for genotyping pathogens has elucidated the epidemiology of many infectious diseases. The most frequently used genotyping techniques (e.g., pulsed-field gel electrophoresis, restriction fragment length polymorphism [RFLP], and randomly amplified polymorphic DNA) yield fragment-based data. Fewer than 100 patterns can be compared visually. For larger numbers, commercially available computer programs can be used to identify a manageable subset of potentially matching patterns, which are then compared visually. This approach is accurate but cumbersome and excessively labor-intensive as the number of isolates exceeds several hundred. Furthermore, the results of computer-assisted matching are not as reproducible as systematic computational methods. These limitations significantly constrain the size, scope, and standardization of molecular epidemiologic investigations. We present an approach by which identical patterns can be identified from large collections of DNA fingerprints.

The number of IS*6110* fingerprints continues to increase, with many studies across the globe producing IS*6110* data to characterize *Mycobacterium tuberculosis* isolates. Such molecular epidemiologic studies provide information about tuberculosis (TB) transmission patterns. Studies in Ethiopia, Tunisia, and The Netherlands (1), South Africa (2), India (3), Denmark and Greenland (4,5), the United States (6), and Tanzania (7), among many others, exploit IS*6110*-based RFLP genetic fingerprints.

We developed an automated computational system, in which a statistical analysis of the error in measuring fragment sizes provides a conceptual framework for comparing sets of fragment lengths. The computational approach to lane comparison—align-and-count method (ACM)—permits calculation of the number of fragments that match between two IS*6110*-based RFLP fingerprints. The parameters of the computational ACM are adjusted to provide the same high

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sensitivity as the labor-intensive visual inspection used over the last 5 years.

We developed an approach to identifying a set of identical fingerprints when the identity of the fingerprints is nontransitive. We also explored improving the specificity among matched fingerprints to reveal additional information in RFLP lanes.

Data Acquisition

Genotyping *M. tuberculosis* isolates with IS*6100*-based RFLP fingerprinting was performed as described in van Embden et al., 1993 (8). Computer-assisted comparison and clustering were performed on the RFLP lanes as described in Woelffer et al., 1996 (9).

Internal standards were used to quantitate fragment sizes visualized with a DNA probe to IS*6110*. Two films' exposures were scanned into Whole Band Analyzer software (BioImage, Ann Arbor, MI, USA); one was obtained when probing for the internal standard, the other when probing for IS*6110*. The resulting images were aligned with three registration marks that gave reference to the original nylon membrane. The Whole Band Analyzer software quantitated fragment lengths for the IS*6110*-visualized bands, which were inspected and edited manually in the software package. The resulting collections of fragment lengths for each lane (bacterial isolate or laboratory strain H37Rv) were exported to our ACM software and compared with other lanes.

Mathematical Methods

The following is a descriptive summary of the principles underlying the analysis (Appendix).

Analysis of Error in Data

The magnitude and characteristics of experimental error were empirically assessed by analyzing variation in the results obtained from a reference strain included in each experiment (gel). The absolute and proportional differences in the measured fragment lengths of biologically identical samples of this strain were calculated; results showed that the error in measurement was proportional to fragment length and greater between than within gels.

Align-and-Count Matching Algorithm

A method for matching was developed to accommodate the empirically defined error. The fragment length data from two lanes were scaled through a range of values, and the maximum number of mutually closest bands falling within a threshold tolerance was reported (Figure 1). The acceptable tolerance was smaller when lanes from the same gel were compared than when lanes from different gels were compared. An animated demonstration of this method is accessible on the Internet (URL for use with a graphics-capable Web browser: http://molepi.stanford.edu/hugh/acm/counting).

A Graph-Theoretic Approach to Identical Fingerprints

We considered pairs of patterns identical if all fragments matched. However, because the results might be nontransitive (A might be identical to B, B identical to C, but A not identical to C), the identification of groups of matched patterns is more complicated. A graph-theoretic approach was used to assemble clusters of matching fingerprints.

Alignment and Analysis of Residual Error

We further aligned collections of lanes determined to match according to the above algorithms. The optimal alignment was defined as that which minimized the proportional error between putatively identical fragments. This analytic step, which is comparable to the experimental approach of rerunning clustered strains in the same gel, improved the ability to distinguish similar, but nonidentical fingerprints.

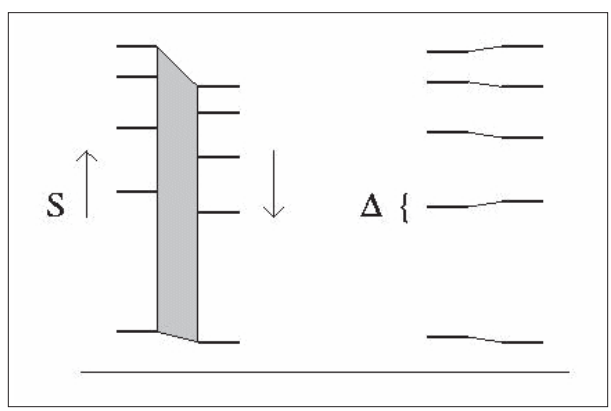

Figure 1. The align-and-count method finds the maximum number of mutually closest bands within a threshold deviation value **Δ**, for a search across a range *S* of scaling values. The two lanes are scaled incrementally, thus searching for the best alignment.

Error in Analysis of H37Rv Data

Investigating the absolute error for pairs of 116 H37Rv lanes (Figure 2), we found that the error was consistently higher in gel-to-gel comparisons than in comparisons of lanes from the same gel. Error was proportional to fragment length for the range of fragment lengths from 0.9 kbp up to at least 5 kbp (Figure 2), which included 90% of the bands in the fingerprint data from San Francisco. The latter empirical result was consistent with the fact that the distance migrated by a DNA fragment on an electrophoresis gel is typically proportional to the logarithm of the fragment length. Furthermore, the error found when we compared one band of a lane to that band in another lane was positively correlated to the error found for the other bands; in other words, if the first band in lane A was larger than the average measurement for that band, it was likely that the other bands in lane A would be larger than the average measurements. This positive correlation was intuitively evident in comparing lane maps (i.e., when comparing graphic representations of the fragment lengths). In Figure 3, the set of lane maps on the left are measurements of a genotype found in San Francisco. With the exception of the fourth lane from the right, they represent a set of identical patterns. Note that the error is mostly a scaling error and that if one fragment is larger than average for that lane, the others are very likely to be larger also. These two observations—that error is proportional to fragment length and positively correlated for bands within a given lane—suggests two classes of error: one is a property of each band; the second is a property of each lane. This analysis motivated us to develop a computational algorithm that scales fingerprints and measures the number of mutually closest bands within threshold sizes of each other for the best alignment, i.e., the scaling that maximizes the number of matching fragments.

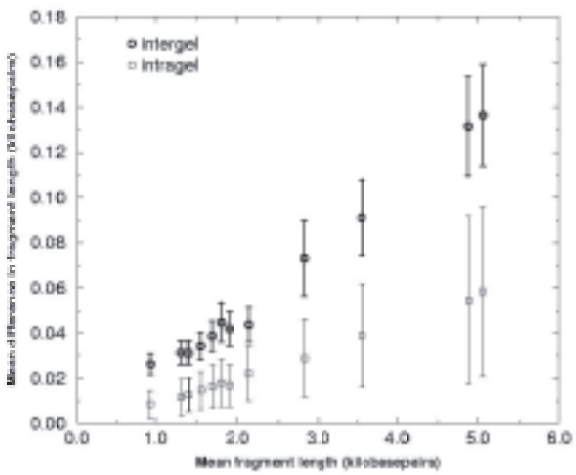

Figure 2. Means and two standard errors of the mean error bars for pairwise comparisons among 116 12-banded H37Rv lanes show that error is consistently larger when comparing lanes between gels than when comparing lanes from the same gel. The x-axis corresponds to *w(b)*, and the y-axis to *d(b)*, as presented in the text. It is evident that error is proportional to fragment length in the range of fragment lengths found in H37Rv. The data exhibit 2% to 3% error for between gel comparisons, but only approximately 1% error on average for within gel comparisons.

Alignment and Residual Error for H37Rv Lanes

By optimally aligning (i.e., minimizing the sum of proportional errors) pairs of H37Rv lanes, we find a distribution of scaling factors and of residual error. Table 1 shows 6,641 pairwise comparisons of 91 replicate lanes (members of each pair are taken from different gels). The mean value of *s*, the scaling factor, for these 6,641 alignments is 0.0212, and the standard deviation of *s* is 0.0189. The reduction in error due to

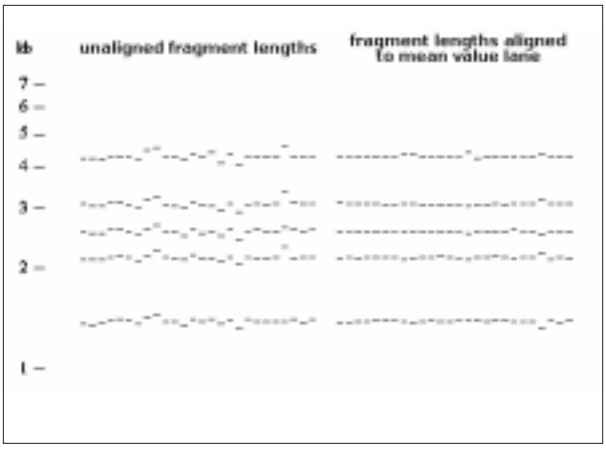

Figure 3. Additional alignment of very similar patterns can identify clearly distinct patterns. Measurement noise obscures the detailed relationships between 26 patterns that were identified from 1,335 as being very similar. However, after alignment to a consensus pattern, a clearly distinct pattern (an outlier from the other members of this autocluster) can be readily identified. Fragment lengths are given in kilobasepairs (kb).

Table 1. Pairwise comparisons (n=6,641) of lanes across gels characterize unaligned proportional error and residual error

H37Rv band b*	Mean kilo-bases $\overline{w}_b$	Unaligned pairwise proportional error mean $\overline{r}(b)$	Unaligned pairwise proportional error s.d.	Aligned pairwise proportional error mean $\overline{r}^a(b)$	Aligned pairwise proportional error s.d.
1	5.029	0.0253	0.0196	0.0099	0.0079
2	4.853	0.0249	0.0202	0.0084	0.0075
3	3.533	0.0253	0.0303	0.0109	0.0203
4	2.814	0.0237	0.0283	0.0105	0.0182
5	2.153	0.0202	0.0186	0.0086	0.0125
6	1.892	0.0210	0.0204	0.0100	0.0085
7	1.800	0.0226	0.0220	0.0092	0.0082
8	1.684	0.0227	0.0193	0.0083	0.0083
9	1.541	0.0221	0.0198	0.0079	0.0081
10	1.397	0.0225	0.0200	0.0093	0.0078
11	1.314	0.0231	0.0200	0.0111	0.0090
12	0.0936	0.0281	0.0249	0.0142	0.0157
mean		0.0235	0.0219	0.0099	0.0110

*Symbols as in Appendix.

alignment is approximately twofold, to approximately 1% with a standard deviation also of approximately 1% (Table 1); therefore, a search range, S, of 0.10 will allow for virtually every incidence of scaling error in the data. (Assuming normally distributed scaling and noting that 0.10 lies more than four standard deviations about the mean scaling error, we conclude that scaling error will not be compensated in fewer than 1 out of 10,000 independent pairwise comparisons.) Employing a deviation tolerance, Δ, of 0.045 should ensure a sensitivity very close to 100% for matching individual bands, after alignment. (Assuming normally distributed differences between replicate band measurements and a deviation tolerance of 4.5%, which is approximately 3.5 standard deviations above the mean fragment length error after alignment, one should falsely conclude two identical bands do not match with an approximate probability of 0.0002.) These parameter values, together with the number of incremental searches, I, set to 100, empirically gave results agreeing closely with visual inspection by experienced researchers who matched entire lanes. Similarly, one may use analysis of within gel lane error to determine the parameter values for the ACM to match lanes from the same gel.

Both the range of scaling factors and the threshold deviations are derived from empirical investigation of the San Francisco data. An adjustment for larger error in measurement is included for the more rare larger fragment length bands. In applying the ACM to San Francisco bacterial fingerprints, Δ is allowed to increase at a rate of 0.005/kbp above a value of 7 kbp.

San Francisco Bacterial Genetic Fingerprint Comparisons

To evaluate the performance of the ACM, we analyzed (by computer-assisted visual inspection and by ACM) 125 lanes from bacterial isolates obtained in the first half of 1996. We evaluated ACM's performance by first determining whether a 1996 lane matched all bands to lanes in visually defined clusters (from previous years), matched other 1996 lanes, or did not find any identical matches at all. The automated matching of the 1996 lanes agreed nearly perfectly with the visual analysis; the few conflicting results were due to inconsistencies in the existing data (e.g., two bands of nearly identical size being edited sometimes as one band and sometimes as two). Humans often compensate for such inconsistencies, whereas a computational method would have to have such capabilities explicitly built in.

Using ACM, we analyzed all 890,445 pairwise comparisons of isolates from 1,335 TB cases in San Francisco from 1991 to mid-1996. The autoclusters defined from the pairwise comparisons agreed closely with the clusters defined by computer-assisted visual inspection (Table 2). An example of one autocluster is shown in Figure 3. Without additional alignment, noise in fragment length measurements makes it difficult to determine if these putative clusters include individual patterns which, although similar, are distinct (outliers) or if the cluster contains identifiable subgroups of patterns (subclusters).

We then further analyzed autoclusters and identified more precisely nonidentical genotypes. Refinement of clusters begins with defining a consensus pattern for the cluster (consisting of the collection of mean fragment lengths for each band). Then the fragment lengths for each putative member of the autocluster are aligned to

Table 2. Preliminary autoclustering agrees closely with results obtained by computer-assisted visual inspection (CAVI)

	Clustered by CAVI	Not clustered by CAVI	Total
Autoclustered	540	27	567
Not autoclustered	49	719	768
Total	589	746	1,335

the consensus pattern. After alignment, a pattern can be easily identified as an outlier (Figure 3).

If the analysis of a refined, aligned cluster shows multiple outliers, realignment of subsets of lanes can be used to reveal subclusters of identical patterns. In Figure 4 the distributions of fragment lengths before (a,b) and after (c,d) an initial alignment are presented for an autocluster of 84 2-banders from San Francisco. Given that the aligned bands are clearly split into two distributions, we split the autocluster into two subclusters. A set of 26 fingerprints (group 1) is aligned to its assembled mean-value lane (Figure 4 e,f), as is a set of 58 fingerprints (group 2, Figure 4 g,h). The contrast between the original fragment length data and the two well-aligned groups of fingerprints shows that the higher fragment length band is shifted between the two groups and no clear outliers exist after alignment. Figure 5 shows that alignment greatly improves a difficult-to-resolve clustering issue among four lanes.

Preliminary investigation with a polymorphic GC-rich sequence (PGRS) fingerprinting method shows that the two subclusters exhibit distinct fingerprints, which further validates the increased specificity in IS*6110* fingerprints. Of 81 PGRS genotyped autoclustered 2-banders, 63 fall into eight visually defined clusters, the remaining being unique PGRS patterns among the members of the IS*6110* 2-banded autocluster. Each cluster consists of isolates that all fall into one or the other IS*6110*-refined subcluster.

Conclusions

We have developed and validated a systematic approach to pairwise comparison and clustering of identical patterns in a large data set of DNA fragment-based genotypes. Incorporating a control pattern in each experiment allows the nature and magnitude of error in DNA fragment length measurements to be determined. An analysis of measurement error provides parameter values to use with algorithms that accommodate these errors. Relative scaling of entire lanes, an important characteristic of the error generated in quantitating fragment lengths from RFLP patterns used to type *M. tuberculosis* isolates, arises in part from aligning two images of a gel, one for internal lane size standards and one for data fragments; the image of internal standards and the image of data fragments are registered by three marks. Error in registration of the two images occurs, leading to the scaling effect in the fragment lengths reported. We strongly suggest that software for analyzing internal lane size standards and data fragments from separate images permit (and encourage) use of more than three registration marks. While internal standards compensate for idiosyncrasies in lane-specific fragment mobility, the limitations imposed by poor registration methods can result in increased scaling error. We have demonstrated that allowing for scaling, as in ACM, greatly assists in automating matching; incorporating alignment of pairs of lanes into the method has provided fully automated lane matching that agrees closely with results of the well-established method of computer-assisted visual comparison. We successfully address the nontransitivity of pairwise identity and once again use scaling of lanes to ensure the reliability of automated clustering of identical patterns.

Alignment of DNA fragment patterns removes noise in clusters of fingerprints, showing further specificity in genotyping. This is analogous to the experimental approach of rerunning similar patterns on a single gel to reduce intergel noise. Mathematical transformation of the fragment length data yields similar information for far less cost in labor and materials. Further automated clustering within sets of aligned patterns could exploit the fact that we assume putative homology of fragments. As the aligned patterns have the same number of fragments, the residual error presents a multidimensional clustering problem; each pattern may deviate from the mean-value pattern in any of the fragment lengths. This clustering may prove more straightforward than the more general problem of clustering among patterns differing in numbers of fragments.

Numerous commercial packages of computer software are available to compare and match DNA fragment patterns. The availability of these systems fosters unquestioning application of turnkey pattern matching and clustering methods, which are not always fully documented or validated (for fragment-based genotypes) in the scientific literature. While possibly acceptable for small studies when visually validated, this approach to data analysis is risky for large studies. The methods for matching DNA fragment patterns presented in this paper should be an adjunct to software packages that quantitate fragments. We provide a systematic approach to analysis of fragment length

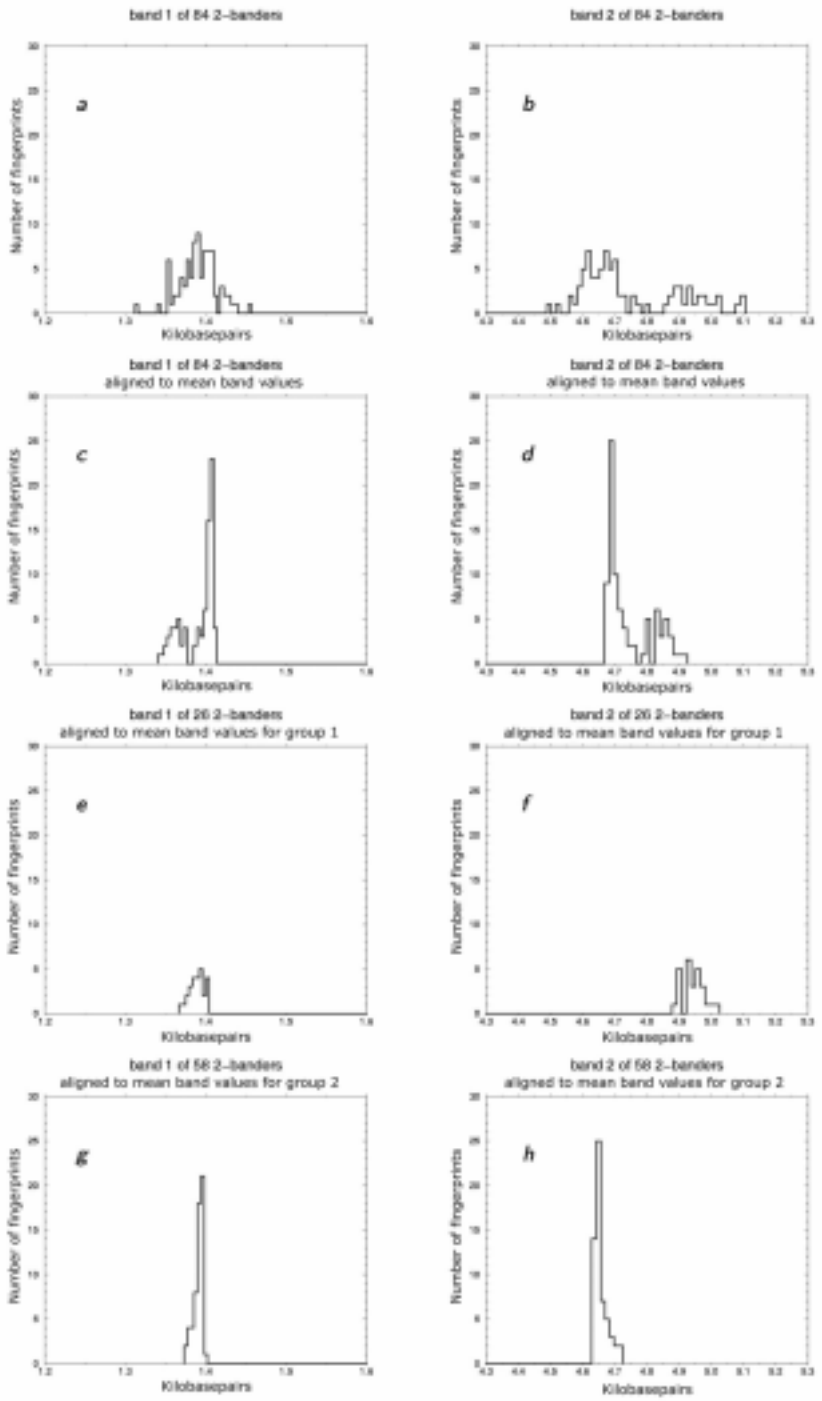

Figure 4. Histograms of the fragment lengths for 84 two-banded patterns connected by identity (autoclustered with in-house software) exhibit enough spread in values to make detecting outliers and band shifts difficult (a,b). Aligning the 84 lanes to the mean-value lane for this collection reveals that the lanes do not align well, but instead shows bimodal distributions for the fragment lengths (c,d). Dividing the 84 fingerprints into two sets and separating the distinct distributions detected when aligning all 84 fingerprints show that 26 fingerprints align well to their mean-value lane (e,f), and the remaining 58 also align well to their respective mean value lane (g,h). The smaller fragment length fragment does not appear shifted between the two sets of 2-banders (comparing e to g), but the larger fragment is clearly shifted (comparing f to h).

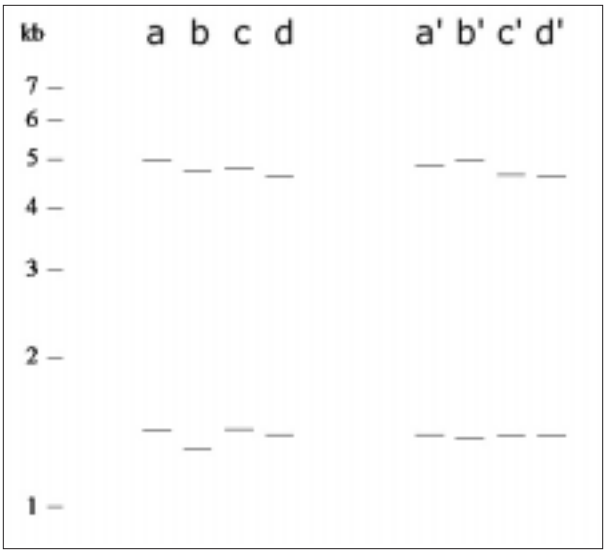

Figure 5. Prior to alignment of two sets of 2-banders, lanes are difficult to cluster (lanes a-d are from the distributions in Figures 4a and 4b). Subsequent to alignment, lanes are much easier to cluster (lanes a′ and b′ are specific examples from the distributions in Figures 4e and 4f; lanes c′ and d′ likewise correspond to Figures 4g and 4h). Fragment lengths are given in kilobasepairs (kb).

estimates, appropriate whether the numerical data are generated by hand using a ruler and arithmetic or are output by a multithousand dollar gel analyzer. This approach can thus be used by molecular epidemiologists working with both large and small budgets around the world. We anticipate that our methods can be incorporated into existing commercial software packages with broad distribution and encourage similar documentation in peer-reviewed journals of other methods provided in software packages.

In addition, our focus on the use of fragment length data, as opposed to the comparison of actual images, will foster comparison of data generated in different laboratories that use different proprietary software. We are working on a World-Wide Web–based system to facilitate IS*6110* genotype data sharing.

The availability of a precise and validated method to count the number of matching fragments in a pairwise fashion among very large numbers of patterns now permits an assessment of the importance and usefulness of approaches that exploit fingerprint similarity. In conjunction with the growing understanding of the underlying biology of IS*6110* instability and the relevant statistical issues, this may greatly expand the scope of TB molecular epidemiology.

A general question arises when comparing molecular fingerprints: how many bands need to match to indicate a close biologic relationship? Aside from the issues of defining biologic "closeness"—too often ignored in epidemiologic studies—technical issues are relevant to ACM. The usefulness of the matched band information output by ACM depends on at least several factors: the underlying band size distribution from which fingerprint bands are sampled, the independence of sampled bands, measurement error (both scaling and independent band error), the length of the gel, and the number of fragments. At one extreme, where error is large and few bands are observed, even in the case of a perfect match, statistical analysis may fail to reject coincidental band matches. By using computer simulation and sets of assumptions regarding band size distributions, one may learn about the role of coincidental band matching. We are actively researching these issues for IS*6110* fingerprint comparisons. Furthermore, we intend to provide a general computational framework in which one may assess error in laboratory measurement, the appropriateness of ACM for analyzing data (and appropriate parameter values to use with the method), and the role of coincidence in band matching. Information regarding computer programs for various tasks, including ACM matching itself, will be made available on the Internet at http://molepi.stanford.edu/hugh/acm.

A study of *M. tuberculosis* isolates from northern Tanzania demonstrates the utility of partial matching (3). The study brings into focus difficulties inherent in employing one-parameter tolerance for DNA fragment-based genotype matching, a technical issue effectively addressed by ACM. Gillespie et al. (7) also call attention to the poor specificity of low copy number IS*6110*-based fingerprints, exacerbated by the use of the Dice coefficient. We are pursuing alternative similarity measures that use the numbers of matching fragments identified by ACM and are tailored to the needs of epidemiologic investigations. Dendogram clustering methods often provided in software targeted at DNA-fragment genotype management and analysis could in some instances fail to reconstruct the correct relationships among infectious organism isolates, even when presented perfect clock-like

genetic distances. This may result in part from the fact that fast-evolving markers are characterized for isolates sampled over a period; samples are not contemporaneous. In conjunction with our efforts to define similarity measures, we are also working to modify phylogenetic inference tools used in clinical and molecular epidemiologic settings to better handle the data typically analyzed in molecular fingerprint management and analysis software.

Appendix

Analysis of Error in Data

To characterize error in fragment length measurement, pairs of 12 band 7H37Rv lanes are compared; the difference between fragment lengths of each band is calculated as follows. Let $w_{i,b}$ be the measured fragment length of band b of lane i. In general, let B be the number of bands. To compare lanes i and j, we can calculate the absolute difference between the measured lengths,

$$d_{i,j}(b) = |w_{i,b} - w_{j,b}|.$$

Let $\delta_{i,j}$ be an indicator that equals 1 when lanes i and j are from the same electrophoresis gel and 0 otherwise. Let n be the total number of replicate lanes. The mean absolute difference for a fragment over all pairwise comparisons of lanes from different gels is found by,

$$\overline{d(b)} = \frac{1}{t} \sum_{i=1}^{i=n-1} \sum_{j>i}^{j=n} d_{i,j}(b) \cdot (1 - \delta_{i,j}),$$

where $t = \sum_{i=1}^{i=n-1} \sum_{j>i}^{j=n} (1 - \delta_{i,j})$ is the number of pairs of lanes from different gels.

Similarly, we calculate the proportional difference between measurements for a fragment,

$$r_{i,j}(b) = \frac{|w_{i,b} - w_{j,b}|}{(w_{i,b} + w_{j,b})/2},$$

and its mean for comparisons between lanes from different gels,

$$\overline{r(b)} = \frac{1}{t} \sum_{i=1}^{i=n-1} \sum_{j>i}^{j=n} r_{i,j}(b) \cdot (1 - \delta_{i,j}).$$

Calculating fragment measurement errors for the $u = \sum_{i=1}^{i=n-1} \sum_{j>i}^{j=n} \delta_{i,j}$ pairwise comparisons of lanes from the same gel are also performed.

Align-and-Count Matching Algorithm

The Align-and-Count Method (ACM) for counting the matching bands between two lanes is defined as follows. Consider two lanes, lane A with m bands,

$$w_{A,x}, x = 1,...,m,$$

and lane B with n bands,

$$w_{B,y}, y = 1,...,n.$$

We count the mutually closest measured fragment lengths within a proportional deviation factor, Δ, over a range of alignments (Figure 1). Alignments are searched by scaling the fragment lengths. Multiplying the fragment lengths in a lane by a scaling factor reflects the phenomenon that error in fragment lengths is proportional to fragment length and is positively correlated for fragments in a lane.

Define

$$\text{Match } (w_{A,x}, w_{B,y}) = \begin{cases} 1 & \text{if } |w_{B,y} - w_{A,x}| \le \Delta \text{ and } x \text{ and } y \text{ are mutually closest bands} \\ 0 & \text{otherwise} \end{cases}$$

By mutually closest we mean that there is no band in lane B closer to band x of lane A than band y, and there is no band in lane A closer to band y of lane B than band x.

Lanes are incrementally scaled (I increments), and the maximum number of matching bands is reported. Specifically, the Jth increment in the search scales the lanes as follows:

$$\Omega_{A,x}(J) = w_{A,x} * (1 + (J - I/2) * S), \quad x = 1, \ldots, m,$$

and

$$\Omega_{B,y}(J) = w_{B,y} * (1 + (I/2 - J) * S), \quad y = 1, \ldots, n.$$

Here S defines the range of scaling factors (Figure 1). In this way the lanes slide past each other as J goes from 1 to I, always scaling the bands proportionally. At one extreme, lane A is

scaled (S/2 * 100)% larger while B is scaled (S/2 * 100)% smaller. At the other extreme the scaling is reversed.

The number of matching bands reported, k, is the maximum of K(J) over J = 1,...,I:

$$K(J) = \sum_{x=1}^{x=m} \sum_{y=1}^{y=n} \mathrm{Match}(\Omega_{A,x}(J), \Omega_{B,y}(J)).$$

We have described a method to find the number of matching bands k, when comparing a lane with m bands and a lane with n bands. This algorithm has three parameters: S, the range of scaling factors to align the lanes, Δ, the cutoff proportional difference for mutually closest bands to be considered matches, and I, the number of increments used to search the range of scaling. Note that Δ, in general, may be a function of the fragment lengths of mutually closest bands under inspection.

A Graph-Theoretic Approach to Identical Fingerprints

If all bands in lane j match their aligned counterparts in lane i, the two lanes are defined as identical. A common feature of ACM and all band-sharing approaches is that identity may not be transitive. This situation may arise when accumulated errors exceed tolerance or (proportionally) small changes in fragment size occur in biologic samples.

To analyze identical fingerprints, we define sets of fingerprints connected by identity. Let each of T fingerprints exhibiting the same number of bands be a node in a graph; let an edge joining two nodes indicate that two fingerprints are identical. By an algorithm attributable to Dijkstra (10), we determine the number of steps, P, of identity between two fingerprints when such a pathway exists. The algorithm finds the shortest path between two nodes. We use a code modified from that presented in Tenenbaum et al. (10). From this analysis we assemble collections of fingerprints connected by identity, which serve as tentative clusters of identical fingerprints. We refer to these tentative clusters as autoclusters. The distributions of fragment lengths for these collections of lanes are subsequently plotted, allowing outliers and band-shifts to be identified.

Alignment and Analysis of Residual Error

We aligned two replicate fingerprint patterns by using the following least squares regression formula. We minimize

$$Q(s) = \sum_{b=1}^{B} \Big(\log(w_{i,b}) - \log(s \cdot w_{j,b})\Big)^2$$

with respect to the scaling factor s by which lane j is aligned to lane i. Log-transformed fragment lengths are used to reflect the fact that measurement error is proportional to fragment length. The minimization has the closed form solution

$$s = \exp\Big(1/B \sum_{b=1}^{B} \log(\frac{w_{i,b}}{w_{j,b}})\Big).$$

Residual error in band size measurement after lane alignment is evaluated in a fashion analogous to that used for unaligned lanes. For example, the proportional difference between band b of lane j aligned to lane i and band b of lane i is

$$r^a_{i,j}(b) = \frac{|w_{i,b} - s \cdot w_{j,b}|}{(w_{i,b} + s \cdot w_{j,b})/2}.$$

For alignment of sets of lanes, the mean fragment length is calculated for each fragment. A mean-value lane is constructed from these mean lengths. Each lane is then aligned to the mean-value lane. The alignment is plotted and analyzed for outliers, providing refined clusters of identical fingerprints.

Acknowledgments

We thank Philip Hopewell, for his interest in and support for continued development of *M. tuberculosis* genetic fingerprinting in TB research; Mike Cummings, for his participation in numerous meetings regarding analysis of the fingerprint data, his input and discussions in the evolution of ideas leading to the work presented herein, and his comments on manuscripts; Melvin Javonillo, for help in accessing fingerprint data; Daniel Ruderman, for help with the issue of logarithmic alignment; and Marcel Behr and Sam Warren, for comments on manuscripts.

This research was supported by NIH grants AI35969, AI34238.

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Legal Issues Associated with Antimicrobial Drug Resistance

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An effective public health strategy against the development of antimicrobial drug resistance needs to be informed by legal as well as scientific analysis. This article describes some legal issues arising from current efforts against antimicrobial resistance and underscores the interdependence between law and public health in these efforts.

The development of antimicrobial resistance in many pathogenic microbes poses one of the most serious problems in the control of infectious diseases (1-3). Antimicrobial resistance results from the ability of microbes to adapt to anthropogenic pressures; therefore, it is not a passing trend but likely a permanent feature in the fight against infectious diseases (4). This article highlights the legal issues involved in addressing the problem of antimicrobial resistance.

Law and Global Public Health

Globalization interferes with infectious disease control at the national level (1,5-7). While microbes move freely around the world, unhindered by borders, human responses to infectious diseases are conditioned by jurisdictional boundaries. Therefore, public health responses to infectious diseases must constantly navigate the mazes created by international and national law. Although national law now dominates legal approaches to infectious diseases, the global nature of the emerging infectious disease problem points towards a larger future role for international law.

Especially in federal systems, countries often divide authority for public health among various levels of government. In the United States, for example, states have primary power for public health because the Constitution did not grant the federal government explicit public health powers (8). While the federal government has authority to act in the public health context (9), its statutes and regulations derive from other federal powers. Most U.S. public health law is at the state level. The emerging infectious disease threat points to a larger role not only for international but also for federal law. Federal agencies create networks that allow the state and national governments to cooperate on issues such as antimicrobial resistance, but public health law remains primarily a state domain; therefore, state laws on public health may need to be reevaluated in the context of emerging infectious disease control (10).

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Public Health Strategies to Address Antimicrobial Resistance

The dominant public health strategy against antimicrobial resistance contains improved surveillance of resistant pathogens, as well as rational use and increased research and development of new antimicrobial drugs (1). These elements fit within the larger strategy to address emerging infectious diseases, which stresses surveillance, applied research, prevention and control, and infrastructure development (1).

Surveillance

Surveillance is critical to the control and prevention of infectious diseases (1). Since no national or global surveillance system exists for monitoring antibiotic resistance, improved surveillance is a top priority (11). Surveillance is also useful in addressing the threat of biological weapons and protecting the community from highly contagious infectious diseases. Law is critical to surveillance not only because reporting information must be a legal duty, but also because law is needed to deal with the tensions

sometimes arising between individual privacy rights and the community's interest in being protected from infectious diseases.

Reporting

International and domestic surveillance systems are based on the legal duty to report certain public health information. The International Health Regulations mandate, for example, that member states of the World Health Organization (WHO) report outbreaks of plague, cholera, and yellow fever to WHO (12). Similarly, state public health departments in the United States legally mandate the reporting of cases of certain diseases from health-care providers to public health agencies (13).

Existing laws at the international and national level require reporting of a limited number of diseases, do not require systematic reporting of antimicrobial resistance, and receive inadequate compliance or noncompliance. WHO, however, has proposed including surveillance of antimicrobial resistance in the revisions of the International Health Regulations (14-16) and requiring drug resistance reporting.

In the United States, surveillance of antimicrobial resistance has been described as woeful (17). In 1992 less than $55,000 was spent on antibiotic resistance surveillance for human pathogens in the United States at the local, state, and federal levels combined (11). While the need for improved surveillance of antimicrobial resistance is recognized (1), state reporting laws have not changed much. For example, although the Council of State and Territorial Epidemiologists recommended in 1995 that states make drug-resistant *Streptococcus pneumoniae* reportable (18), not all states have (D. Bell, pers. comm.).

Several factors explain the lag of state responses to federal recommendations on resistance surveillance. First, state laws on infectious diseases often do not keep pace with new scientific findings (10). In addition, many states do not have within their legal systems the flexibility needed to respond to new threats—as was the case, for example, when the Council of State and Territorial Epidemiologists recommended that *Escherichia coli* O157:H7 be made reportable (13). Second, adding new surveillance responsibilities is difficult because of inadequate resources (13). These two factors indicate inadequate emphasis on public health within many state governments. The structure of public health law in the United States can, therefore, impede antimicrobial resistance strategies. As at the international level, if reporting is not required, surveillance for antimicrobial resistance is compromised.

Changes in diagnostic testing in the United States also raise public health and legal concerns about surveillance. Privatization of laboratory services by state legislatures may compromise national surveillance of emerging infectious diseases and investigation of outbreaks because many surveillance systems rely on data from state laboratories (13). In addition, because of economic pressures, hospitals increasingly rely on testing done in areas outside their jurisdiction; therefore, accurate information on infectious diseases in their areas may not be available. Diagnostic testing done by private laboratories may require closer federal regulation to protect the quality of needed surveillance data (D. Bell, pers. comm.).

Creation of a legal duty does not ensure the success of a policy. WHO member states have routinely ignored required outbreak reporting of plague, cholera, and yellow fever (4,19,20). Reporting of diseases within U.S. states is sometimes poor, haphazard, and unhelpful (21). Fulfillment of legal duties often hinges on sufficient resources. In many developing countries public health systems may be inadequate (22). Thus, financial and technical leadership is needed from national governments towards local authorities and from international organizations towards developing countries. In addition to legal requirements, national regulatory barriers may also hinder global surveillance. Such surveillance will require many countries to import and use equipment, software, and reagents to detect and report on antimicrobial resistance. Eliminating barriers could improve prospects for global surveillance. A precedent can be found in the proposed Convention on the Provision of Telecommunication Resources for Disaster Mitigation and Relief Operations, which obligates the parties, where possible, to lower or remove regulatory barriers for using telecommunication resources during disasters (23).

Privacy Issues

Surveillance systems sometimes have to balance the privacy of the patient with the need for useful scientific and medical information and the need of the community to be protected from

the spread of infectious diseases (9). As the HIV/AIDS epidemic has demonstrated, the privacy issue is particularly acute in sexually transmitted diseases. Because in some sexually transmitted diseases, e.g., gonorrhea, the infectious organisms are developing resistance to antibiotics, the inherent privacy concerns are compounded by surveillance-related privacy concerns. Increased surveillance for antimicrobial resistance may heighten privacy concerns with respect to other diseases, such as multidrug-resistant tuberculosis (MDRTB).

Different systems of law deal with privacy concerns differently, which could create legal problems for global surveillance of antimicrobial resistance. The differences between U.S. and European Union policies illustrate this difficulty. In the United States, the privacy of health-related information is of concern. Health information gathered by public health agencies is regulated largely by the Constitution and by state statutes. While the Constitution "requires reasonable levels of privacy and security when the government collects personally identifiable data through . . . disease reporting" (9), a recent survey of U.S. state legislation on public health information privacy concluded that many states' safeguards of public health privacy are insufficient (24). Laws protecting private sector records are even weaker (9). Some states regulate private dissemination of health-related information through physician-licensing systems, common-law tort rules on invasion of privacy, or statutes (25). Nevertheless, such legal regulations may not adequately protect privacy, as health-related information is increasingly manipulated electronically by health-care providers, health maintenance organizations, and insurance companies (9). Lack of laws adequately protecting the privacy of health-care information has led to calls for federal regulation (9,25,26), and some legislative proposals have been introduced in Congress (27).

The European Union has a strict law forbidding the processing of health information data without the written permission of the patient (28). This law places other strict conditions on the use of health data directly affecting European Union surveillance efforts. The contrast between American and European legal protection for health-related information may affect global surveillance efforts because European law permits states to withhold personal data from those who cannot adequately protect these data (26).

A particularly important development is the growth of privately owned infectious disease information. Private initiatives are building global information-sharing networks on various disease issues through the Internet and other information technologies (4,29,30); private companies are starting to monitor and test bacterial resistance globally (31); and some for-profit companies gather and sell epidemiologically useful information. These private efforts raise legal questions: privacy issues arise with the dissemination of epidemiologic data by private companies; this dissemination is treated differently in different countries; jurisdictional problems arise regarding legal regulation of information sharing in cyberspace (32) (the quality of health information on the Internet, for example, is being questioned [33-35]); and legal (and ethical) concerns arise with the practice of selling epidemiologic data, especially with data gathering, and whether governments can compel disclosure of privately gathered information in the interest of public health.

Biological Weapons

While most of the pathogenic agents considered to be the most likely candidates for use as biological weapons do not exhibit resistance (36), the potential use of resistant pathogens as weapons is of concern because resistance blunts one of the few lines of defense against a biological weapons attack. For the U.S. Department of Defense, antibiotic resistance is one of the criteria for characterizing suspicious outbreaks of infectious disease that might point to a possible biological weapons event (37).

The main source of international law on biological weapons, the 1972 Biological Weapons Convention, prohibits the development, production, and stockpiling of biological and toxin weapons (38). Negotiations are under way to strengthen the Biological Weapons Convention through a protocol that both establishes compliance procedures and commits states to improving domestic and global surveillance of infectious diseases (37). Calls for reform are also being made in domestic legal systems (37). The comprehensive statutory and regulatory system in the United States that governs the acquisition, use, and transfer of biological agents that pose a threat to public health (39) might serve as a model for legislation in other countries.

Personal Control Measures

The authority of public health officials to detain or isolate persons infected with highly contagious and resistant pathogens in order to protect the community is another important legal issue. MDRTB, for example, is a threat to public health because it is highly infectious (10). To deal with its TB epidemic, New York City has issued dozens of orders to detain MDRTB patients for isolation and compulsory treatment (40). Controversy exists about the proper scope of detaining patients for infectious disease control purposes (13). While U.S. courts have upheld detaining infected patients to protect public health (41), governments today face heightened judicial scrutiny of personal control measures in the public health context (10,42). In one case in New Jersey, the court held that public health authorities, in applying a 1912 TB control statute, had to comply with contemporary notions of due process and the Americans with Disabilities Act in order to detain and isolate a patient with MDRTB (43). At a time when antimicrobial resistance may have created a greater need for personal control measures for public health (e.g., with MDRTB), the status of U.S. law on the scope and nature of the government's power to undertake such measures seems unsettled (13). U.S. public health law may need to be modified to allow public health officials to control demonstrated threats of risk through flexible policy options that minimize infringements on individual rights (10).

The proper scope of personal control measures may appear pertinent only to industrialized countries, given that only those countries can identify resistant pathogens and their human hosts. In addition, the notion of personal control measures against drug-resistant malaria patients in Africa seems far-fetched, given the scale of the problem. Nevertheless, the importance of international human rights law to effective public health policies—as seen in the context of HIV/AIDS (44)—demonstrates that complacency towards individual rights in any public health policy is dangerous legally and medically.

Rational Use of Antimicrobial Drugs

Antimicrobial drug misuse in both industrialized and developing countries is a problem in connection not only with human treatment, but also with food production (11,45,46). More rational use of antimicrobial drugs in every country—for disease treatment and food production—must be at the core of the response to antimicrobial resistance.

Education has been suggested as the primary tactic for improving antimicrobial drug use (1,16). WHO has recommended "a code of practice for prudent use of antimicrobials in food animal production" (47).

However, the global scope of antimicrobial resistance indicates that an integrated strategy operating at both the national and international legal levels is needed. Such an integrated strategy faces political and legal challenges. Building effective national legal regimes regarding prudent antimicrobial use in many developing countries is unrealistic absent financial, political, and legal support from the international community (46). International legal harmonization of principles for prudent antimicrobial drug use will have to include monitoring and enforcement, as well as financial, technical, and legal assistance by industrialized countries to developing countries.

The international legal strategy needed will be difficult to create. WHO's limited powers to adopt regulations (48) do not seem to extend to creating regulations regarding the use of drugs. WHO has not, for example, proposed revising the International Health Regulations to rationalize the use of antimicrobial drugs. WHO has authority to adopt a convention on the use of antimicrobial drugs (49), but it has not done so (4). As illustrated by its proposal for a code of practice, rather than regulations, for antimicrobial use in food animal production, WHO historically has preferred not to use international legal powers to advance global health. Lessons from international environmental efforts suggest that international law must play a major role in setting international standards for implementation domestically and creating the political, technical, and financial conditions necessary to integrate international and national law (19).

The misuse of antibiotics in food production also raises concerns under international trade law. If antimicrobial drugs routinely used by food producers lose their effectiveness against animal-borne or plant-borne diseases, exports of contaminated food may be restricted by countries applying sanitary and phytosanitary measures (SPS measures) under the World Trade Organization (WTO) and other international trade agreements to keep such food out of their

territories as threats to human, animal, or plant health (50). In addition, exports might be disrupted by countries applying SPS measures under international trade agreements against products suspected of containing harmful residues of antimicrobial drugs. The Codex Alimentarius Commission already sets standards for residues from veterinary drugs in food through its Committee on Residues of Veterinary Drugs in Foods, and WHO has recommended that this Codex committee discuss antimicrobial resistance (47). Codex standards have become important in international trade law through the WTO Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) (50), which uses these standards as a basis for international harmonization of SPS measures. The importance of Codex food safety standards to international trade law was seen in the Beef Hormones Case, in which the WTO held that the European Union violated the SPS Agreement for not providing scientific justification for a beef hormone regulation stricter than the relevant Codex standards (51-53).

The inability of many governments to regulate antimicrobial drug use and the consequent misuse of these drugs raise the possibility that countries might use trade restrictions on food produced with improper use of antimicrobial drugs. In this situation, the food products might have residue levels below those established as maximums by Codex, but the trade restriction is intended to pressure the exporting country to improve regulation of antimicrobial drug use and the process by which the product is made. In the context of environmental protection, trade restrictions seeking to change a production process in another country, rather than to protect against health dangers from a particular product, have been ruled incompatible with international trade law (54-56). Although "relevant processes and production methods" (SPS Agreement, art. 5.2) form part of a risk assessment under the SPS Agreement, the risk must be a specific health risk from the product (e.g., highly inconsistent residue levels created by inadequate antimicrobial regulation) rather than fear of the health consequences of antimicrobial misuse. To avoid losing trade restrictions as part of a general strategy to combat antimicrobial misuse, legitimate trade restrictions against countries that systematically neglect recognized principles and practices for antimicrobial use might be considered; such a move would elevate the status of Codex's Code of Practice for Control of the Use of Veterinary Drugs and Guidelines for the Establishment of a Regulatory Programme for Control of Veterinary Drug Residues in Foods, as the SPS Agreement has elevated the importance of Codex's Maximum Residue Levels for Veterinary Drugs in Foods.

The International Conference on Harmonization, a multilateral effort between the United States, Japan, and the European Union to harmonize pharmaceutical regulatory systems, is another forum for discussing antimicrobial drug resistance. If national regulatory systems begin to grapple with the overuse of antimicrobial drugs, the International Conference on Harmonization might provide a forum to discuss a harmonized approach to more rational drug use in the United States, Japan, and the European Union. As structured, the Conference does not include other countries where overuse of antimicrobial drugs is a serious problem.

National laws for improving antimicrobial drug use face difficulties in industrialized countries; realistic national strategies confront legal and political hurdles. In the United States, state legislatures probably have the power to regulate how physicians prescribe antimicrobial drugs, but any attempt to legislate more rational use of drugs might evoke negative reactions from physicians and their medical associations, who might oppose the government's efforts to interfere with their professional judgment (57). If formal legislative regulation would not prove feasible, an alternative would be self-regulation by the medical and veterinary professions through practice guidelines, for example (57). A peer review process to monitor antimicrobial drug use has also been recommended (1). Managed care organizations may be included in the effort to control misuse of antimicrobial drugs, given their power and economic incentives to curb such misuse by physicians.

Formal regulation of antimicrobial use may be needed, which would involve monitoring and enforcement. In the United States, Congress could regulate use of antimicrobial drugs by monitoring interstate commerce in these products. Congress probably does not have the authority to regulate antimicrobial prescription practices directly; such authority rests with the states. The U.S. Food and Drug Administration (FDA) has authority to restrict the postapproval

marketing of new drugs designed for treating serious or life-threatening illnesses and has indicated that these regulations can be used specifically in cases of new antimicrobial drugs (17,58). Restricted distribution is, however, a disincentive to the development of new drugs, and the regulations do not address misuse of existing products. Another regulatory strategy available to the FDA for dealing with resistance in existing products is to modify labeling requirements; modifying labels is, however, somewhat cumbersome (17). Effective regulatory changes in the United States will be jeopardized by the lack of similar changes in other countries.

Perhaps the most powerful U.S. federal strategy would be to make implementation of state policies to curb the misuse of antimicrobial drugs mandatory before states receive federal funds earmarked for public health. Although states might argue that this would encroach on their traditional public health rights and powers, such a federal enactment would be constitutional. In addition, using federal funds to improve antimicrobial use policies nationwide fits with the need for federal political leadership as well as financial and technical assistance. State governments might, therefore, welcome federal money conditioned on implementing policies the money supports. Pharmaceutical companies, worried about the federalization of policies affecting their economic relationships with local and state health-care providers, might oppose these mandates. In addition, federal leadership in this way would also run counter to trends in other areas, such as welfare reform, which are moving responsibilities from the federal to the state level.

In countries where governments subsidize the purchase of antimicrobial drugs, legislative or regulatory changes in these subsidies could lead to a decline in the use of the drugs. When Iceland ended government subsidies of antimicrobial drugs, their use in Iceland declined, while sales kept increasing in other Nordic countries (59). Although Iceland's legislative change was made for political, not medical reasons (59), it illustrates the possible impact of legislative and regulatory controls on the use of antimicrobial drugs.

Improving physicians' awareness of antimicrobial resistance does not address, however, misuse of these drugs by patients (60). MDRTB has developed largely because of improper adherence by patients to TB therapy (10); directly observed therapy was initiated as a result. Because patient misuse of antimicrobial drugs also contributes to the public health crisis, proper completion of antimicrobial therapy must also be addressed.

New Antimicrobial Drug Research and Development (R&D)

As the antimicrobial arsenal shrinks, new drug R&D becomes critical (1). Legislation at the domestic level would ensure adequate funding for public sector involvement. For diseases that pose an especially large or complex problem (e.g., malaria), international law can play a role by structuring international cooperation through such institutions as WHO or the World Bank. The recently proposed Multilateral Initiative on Malaria, an international multiagency malaria control program, involves, for example, plans to coordinate R&D on antimalarial products supported by WHO and the World Bank (61-63).

While public involvement and funds are important, the real engine of pharmaceutical development is the private sector. However, pharmaceutical companies have only recently brought new antimicrobial drugs forward for regulatory approval. Critical disincentives continue to constrain private R&D investment in new drugs: intellectual property protection, regulatory approval procedures, and perceived antitrust law limitations on collaborative R&D.

Intellectual Property Protection

Pharmaceutical companies fear that their R&D efforts can be undermined by loss of intellectual property rights. Lack of secure patents deters pharmaceutical companies from some R&D activities on new drugs. While the WTO Agreement on Trade-Related Aspects of Intellectual Property Rights offers pharmaceutical companies better international legal rules on patent protection (64), the loss of patented agents remains a concern. Many WTO member states, especially in developing countries, have to upgrade their national laws to fulfill the agreement obligations, a process that could take years. International law on intellectual property protection is, thus, a critical piece of the overall strategy against antimicrobial resistance.

Other patent issues are relevant to the problem of antimicrobial resistance. Pharmaceutical companies that developed antibiotics years ago but never commercially exploited them might pursue more antimicrobial R&D if their earlier antibiotics (now without patent protection) were

given extra legal protection, either under patent law or a legal regime like the Orphan Drug Act (17). The efficacy of such legal strategies depends, of course, on the number of promising antibiotics potentially in the R&D pipeline.

Regulatory Approval Procedures

Another legal deterrent to antimicrobial development is the varied, complex, and costly regulatory approval procedures pharmaceutical companies face in the United States and other countries. Unilateral efforts are being made in the United States and the European Union to streamline drug approval regimes, and the International Conference on Harmonization represents an international effort at harmonization.

Another approach involves creating "expedited approval of new antimicrobials" (1). FDA, which has already created accelerated approval rules for drugs that treat serious or life-threatening illnesses, has indicated that these rules could be used to review new antibiotic treatments (17).

Antitrust Law Limitations on Collaborative R&D

In some circumstances, collaborative R&D efforts by a number of pharmaceutical companies might be indicated. Pharmaceutical companies have, however, noted the difficulties that antitrust laws create for collaborative R&D efforts. For example, in response to calls for collaborative R&D on antimalarial drugs, pharmaceutical companies are concerned about sharing intellectual property and about laws against cartels (65). Both U.S. antitrust law and European Union competition law, however, permit collaboration in certain areas (66,67). The Inter-Company Collaboration for AIDS Drug Development and the developing Multilateral Initiative on Malaria set precedents and might be replicated in the area of antimicrobial resistance.

Conclusion

Strategies to address antimicrobial resistance as a public health and legal challenge must consider three levels of interdependence: among the antimicrobial drug surveillance, use, and R&D components of the public health strategy; among the levels of law—national and international; and between the public health and legal aspects of dealing with antimicrobial resistance.

Each element of the public health strategy against antimicrobial resistance affects and depends on the other elements. More rational use of antimicrobial drugs and increased R&D depend on accurate surveillance. Limitations on the use of new drugs negatively affect the incentives pharmaceutical companies have to develop new drugs. Continued misuse of antimicrobial drugs places more pressure on both surveillance and new R&D. The effectiveness of new drugs will have to be measured by accurate surveillance and will continue to be undercut by their misuse. The lack of new R&D will force continued use of existing products, reducing their effective life-span. Thus, any public health strategy addressing antimicrobial resistance must consider all three components.

Because antimicrobial resistance is a global problem, national legal reforms taken in one or a few countries would suffer if other countries did not take similar actions. For example, since drug-resistant pathogens travel easily in today's world, national legal reforms to rationalize antimicrobial use in a few countries might be subverted if such misuse is not curtailed in many other countries. The creation of new international legal duties would likewise be undermined if such duties were not translated into national law. Thus, any legal strategy against antimicrobial resistance must be pursued at both the national and international levels.

Achieving the public health objectives of an antimicrobial resistance strategy involves, at each stage, legal considerations and often calls for legal decisions. National and international law are integral to the public health mission in every country; the interdependence of public health and law forms part of the large multidisciplinary challenge created by the global problem of emerging infectious diseases.

Confronting antimicrobial resistance requires not only a scientific and public health strategy but also a legal strategy. Including law in the developing discourse will broaden and strengthen the strategy for combating antimicrobial resistance.

Acknowledgments

I thank Dr. Polly F. Harrison, Terry P. O'Brien, Dr. David Bell, Fred Cate, Beth Cate, Roger Dworkin, Ken Dau-Schmidt, Larry Gostin, Mary Ann Torres, Cynthia Baraban, and members of the Institute of Medicine's Forum on Emerging Infections for their help and encouragement

during the preparation of this article; I also thank the anonymous reviewers of Emerging Infectious Diseases for their comments and suggestions.

An earlier draft of this article was originally presented as a working paper to the Institute of Medicine's Forum on Emerging Infections in July 1997. Research for this article was supported by a research grant from the Indiana University School of Law-Bloomington.

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Rickettsial Pathogens and their Arthropod Vectors

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Rickettsial diseases, important causes of illness and death worldwide, exist primarily in endemic and enzootic foci that occasionally give rise to sporadic or seasonal outbreaks. Rickettsial pathogens are highly specialized for obligate intracellular survival in both the vertebrate host and the invertebrate vector. While studies often focus primarily on the vertebrate host, the arthropod vector is often more important in the natural maintenance of the pathogen. Consequently, coevolution of rickettsiae with arthropods is responsible for many features of the host-pathogen relationship that are unique among arthropod-borne diseases, including efficient pathogen replication, long-term maintenance of infection, and transstadial and transovarial transmission. This article examines the common features of the host-pathogen relationship and of the arthropod vectors of the typhus and spotted fever group rickettsiae.

Rickettsial diseases, widely distributed throughout the world in endemic foci with sporadic and often seasonal outbreaks, from time to time have reemerged in epidemic form in human populations. Throughout history, epidemics of louse-borne typhus have caused more deaths than all the wars combined (1). The ongoing outbreak of this disease in refugee camps in Burundi involving more than 30,000 human cases is a reminder that rickettsial diseases can reemerge in epidemic form as a result of the catastrophic breakdown of social conditions (2). In addition to explosive epidemics, sporadic but limited outbreaks of louse-borne typhus and other rickettsial diseases have been reported throughout the world. In the United States, a drastic increase of murine typhus in the 1940s, Rocky Mountain spotted fever (RMSF) in the late 1970s, and the human ehrlichioses in the 1990s attests to the potential emergence of these infections in populations at risk (3).

The rickettsiae's obligate intracellular existence in both mammalian and arthropod hosts serves as an excellent model for the study of complex host-parasite interactions. Rickettsial associations with obligate blood-sucking arthropods represent the highly adapted end-product of eons of biologic evolution. The ecologic separation and reduced selective pressure due to these associations may explain rickettsial genetic conservation. Their intimate relationships with vector hosts (Table 1) are characterized by efficient multiplication, long-term maintenance, transstadial and transovarial transmission, and extensive geographic and ecologic distribution. Although rickettsiae have a symbiotic relationship with their arthropod hosts, in some instances, they act as true parasites—for example, members of the *Wolbachia* and *Orientia tsutsugamushi* alter reproduction and manipulate cellular processes in their arthropod hosts (4), and the agent of epidemic typhus, *Rickettsia prowazekii*, kills its vector, the human body louse (5).

Although rickettsiae are maintained in nature through arthropod vectors, they frequently infect vertebrates, which in turn allow new lines of vectors to acquire infection from the rickettsemic hosts. The involvement of vertebrates, including humans, in the rickettsial cycle is variable and in some cases complicated (Figure). With the exception of epidemic typhus, humans are not essential in the rickettsial cycle. Humans acquire rickettsial infection from the infected vectors. While tick-borne rickettsiae are transmitted to humans by tick salivary secretions, flea- and

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Table 1. Epidemiologic features of the pathogenic rickettsiae[a]

Rickettsia species	Disease	Natural cycle[b] Vectors	Hosts	Geographic distribution
Typhus group:				
Rickettsia prowazekii	Epidemic typhus	Human body lice	Humans	Worldwide
	Recrudescent typhus	None	Humans	Worldwide
		Lice, fleas	Flying squirrels	Eastern USA
R. typhi	Murine typhus	Fleas	Rodents	Worldwide
		Fleas	Opossums	USA
R. felis	Murine typhuslike	Fleas	Opossums	USA
Spotted Fever group:				
R. rickettsii	Rocky Mountain spotted fever	Ticks	Small mammals, dogs, rabbits, birds	North & South America
R. conorii	Boutonneuse fever	Ticks	Rodents, dogs	Africa, Southern Europe, India
R. sibirica	North Asia tick typhus	Ticks	Rodents	Eurasia, Asia
R. japonica	Japanese spotted fever	Ticks	Rodents, dogs	Japan
R. australis	Queensland tick typhus	Ticks	Rodents	Australia
R. akari	Rickettsialpox	Mites	House mice, rats	Worldwide
Ehrlichioses group:				
Ehrlichia chaffeensis	Human monocytic ehrlichiosis	Ticks	Humans, deer	USA, Europe
Ehrlichia Sp	Human granulocytic ehrlichiosis	Ticks	Humans, deer, rodents	USA, Europe
Others:				
Coxiella burnetii	Q fever	Ticks	Small mammals, sheep, goats, cattle, dogs	Worldwide
Orientia tsutsugamushi	Scrub typhus	Mites	Rodents	Asia, Indian subcontinent, Australia

[a]Not listed are *R. helvetica, R. honei,* and *R. slovaca* (6).
[b]Evidence for arthropod serving as a vector or vertebrate serving as a host is based on the rickettsial isolation from field-collected specimens, experimental studies, and indirect evidence for rickettsial presence or exposure to rickettsiae (hemolymph test and serosurveys).

louse-borne rickettsiae are transmitted to humans through contamination of broken skin and mucosal surfaces by infected vector feces.

Although rickettsiae have common features with their vertebrate and invertebrate hosts, they differ considerably in terms of arthropod vectors, geographic distribution, and virulence (Table 1). In this article, we focus on the members of the typhus group (TG) and spotted fever group (SFG) rickettsiae to construct a conceptual framework of the natural history of human rickettsioses and evaluate feeding behavior of the vectors with regard to rickettsial maintenance and transmission.

Tick-borne Rickettsial Pathogens

The development and extensive use of the hemolymph test (which has been fundamental to tick and rickettsial surveys), improved isolation methods, and the application of molecular techniques have helped identify 14 valid, relatively distinct SFG rickettsiae (Tables 1, 2). Except for mite-borne *R. akari*, all SFG rickettsiae are transmitted by ixodid ticks (Tables 1, 2). In addition to *R. rickettsii*, the etiologic agent of RMSF, eight other tick-borne rickettsial species are human pathogens (Table 1; 6). The remaining SFG rickettsiae are isolated only from ticks and have low or no pathogenicity

to humans or certain laboratory animals (7). However, some of these rickettsiae could be etiologic agents of as-yet-undiscovered, less severe rickettsioses.

Distribution of SFG rickettsiae is limited to that of their tick vectors. In the United States, a high prevalence of SFG species in ticks cannot be explained without the extensive contributions of transovarial transmission. The transovarial and transstadial passage of SFG rickettsiae within tick vectors in nature ensures rickettsial survival without requiring the complexity inherent in an obligate multihost reservoir system. Although many genera and species of ixodid ticks are naturally infected with rickettsiae, *Dermacentor andersoni* and *D. variabilis* are the major vectors of *R. rickettsii*. SFG infection rates vary considerably by state. For example, the infection rate for adult *D. variabilis* collected from vegetation and mammalian hosts was 2% to 9% in Connecticut, 5% in New York, 6% in Kentucky-Tennessee and Maryland, 8.8% in Arkansas, and 10% in Alabama (5,8,9). Rickettsia and tick surveys indicate that *R. rickettsii* is less prevalent in vector ticks than some other SFG rickettsiae. In most cases, the SFG-positive ticks, including *D. andersoni* and *D. variabilis,* are infected with nonpathogenic rickettsiae rather than with *R. rickettsii* (Table 2). The low prevalence of *R. rickettsii* in SFG-positive ticks is intriguing. Interspecific competition among ticks may result in stable maintenance of SFG rickettsiae through transovarial transmission and may cause the gradual replacement of *R. rickettsii* in the tick population. Very little is known about the process of interspecific competition between prokaryotic microorganisms in ticks. Burgdorfer et al. (10) reported that *D. andersoni* from the east side of Bitterroot Valley in western Montana contained a nonpathogenic SFG-rickettsia, which they named East Side agent. East Side agent has recently been described as a new species, *R. peacockii* (11). This rickettsia is rarely present in tick hemolymph and is readily missed by the standard hemolymph test. The rickettsiae are confined primarily to the tick posterior diverticulae of the midgut, the small intestine, and most importantly, the ovaries. *R. peacockii* is maintained in the tick population through transovarial transmission, and the infected ticks were shown to be refractory to ovarian infection with *R. rickettsii*. However, these ticks acquired experimental infection with *R. rickettsii* and transmitted rickettsiae horizontally (10,11). Thus, ticks constitutively infected with *R. peacockii* and infected experimentally with *R. rickettsii* were unable to transmit *R. rickettsii* to their progeny. In effect, infection of *D. andersoni* with *R. peacockii* blocked the subsequent ability of the ticks to transmit *R. rickettsii* transovarially (10,11). The phenomenon of transovarial interference provided an explanation for the curious long-standing disease focus in Bitterroot Valley. Most cases of RMSF have occurred among residents on the west side of the valley where *D. andersoni* were abundant; on the east side, *D. andersoni* were also abundant and were reported

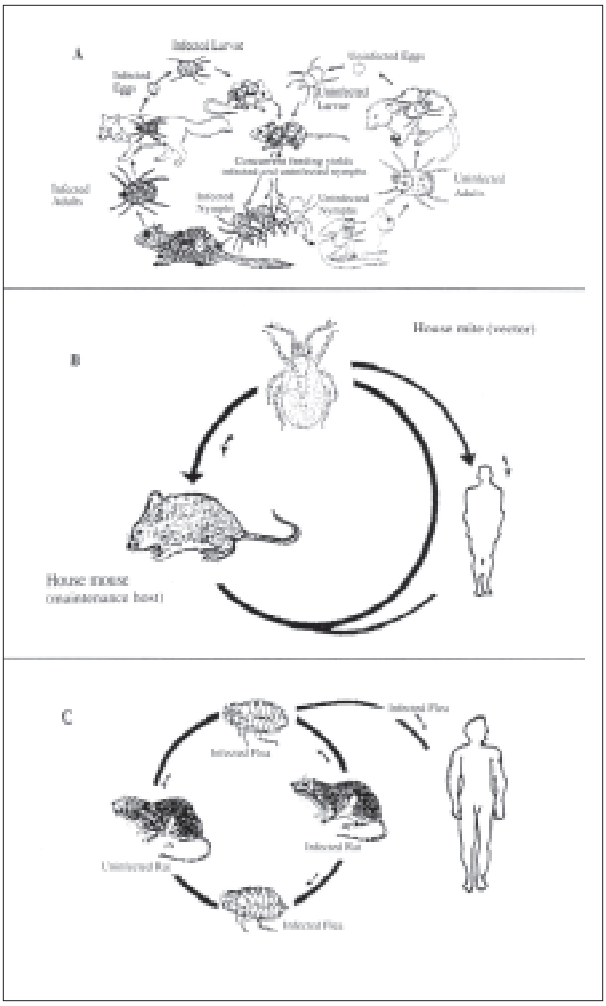

Figure. Composite diagram of the life cycle of Rocky Mountain spotted fever, rickettsialpox, and murine typhus. A. Life cycle of *Rickettsia rickettsii* in its tick and mammalian hosts (7); B. *Rickettsia akari* life cycle; and C. *Rickettsia typhi* life cycle.

Table 2. Epidemiologic characteristics of the North American tick-borne *rickettsia*[a]

Rickettsia species	Disease	Natural cycle	
		Vectors	Hosts
Rickettsia rickettsii	Rocky Mountain spotted fever	*Dermacentor, Amblyomma, Rhipicephalus, Haemaphysalis*	Small mammals, dogs, rabbits, birds
R. akari	Rickettsialpox	*Liponyssoides*	House mice, rats
R. amblyommii		*A. americanum*	Small mammals
R. bellii		*D. andersoni, D. variabilis, D. occidentalis, D. albopictus, H. leporispalustris*	Rodents, dogs
R. canada		*Haemaphysalis*	Rabbits, hares, birds
R. montana		*D. andersoni, D. variabilis*	Rodents, dogs
R. parkeri		*A. americanum, A. maculatum*	Domestic animals, birds, rodents
R. peacockii		*D. andersoni*	Rodents, deer
R. rhipicephali		*R. sanguineus, D. andersoni, D. variabilis, D. occidentalis*	Small mammals

[a]Excluding four as yet undescribed species of SFG rickettsiae (WB-8-2, 364-D, Tillamook, and the *D. parumapertus* agent).

to feed on residents, yet few (if any) locally acquired cases of RMSF occurred there (8,10). In the presence of *R. peacockii, R. rickettsii* could not be maintained transovarially—it could only be transmitted horizontally, and thus long-term maintenance could not be sustained. Transovarial interference by tick-associated symbionts such as *R. peacockii* is unlikely to be confined only to *D. andersoni* ticks on the east side of Bitterroot Valley, Montana. Burgdorfer et al. (10) stated that transovarial interference of *R. rickettsii* in *D. andersoni* ticks may be mediated by other nonpathogenic SFG rickettsiae—*R. montana* and *R. rhiphicephali*. Tick surveys for SFG rickettsiae generally report finding *R. rickettsii* only in a minority of ticks with rickettsiae (Table 3). Most infected ticks harbor nonpathogenic species. Thus, transovarial interference may have epidemiologic significance: it may explain why ticks collected from various geographic regions are not infected with two or more species of SFG rickettsiae.

The tick-rickettsia interrelationships are complex, and the mechanisms used by rickettsiae to survive in unfed overwintering ticks or during molting are poorly understood. How changes (e.g., before and after blood meal) in tick gut physiology influence rickettsial growth, cell division, and differential expression of rickettsial surface protein is not well understood. Although experiments (8,10) have deciphered the phenomenon of reactivation of rickettsial virulence after infected ticks take a blood meal, the underlying molecular events have yet to be elucidated. Other aspects of rickettsia-tick interactions need to be studied. For example, after feeding, a tick larva enters a quiescent period before emerging as a questing nymph the following year. How rickettsiae survive within the tick during this quiescent period and regain infectivity during the nymphal feeding is poorly understood. Although the precise mechanisms of rickettsial reactivation are not known, temperature shift and blood intake are believed to reactivate rickettsiae. As in the *Borrelia burgdorferi-Ixodes scapularis* model, transmission of rickettsiae probably cannot occur until after 24 hours of tick attachment, which allows time for rickettsial growth.

Insect-Borne Rickettsial Pathogens

Unlike SFG, TG rickettsiae are associated with insects (Table 1). Their association with blood-sucking insects such as human body lice (*Pediculus humanus*) and fleas (*Xenopsylla cheopis* and other rodent fleas [13-15]) provides rickettsiae the potential to spread rapidly among susceptible populations. Both fleas and human body lice, intermittent feeders, are capable of multiple feeding and thus of transmitting rickettsiae to several hosts. Outbreaks of epidemic typhus thereby can result from rapid transmission of *R. prowazekii* from human to human by infected lice. Unlike ticks that transmit SFG rickettsiae, lice infected with *R. prowazekii* die within 2 weeks after imbibing infected blood. *R. typhi*, the etiologic agent of murine typhus, does not shorten the life span of fleas (15). Although *R. typhi* and *R. felis* are maintained transovarially in fleas (15-18), there is no evidence that *R. prowazekii* is maintained in human body lice vertically. Since lice die of *R.*

prowazekii infection, the role of the reservoir in maintaining the rickettsiae in nature becomes essential. *R. prowazekii* sequesters in its human host; persistence of rickettsiae occurs despite the strong, long-lasting immunity after infection with *R. prowazekii*. Patients with recrudescent typhus (Brill-Zinsser disease) serve as potential reservoirs capable of infecting lice. Although a search for a zoonotic cycle of *R. prowazekii* in areas with louse-borne typhus epidemics (e.g., Ethiopia and Burundi) proved to be unsuccessful, flying squirrels (*Glaucomys volans*) in the eastern United States are naturally infected with this organism. Flying squirrel ectoparasites (lice and fleas) were implicated in the transmission of *R. prowazekii* between the squirrels and from squirrels to humans (5). Although the distribution of *G. volans* extends to the entire eastern United States as well as to isolated areas of Mexico and Guatemala, the search for an extrareservoir of *R. prowazekii* was not pursued further. Consequently, the importance of the *R. prowazekii* and squirrel system remains unclear. In the absence of a zoonotic cycle, conditions such as widespread lice infestations, active human infection, reactivation of latent infection in patients with recrudescent typhus could easily ignite a resurgence of louse-borne typhus. Louse-borne typhus continues to occur in epidemics following the breakdown of social, economic, or political systems, as exemplified by recent outbreaks in Burundi and remote parts of South America. Therefore, active surveillance to monitor louse-borne typhus and prevent its spread is indicated.

In contrast to louse-borne typhus, murine typhus is prevalent throughout the world and accounts for widespread illness in areas infested with many rats and fleas. Murine typhus occurs in epidemics or has a high prevalence, is often unrecognized and substantially underreported, and although it may be clinically mild, can cause severe and even fatal cases (19). Thousands of human cases used to occur annually in the United States (13,14). Outbreaks have been reported in Australia and recently in China, Kuwait, and Thailand (13-15). The classic cycle of *R. typhi* involves rats (*Rattus rattus* and *R. norvegicus*) and primarily the rat flea, *X. cheopis* (13,14). *X. cheopis* is the main vector, and transmission is affected by contact with rickettsia-containing flea feces or tissue during or after blood feeding. Reported cases of murine typhus in the United States are from south and central Texas and the Los Angeles and Orange County area of California (21-25). However, most of the cases are attributed to opossum-cat flea cycles. Both opossums and domestic cats collected from the case areas were seropositive for *R. typhi* antibodies. The cat flea, *Ctencephalides felis*, which is a competent vector of murine typhus, is the most prevalent flea species (97%) collected from opossums, cats, and dogs in southern Texas; no fleas were recovered from rats in this area. In addition to *R. typhi*, *R. felis* was also found in opossums and their fleas (15). This finding, consistent with surveys in other areas of the country (14,20), further documents the reduced role of rat and *X. cheopis* in the maintenance of murine typhus within endemic-disease areas of the United States. The maintenance of *R. typhi* in the cat flea and opossum cycle is therefore of potential public health importance since *C. felis* is a prevalent and widespread pest that avidly bites humans (12,15).

Table 3. Species composition of tick-borne rickettsiae isolated from hemolymph-positive *Dermacentor* ticks[a]

Rickettsial sp.	California *D. occidentalis* (No. isolates)	Montana *D. andersoni* (No. isolates)	Ohio *D. variabilis* (No. isolates)	Long Island *D. variabilis* (No. isolates)	Maryland *D. variabilis* (No. isolates)
R. rickettsii	0 (0)	9 (10)	18 (4)	0 (0)	8 (2)
R. rhipicephali	96 (79)	44 (47)	0 (0)	0 (0)	0 (0)
R. montana	-	7 (8)	59 (13)	100 (100)	0 (0)
Other SFG[b]	-	-	5 (1)[c]	-	88 (23)[d]
R. bellii	4 (3)	39 (41)	18 (4)	0 (0)	4 (1)
Total number isolates	82	106	22	100	26

[a]Shows a compilation of various statewide surveys, comparing the species composition of SFG rickettsiae in *Dermacentor spp.* ticks that tested positive by immunofluorescence assay.
[b]SFG, spotted fever group.
[c]*R. amblyommii.*
[d]Mouse anti-sera made against Maryland isolates reacted with WB-8-2 (unnamed SFG rickettsiae).

In many parts of the world, murine typhus infection is intimately associated with introduced commensal rodents (*R. rattus*, *R. norvegicus*, and *Mus musculus*) and their ectoparasites, particularly fleas. Although *R. typhi* have been isolated from other commensal rodents and even shrews, they do not seem to play a role in the transmission of murine typhus to humans (13,14). In Rangoon (Myanmar), of four species of murines and one shrew commonly found in buildings, 7% (*M. musculus*) to 30% (*R. rattus*) and 38% (*Bandicota bengalensis*) of those tested were seropositive to *R. typhi*. In contrast, 62% of *R. rattus* collected from buildings in Addis Ababa (Ethiopia) and 49% of those collected in Sarawak (Nepal) were seropositive for murine typhus (Azad et al., unpub. data). Infection rates in *X. cheopis* fleas collected from rats were 7% to 18%. While infection rates vary considerably among indoor rats and their fleas, murine typhus infection seems clearly associated with indoor rat populations throughout the world. However, in the absence of indoor rats, murine typhus infection is maintained in suburban and rural cycles when native animals seek shelter in human habitations where food and hospitable environments are plentiful.

Pathogen-Arthropod Interaction

The process of displacement of pathogenic rickettsiae with nonpathogenic endosymbionts in ticks through transovarial interference is of potential epidemiologic importance. The displacement might occur only if transovarial maintenance of pathogenic rickettsiae harms the host or the maintenance of nonpathogenic organisms confers important advantages to the tick progeny. Burgdorfer et al. (10), in experimental studies with the *D. andersoni*-*R. rickettsii* model, observed that maintenance of this pathogen in ticks over several generations resulted in unusually high death rates among engorged females and reduced numbers and fertility of deposited eggs. If tick infection with pathogenic rickettsiae in nature adversely affected egg maturation, oviposition, and embryogenesis, the balance would favor a tick population infected with the nonpathogenic rickettsiae, and over time such tick populations would displace those infected with *R. rickettsii*. Also, ticks infected experimentally with *R. montana* and *R. rhipicephali* could not maintain *R. rickettsii* through transovarial transmission, which suggests an interference phenomenon. Such a precedent exists—several tick species carry nonpathogenic rickettsiae (e.g., *D. andersoni* in east side Bitterroot Valley of western Montana, *A. americanum* in Maryland) frequently encountered in tick samples from different parts of the United States (8,9).

Recent work in our laboratory with TG rickettsiae suggests that interspecific competition between closely related rickettsiae may control rickettsial establishment in arthropods. Studies have identified both *R. typhi* and *R. felis* in opossums and in their cat fleas in endemic-disease regions in Texas and California (21-25). However, infection with both rickettsiae has not been observed in individual fleas (20-24). The intermittent feeding behavior of cat fleas associates them with various hosts, which increases the likelihood of infection with more than one pathogenic organism. Cat fleas constitutively infected with *R. felis* and experimentally infected with *R. typhi* contained both rickettsial species. While the *R. felis* infection rate in the infected flea population was 86% to 94%, prevalence of dually infected fleas was 13% and 26% (25). While no other relationships involving multiple bacterial infections in arthropods have been studied as thoroughly, the results from the few available studies show that dual infections in arthropod vectors are rare (e.g., human granulocytic ehrlichia was recently identified in 2.2% and 4% of *I. scapularis* ticks coinfected with *B. burgdorferi*) (26).

Whether infection with nonpathogenic rickettsiae presents an advantage to the tick population is difficult to ascertain because of lack of experimental data. There is no experimental evidence for rickettsial-induced postzygotic reproductive incompatibility, parthenogenesis, and feminization of genetic males as observed for members of the genus Wolbachia (4,27). Although a rickettsial relative is associated with male killing in the ladybird beetle (27), we do not know whether any nonpathogenic members of the SFG rickettsiae can induce reproductive incompatibility or sex distortion in ticks. A compilation of data from various laboratories that maintain cat flea colonies indicate that after several generations *R. felis* infection in fleas approaches 100% (28). Whether the high infection rate is the result of selection through reproductive incompatibility remains to be elucidated. Flea samples from opossums, cats, and dogs in different parts of the

United States were infected with *R. felis*. Since it is maintained transovarially, *R. felis* could be used as a marker to follow changes in the infection rates over time.

Several questions remain: 1) why does infection with nonpathogenic rickettsiae prevent establishment of virulent species in the ovaries of infected ticks; 2) what are the molecular bases for transovarial interference; 3) are nonpathogenic rickettsiae selected favorably by their arthropod hosts through reproductive incompatibility; and 4) why are nonpathogenic rickettsiae found more often in ticks than a virulent species.

Summary and Conclusions

Ixodid ticks, fleas, and lice are temporary obligate ectoparasites often found on the same vertebrate hosts. They differ substantially with respect to feeding behavior and digestion of blood meals (30), which can affect rickettsiae transmission from vector to vertebrate hosts. While all stages of ticks and lice are blood feeders, only adult fleas take blood from the host. Fleas and lice feed intermittently and digest blood meals rapidly, while ixodid ticks feed for several days and digest meals very slowly (29,30). SFG rickettsiae are associated with ixodid ticks, while TG rickettsiae are transmitted by fleas and lice. Efficient transovarial and transstadial transmission of rickettsiae in ticks ensures rickettsial survival while maintaining the genetic integrity of the SFG rickettsiae. This mechanism also allows ticks to serve as reservoir host for SFG rickettsiae. The SFG rickettsiae are transmitted by tick bites, whereas TG rickettsiae are deposited with insect feces at the bite site. At each life stage (larva, nymph, and adult), ticks feed only once; if undisturbed, they transmit rickettsiae to a single host, whereas repeated feedings allow fleas and lice to infect several hosts. Thus, vector feeding behavior accounts for the observed differences in disease epidemiology and natural history between tick- and insect-borne rickettsioses. Despite their strong similarities, SFG and TG rickettsiae have major differences in terms of growth, entry, and exit from host cells. Rickettsial characteristics related to vector compatibility were discussed in a recent publication by Hackstadt (31).

The molecular aspects of rickettsia-vector interactions are not well understood largely because only a few laboratories have addressed the mechanisms by which arthropods and rickettsiae interact. These intracellular organisms have long been associated with arthropods, and yet we know very little about their relationship with arthropod vectors.

This work was supported by grants R37 AI 17828 and R01 AI 43006 from the National Institutes of Health.

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Could Myocarditis, Insulin-Dependent Diabetes Mellitus, and Guillain-Barré Syndrome Be Caused by One or More Infectious Agents Carried by Rodents?

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The numbers of small rodents in northern Sweden fluctuate heavily, peaking every 3 or 4 years. We found that the incidence of Guillain-Barré syndrome and insulin-dependent diabetes mellitus, as well as the number of deaths caused by myocarditis, followed the fluctuations in numbers of bank voles, although with different time lags. An environmental factor, such as an infectious agent, has been suggested for all three diseases. We hypothesize that Guillain-Barré syndrome, myocarditis, and insulin-dependent diabetes mellitus in humans in Sweden are caused by one or more infectious agents carried by small rodents. Also, a group of novel picornaviruses recently isolated from these small rodents is being investigated as the possible etiologic agent(s).

Nephropathia epidemica (NE) is a disease caused by Puumala virus (genus *Hantavirus*, family Bunyaviridae). The vector and natural reservoir of Puumala virus is a small rodent, the bank vole (*Clethrionomys glareolus*) (1). In most parts of southern Sweden, bank vole populations are noncyclic, whereas in the north, populations fluctuate on a 3- or 4-year cycle of abundance (2-4). NE is endemic only in the northern part of the country, and the number of human cases cycles with the bank vole population (5-6).

We found the incidence of myocarditis, Guillain-Barré syndrome (GBS), and insulin-dependent-diabetes mellitus (IDDM) to lag behind the population density fluctuations of bank voles, although with different time delays. We hypothesize that in Sweden the bank vole is a vector for one or more infectious agents, which are pathogenic and cause these diseases in humans.

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The Study

Density of Bank Voles

Long-term records (>10 years) on small rodent abundance are scarce. However, in Sweden such data are available from the area with cyclic rodent populations (2, 4-8). We used data from Grimsö (59°40' N, 15°25' E), where small rodents have been collected every autumn since 1973 (6-8). Snap-trapping was performed where possible in six adjacent 5x5-km areas, each with four 1-ha plots. Of 24 permanent 1-ha plots, 20 were trapped. At each trapping, approximately 940 traps were set during three consecutive days, amounting to approximately 2,800 "trap-nights."

Species, date, and location of trapped animals were recorded (4, 6-8). As an index of bank vole abundance, we calculated the number of animals trapped per 100 trap nights (i.e., density). Bank vole abundance fluctuated in cycles with 3- or 4-year intervals between density peaks (Figure 1).

Incidence of Disease

We used the national census to calculate (according to counties and age groups) annual

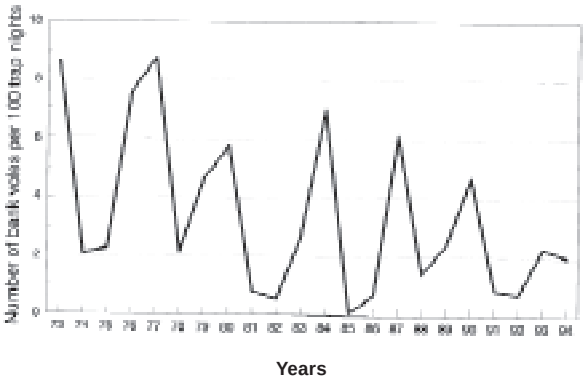

Figure 1. Bank vole abundance in Grimsö, 1973–1994. Untransformed data.

disease incidences per 100,000 population. Because of the lack of long-term records on noncyclic small rodent populations, we analyzed data from the counties with cyclic rodent populations: Norrbotten, Västerbotten, Västernorrland, Jämtland, Gävleborg, Kopparberg, Värmland, Örebro, Västmanland, Uppsala, Stockholm, Södermanland, Göteborg, Älvsborg, Jönköping, and Kronoberg (2-3). We excluded Stockholm and Uppsala counties as they are predominantly urban and on the border between counties with cyclic and noncyclic rodents.

Myocarditis

Myocarditis is a clinical condition in which the heart is infiltrated by inflammatory cells. Diagnosis is very difficult during the acute stage and is often made by postmortem microscopic investigation of the myocardium. All causes of death in Sweden are registered by the Swedish Cause-of-Death Statistics, Statistics Sweden, S-115 81 Stockholm, Sweden. Our study included all deaths between 1970 and 1986, in the age group 11 to 46 years, caused by myocarditis (ICD 8 code 422), if the diagnosis was given as either the direct (primary) cause of death or as the first out of six recorded contributing causes of death. The age group 11 to 46 years was chosen because congenital or arteriosclerotic cardiovascular disease is less common in this age group. In 201 (92%) of 218 cases, the diagnoses were based on clinical or forensic autopsies. The remaining diagnoses were based on clinical examination before death. We did not use data collected after 1986, as the disease classification changed from ICD 8 to ICD 9 in 1987, making comparison with the period after 1986 difficult.

Guillain-Barré Syndrome

In different parts of Europe (9), reported incidence of GBS varies considerably depending on study method. Our data, from the hospital discharge registry, Swedish National Board of Health and Welfare, S-106 30 Stockholm, Sweden, are considered adequate for epidemiologic surveillance of GBS (9). We selected patients hospitalized with the diagnosis GBS (ICD 8 code 354) and gathered information on age, sex, county of residence, county of hospitalization, diagnosis, date of admission and date of discharge. We only analyzed data of GBS patients 46 years of age and younger, as the diagnoses in older patients frequently are obscured by nonspecific illnesses and other problems (10).

Insulin-Dependent Diabetes Mellitus

IDDM was studied in Medelpad (part of Västernorrland County), an area with cyclic rodent populations. The patients were identified through registries of patients with diabetes at the only hospital and at 16 out of the 17 health-care centers, as well as through common registries of diagnosis at one health-care center. The World Health Organization (WHO) diagnostic criteria were applied. We analyzed case data in all age groups because precision in the diagnosis of IDDM is not age related.

Statistical Analysis

A cross-correlation function (CCF) (11) can indicate any direct or delayed dependence between two different time series. A CCF graph is a plot of positive and negative correlation coefficients between pair-wise values from the two series. The correlations have been calculated between the two series for different time shifts (lags), with lag 0 meaning no time shift, lag -1 meaning the first series has been shifted backwards one time unit, and lag 1 meaning the first series has been shifted forward by one time unit.

We used CCFs, calculated in Statistical Package for the Social Sciences (SPSS) (12), to indicate any temporal association between the incidence in cyclic versus noncyclic areas of GBS and of deaths from myocarditis, respectively. We also used CCFs to indicate any direct (at lag 0) or delayed dependence (at negative lags) of the different disease incidences on vole density. At negative lags, the vole time series is the leading indicator that is shifted "backwards" along the time scale, relative to the disease series. Thus,

the correlation coefficients at negative lags are in focus, as they indicate any disease incidence dependence on past vole densities, i.e., on densities 1, 2, or 3 years ago. At positive lags, the disease time series is shifted "backwards" relative to the vole time series. The correlation coefficients at positive lags may be large because of the similarities between the two series, but we see no causal relationship between vole density and past disease incidence.

Log transformation of the time series was used because of the variable amplitudes of the fluctuations in the disease and vole series. In the case of IDDM, we also ran one CCF with the disease and vole time series difference-transformed by one year.

Findings

Myocarditis

A total of 218 patients, ages 11 to 46 years, died of acute myocarditis in 1970 to 1986; 148 in counties with cyclic rodent density and 70 in counties with noncyclic rodent density. Sex ratio was 2:1 (146 male, 72 female patients). Myocarditis death incidences in cyclic and noncyclic areas (Figure 2) were not correlated as revealed by cross-correlation of the disease series (CCF not shown; n = 17 computable 0-order correlations). Cross-correlation of the annual incidence of myocarditis deaths in 1970 to 1986 in the cyclic area with bank vole abundance (Figure 3) showed that the incidence of myocarditis was most highly correlated with vole abundance in the previous year, according to the high positive and significant correlation at lag -1 in the CCF (Figures 4, 5) (r = 0.635, $p < 0.05$, n = 13).

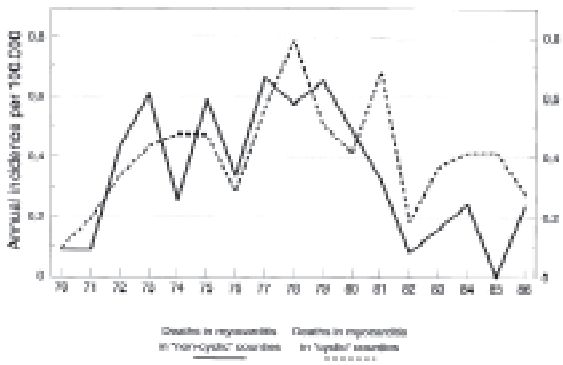

Figure 2. Incidence of death from myocarditis, 1970–1986. Untransformed data.

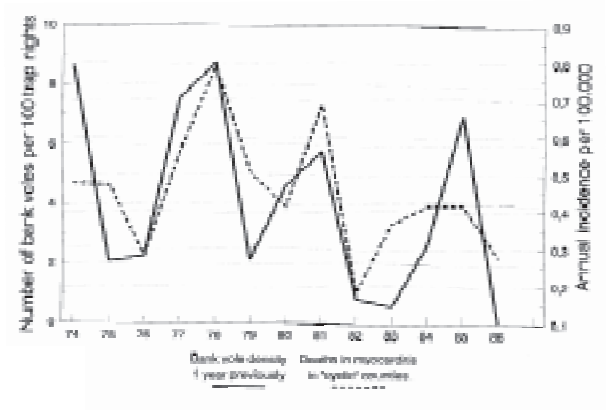

Figure 3. Myocarditis deaths, 1974–1986 relative to bank vole abundance 1 year previously (vole data from 1973-1985). Untransformed data.

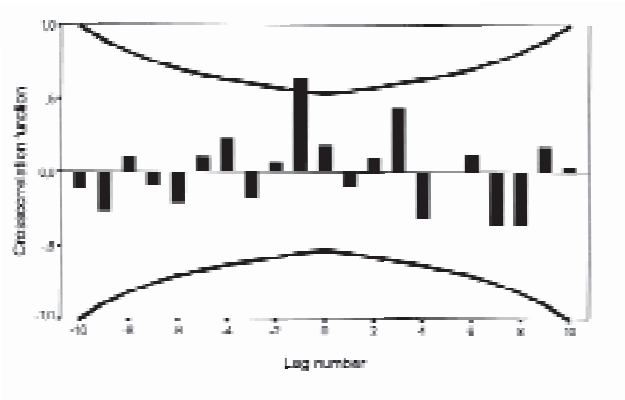

Figure 4. Cross-correlation function of incidence of death from myocarditis with bank vole abundance, 1973–1986. Time series are log transformed; n = 14 computable 0-order correlations. Lines represent $\pm$ 2 SE. The standard error is based on the assumption that the series are not cross-correlated and one of the series is white noise.

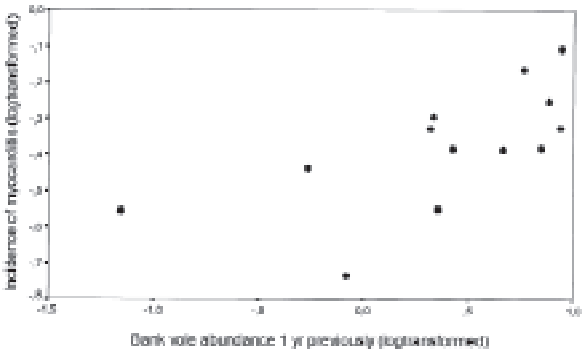

Figure 5. Myocarditis deaths, 1974–1986, relative to bank vole abundance 1 year previously (vole data from 1973-1985). Log transformed data. r = 0.635, n = 13.

Guillain-Barré Syndrome

In five cyclic counties and two noncyclic counties, where complete data were available, 258 GBS patients (≤ 46 years of age) were hospitalized in 1973 to 1982; the sex ratio was 1.1:1 (135 male, 123 female patients), 104 from the cyclic and 154 from the noncyclic area.

No correlation was found between GBS in the cyclic and noncyclic areas by cross-correlation of the disease series (CCF not shown; n = 10 computable 0-order correlations). Cross-correlation of the annual incidence of GBS in cyclic areas in 1973 to 1982 with bank vole abundance (Figure 6) suggested that the incidence of GBS co-varied with current vole abundance, as shown by the high positive and significant correlation at lag 0 in the CCF (Figures 7, 8) (r = 0.757, $p < 0.05$, n = 10).

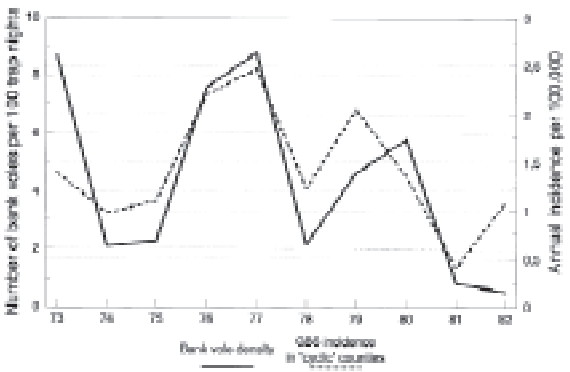

Figure 6. Time series of Guillain-Barré syndrome incidence, 1973–1982, relative to bank vole abundance in the same years. Untransformed data.

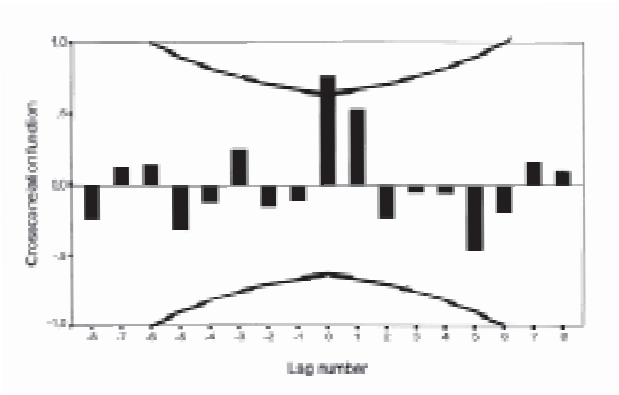

Figure 7. Cross-correlation function of Guillain-Barré syndrome incidence with bank vole abundance, 1973–1982. Time series are log transformed; n = 10 computable 0-order correlations. Lines represent ± 2 SE. The standard error is based on the assumption that the series are not cross-correlated and one of the series is white noise.

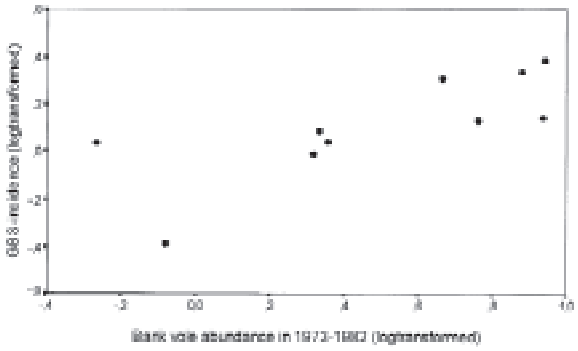

Figure 8. Guillain-Barré syndrome incidence, 1973–1982, relative to bank vole abundance in the same years. Log transformed data. r = 0.757, n = 10.

Insulin-Dependent Diabetes Mellitus

A total of 318 cases of IDDM were recorded in 1971 to 1991, representing a sex ratio of 1.3:1 (179 male, 139 female patients). No cross-correlation was found between the annual incidence of IDDM in the cyclic area and bank vole abundance (Figure 9)(CCF not shown; n = 19 computable 0-order correlations), as was found for myocarditis and GBS with vole abundance. On the other hand, after the alternative transformation (to log transformation) by differencing the IDDM and vole time series by 1 year, changes of the IDDM incidence from one year to the next covaried with the changes of vole abundance 2 years previously. This was suggested by the high positive and significant correlation at lag -2 in the CCF (Figures 10,11) (r = 0.595, $p < 0.05$, n = 16).

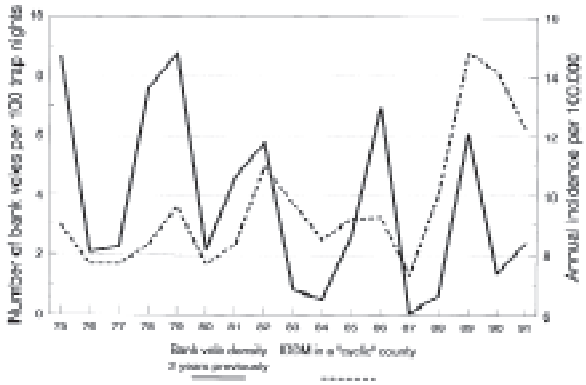

Figure 9. Time series of insulin-dependent diabetes mellitus incidence in 1975-1991 relative to bank vole abundance 2 years previously (vole data from 1973-1989). Untransformed data.

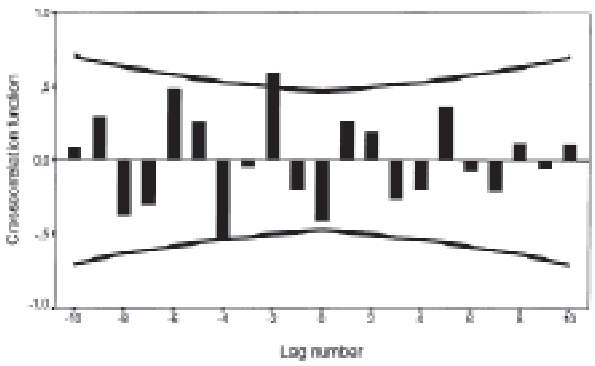

Figure 10. Cross-correlation function of insulin-dependent diabetes mellitus incidence with bank vole abundance, 1973–1991. Time series are differenced (1); n = 18 computable 0-order correlations. Lines represent $\pm$ 2 SE. The standard error is based on the assumption that the series are not cross-correlated and one of the series is white noise.

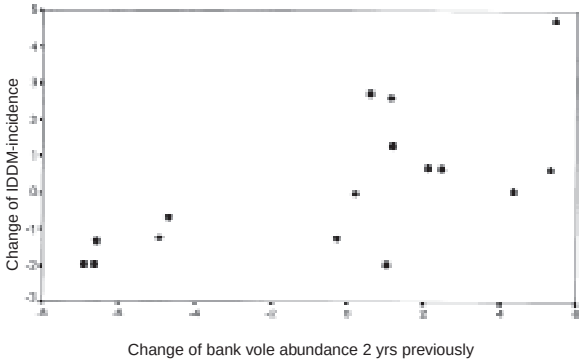

Figure 11. Change of insulin-dependent diabetes mellitus incidence, 1975–1991, relative to change of bank vole abundance 2 years previously, after transformation of time series by differencing (1) (vole data from 1973-1989). r = 0.595, n = 16.

Calculation of Confidence Limits

The confidence limits ($\pm$2 SE) for the most important cross correlations, at lag 0 for GBS (Figure 7), lag -1 for myocarditis (Figure 4), and at lag -2 for IDDM (Figure 10), were calculated in an alternative way to the default calculation in SPSS (12) according to formula 11.1.11, p. 377 (11). The alternative calculations were made according to formula 11.1.7, p. 376 (11). These calculations were made assuming that 1) the three disease time series represented white noise (i.e. contain no autocorrelation); 2) the vole time series contained autocorrelation, but it was zero after lag 5; 3) for the CCF of GBS with the vole time series, correlation existed at lag 0 and +1 but was zero at other lags; 4) for the CCF of myocarditis with the vole series, correlation existed at lag -1 but was zero at other lags; and 5) for the CCF of the differenced IDDM with the differenced vole time series, correlation existed at lag 0 to lag -4 but was zero at other lags and the differenced vole time series contained autocorrelation up to, but not after, lag 7. The calculations of the alternative confidence limits showed that the most important cross-correlations were significant at $p < 0.05$. The confidence limits for GBS at lag 0 were 0.757 $\pm$ 0.542; for myocarditis at lag -1, 0.635 $\pm$ 0.450; and for the differenced IDDM at lag -2, 0.595 $\pm$ 0.478.

Conclusions

Zoonotic disease reservoir or vector population density data can indicate a link between the etiologic agent and its reservoir/vector and between the agent and the disease. This type of data linked rodents to NE in Sweden in the 1930s and to Korean hemorrhagic fever in the 1950s (13-14). In a previous study, we found that the Puumala virus infection rate in bank voles in the spring tracked the vole density in the previous autumn and that infection rate peaked as vole population declined (5). We believe that this explains the approximately 9-month time lag in the incidence of NE in the spring relative to vole abundance in the previous autumn, although the incubation period in humans is only 2-6 weeks.

Our present data show significant temporal correlations of bank vole population density with incidence of death from myocarditis (Figures 3–5) and incidence of GBS (Figures 6–8). Our data also show a positive and significant correlation between changes in IDDM incidence and changes in bank vole density (Figures 9–11). We hypothesize that these three diseases are caused or triggered by one or more infectious agents carried by bank voles in Sweden. In this context, several sudden deaths among Swedish orienteers were recognized during 1989 to 1992. Six of approximately 200 elite orienteers died of acute myocarditis (15). Orienteering involves finding the fastest/shortest way between several checkpoints, often in forested areas. Because of extensive exposure to nature, it has been speculated that the sudden deaths of the orienteers were caused by a vector-borne (rodent or arthropod) infectious agent.

If our hypothesis is correct, the different time lags of different diseases relative to vole abundance, as seen in the present study, may depend in part on the specific infection rate dynamics of any agent in the bank vole population as seen in the case of NE (5). The time elapsed from infection to disease onset or death may also be important in IDDM and myocarditis.

The disease time series presented here were not very long, and the results should therefore be treated with caution. The present findings do not prove any direct link between the diseases investigated and the bank vole. Many infectious diseases and other biological phenomena have a cyclic variation, and the relationships presented here may be spurious or may involve another reservoir or vector population (e.g., small rodents and small game species that fluctuate synchronously with the bank vole at these latitudes) (4-5,7-8,16-17).

The identification of the etiologic agent or agents is critical to "prove" our hypothesis. However, as seen in several zoonotic diseases, humans are often dead-end hosts, and it may be easier to isolate the etiologic agent from the reservoir or vector than from the patient. Also, because of the time elapsed between primary infection and onset of disease, the infectious agent may be present only in very small amounts or absent. It is also possible that several human diseases with no etiologic agent identified are caused by agents that are difficult to cultivate, at least by standard techniques.

Thus, because we need to identify the etiologic agent(s) and it is difficult to isolate any virus from humans, we have initially attempted to isolate new viruses from small rodents, the primary suspects. The bank vole is the most common wild-living Swedish mammal. Its abundance varying by >300 times (4), this rodent is likely to have a high and highly variable potential over time to transmit any etiologic agent to humans. In addition, bank voles are known to enter buildings, thus transporting disease risk closer to humans.

Three novel virus isolates from bank voles, resembling picornaviruses in size and morphology, have been found. The first isolate was named Ljungan after the Ljungan river in Medelpad County, Sweden, where the animals were trapped. The second and third isolate originated from animals trapped in Västerbotten County. The amino acid sequences of predicted Ljungan virus capsid proteins were closely related (approximately 70% similarity) to the human pathogen echovirus 22. Partial 5' noncoding region sequence of Ljungan virus was most closely related to cardioviruses. Two additional isolates were serologically and molecularly related to the prototype (Niklasson et al., unpub. observation). Echovirus 22 is a known human pathogen, and other known cardioviruses can induce not only myocarditis but also neurologic diseases and IDDM in several species of animals.

We hope to elucidate the role of Ljungan virus as a human pathogen by serologic assays for Ljungan virus, diagnostic polymerase chain reaction based on generated sequence data, and specific antisera for viral antigen detection. Studies are also under way to identify and determine the role of other viruses of these small rodents in inducing myocarditis, GBS, and IDDM in humans.

Acknowledgments

We thank Sara Sjöstedt for statistical help and Stiftelsen Olle Engkvist, Byggmästare and by the Swedish Environment Protection Agency (via the National Environmental Monitoring Programme) for financial support for the vole trapping.

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Multidrug-Resistant *Mycobacterium tuberculosis*: Molecular Perspectives

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Multidrug-resistant strains of *Mycobacterium tuberculosis* seriously threaten tuberculosis (TB) control and prevention efforts. Molecular studies of the mechanism of action of antitubercular drugs have elucidated the genetic basis of drug resistance in *M. tuberculosis.* Drug resistance in *M. tuberculosis* is attributed primarily to the accumulation of mutations in the drug target genes; these mutations lead either to an altered target (e.g., RNA polymerase and catalase-peroxidase in rifampicin and isoniazid resistance, respectively) or to a change in titration of the drug (e.g., InhA in isoniazid resistance). Development of specific mechanism–based inhibitors and techniques to rapidly detect multidrug resistance will require further studies addressing the drug and drug-target interaction.

In the last decade, tuberculosis (TB) has reemerged as one of the leading causes of death (nearly 3 million deaths annually) (1). The estimated 8.8 million new cases every year correspond to 52,000 deaths per week or more than 7,000 each day, which translates into more than 1,000 new cases every hour, every day (2,3). These death rates, however, only partially depict the global TB threat; more than 80% of TB patients are in the economically productive age of 15 to 49 years. The emergence of AIDS and decline of socioeconomic standards contribute to the disease's resurgence in industrialized countries (4). In most developing countries, although the disease has always been endemic, its severity has increased because of the global HIV pandemic and extensive social restructuring due to rapid industrialization and conflicts. A major public health problem worldwide, TB is now a global emergency (Figure 1).

Short-course chemotherapy forms the backbone of antitubercular chemotherapy (5). Proper prescriptions and patient compliance almost always cure. In fact, TB incidence was steadily declining in most industrialized countries, until the trend was reversed (6). Further contributing to the increased death rate is the emergence of new strains of *M. tuberculosis* resistant to some or all current antitubercular drugs. The resistance is attributed primarily to improper prescriptions or patient noncompliance and is often a corollary to HIV infection (7-9). Multidrug-resistant TB (MDRTB), associated with high death rates of 50% to 80%, spans a relatively short time (4 to 16 weeks) from

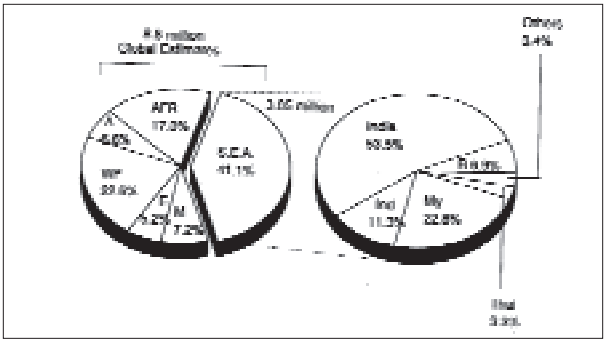

Figure 1. Global incidence of tuberculosis. Of the estimated 8.8 million cases worldwide, more than 40% of the cases are in Southeast Asia; India has approximately 53.3% of those cases. A, Americas; Afr, Africa; WP, Western Pacific; E, Europe; M, Eastern Mediterranean; and SEA, Southeast Asia; Ind, Indonesia; B, Bangladesh; Thai, Thailand; My, Myanmar. *Others include Bhutan, 0.05%; Nepal, 1.2%; Maldives, 0.001%; Sri Lanka, 1%; DPR Korea, 1.2%. (Data from reference 2).

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diagnosis to death (10). Delayed recognition of drug resistance, which results in delayed initiation of effective therapy, is one of the major factors contributing to MDRTB outbreaks, especially in health-care facilities (11,12). In most countries, MDRTB has increased in incidence and interferes with TB control programs, particularly in developing countries, where prevalence rates are as high as 48% (13,14). The high infection and death rates pose an urgent challenge to rapidly detect cases.

In the past few years, genetic and molecular insights have unraveled the mechanisms involved in the acquisition of drug resistance by *Mycobacterium tuberculosis* (MTB), concomitant with the development of various molecular strategies to rapidly detect MDRTB. In this review, we examine the status of the mechanisms of resistance to antitubercular drugs.

MDRTB and the Mechanisms of Resistance

Currently TB is treated with an initial intensive 2-month regime comprising multiple antibiotics—rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), and ethambutol (EMB) or streptomycin (SM)—to ensure that mutants resistant to a single drug do not emerge (15). The next 4 months, only RIF and INH are administered to eliminate any persisting tubercle bacilli. INH and RIF, the two most potent antituberculous drugs, kill more than 99% of tubercule bacilli within 2 months of initiation of therapy (16,17). Along with these two drugs, PZA, with a high sterilizing effect, appears to act on semidormant bacilli not affected by any other antitubercular drugs (18). Using these drugs in conjunction with each other reduces antitubercular therapy from 18 months to 6 months. Therefore, the emergence of strains resistant to either of these drugs causes major concern, as it leaves only drugs that are far less effective, have more toxic side effects, and result in higher death rates, especially among HIV-infected persons.

The phrase "MDR state" in mycobacteriology refers to simultaneous resistance to at least RIF and INH (19) (with or without resistance to other drugs). Genetic and molecular analysis of drug resistance in MTB suggests that resistance is usually acquired by the bacilli either by alteration of the drug target through mutation (20) or by titration of the drug through overproduction of the target (21). MDRTB results primarily from accumulation of mutations in individual drug target genes (Table). The probability of resistance is very high for less effective antitubercular drugs such as thiacetazone, ethionamide, capreomycin, cycloserine, and viomycin (10^{-3}); intermediate for drugs such as INH, SM, EMB, kanamycin, and p-amino salicylic acid (10^{-6}); and lowest for RIF (10^{-8}) (22,23). Consequently, the probability of a mutation is directly proportional to the bacterial load. A bacillary load of 10^9 will contain several mutants resistant to any one antitubercular drug (24). Because the mutations conferring drug resistance are chromosomal, the likelihood of a mutant being simultaneously resistant to two or more drugs is the product of individual probabilities; thus the probability of MDR is multiplicative. Resistance to a drug does not confer any selective advantage to the bacterium unless it is exposed to that drug (19). Under such circumstances, the sensitive strains are killed

Table. Gene loci involved in conferring drug-resistance in *Mycobacterium tuberculosis*

Drug	Gene	Product	Reported frequency in resistant strains[a] **(%)**	Reference
Rifampicin	*rpo*B	B-subunit of RNA polymerase	>95	45-48,68-71
Isoniazid	*kat*G	Catalase-peroxidase	60-70	39-48
	*oxy*R-*ahp*C	Alky hydro-reductase	~20	36
INH-Ethionamide	*inh*A	Enoyl-ACP reductase	<10	46-48
Streptomycin	*rps*L	Ribosomal protein S12	60	46-48
	rrs	16s rRNA	<10	113-117
Fluoroquinolone	*gyr*A	DNA gyrase	>90	107
Pyrazinamide	*pnc*A	Amidase	70-100	92-94
Ethambutol	*emb*CAB	EmbCAB	69	88

[a]Mutation frequencies are as determined by sequencing and polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis.

and the drug-resistant mutants flourish. When the patient is exposed to a second course of drug therapy with yet another drug, mutants resistant to the new drug are selected, and the patient may eventually have bacilli resistant to two or more drugs. Serial selection of drug resistance, thus, is the predominant mechanism for the development of MDR strains; the patients with MDR strains constitute a pool of chronic infections, which propagate primary MDR resistance. In addition to accumulation of mutations in the individual drug target genes, the permeability barrier imposed by the MTB cell wall can also contribute to the development of low-level drug resistance. Studies addressing resistance to SM have found evidence of such a two-step mechanism for the development of drug resistance (119,120).

Resistance to INH

INH (isonicotinic acid hydrazide, 4-pyridinecarboxylic acid hydrazide), highly active against the MTB complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*), has very low MICs (0.02 µg/ml to 0.06 µg/ml) (25). The mechanism of action of INH, as well as mechanisms conferring INH resistance, are complex and not completely understood (Figure 2). However, evidence suggests that INH inhibits the biosynthesis of cell wall mycolic acids (long-chain α-branched ß-hydroxylated fatty acids), thereby making the mycobacteria susceptible to reactive oxygen radicals and other environmental factors. Activation of INH to an unstable electrophilic intermediate requires the enzyme catalase-peroxidase (KatG, coded by *kat*G) and an electron sink (hydrogen peroxide) (26), although hydrazine formed after INH spontaneously decomposes may also mediate activation of INH (27). Nevertheless, KatG is the only enzyme capable of activating INH, and consequently, KatG mutant MTB strains are invariably INH resistant.

Early studies by Middlebrook demonstrated that INH resistance was associated with loss of catalase activity (28). Genetic studies demonstrated that transformation of INH-resistant *M. smegmatis* and MTB strains with a functional KatG restored INH susceptibility and put forth the hypothesis that *kat*G deletion may cause INH resistance in MTB (29,30). However, in the absence of a peroxide-inducible genetic response, mediated in most bacteria by the transcription factor OxyR (31), KatG is the only peroxide-inducible MTB protein (32). Consequently, MTB

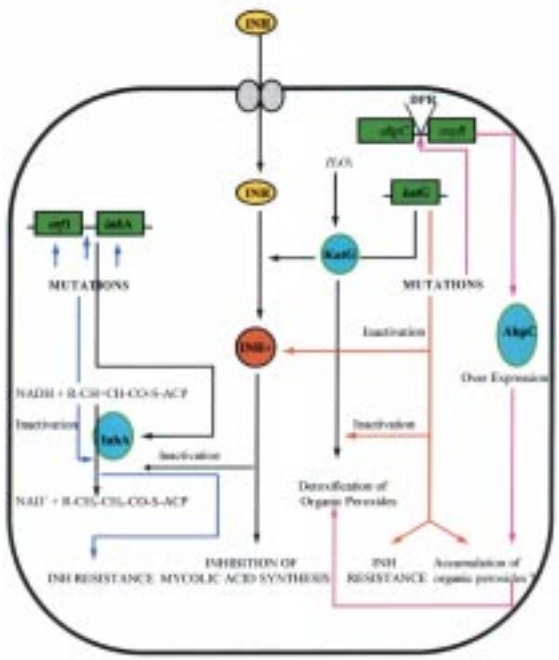

Figure 2. Mechanism of action of isoniazid (INH); acquisition of resistance and combating oxidative stress. DPR, divergent promoter region.

resistance to INH is paradoxical; it has to sacrifice KatG function. MTB's ability to adapt to the loss of KatG function and combat organic peroxides is remarkable. Studies conducted by Sherman et al. demonstrated that all KatG mutant MTB strains overexpressed a 22-kD protein at levels significantly higher than INH-sensitive strains (33). Sequence analysis confirmed that this protein was similar to the earlier reported MTB AhpC protein. AhpC can detoxify organic peroxides and is homologous to other bacterial and eukaryotic proteins with alkyl hydroperoxidase and thioredoxin-dependent peroxidase activities (34,35). The 5' regions (39 to 81 bp upstream from the *ahp*C start codon) of each AhpC-upregulated (and *kat*G mutant) isolate contained mutations that could increase promoter activity; it was proposed that compensatory mutations in the *ahp*C promoters were selected in *kat*G mutant strains to combat oxidative stress (33). Subsequent studies using immunoblotting experiments demonstrated the consistency of AhpC upregulation among clinical isolates with complete deletion of *kat*G (36,37). *kat*G mutant isolates with variable residual KatG

activity did not have this strict linear relationship. Characterization of the *oxy*R-*ahp*C region further demonstrated that mutations responsible for AhpC upregulation occurred at low frequencies and were primarily G>C to A>T transitions localized in the *oxy*R-*ahp*C intervening region (36). Although the sequence alterations in the *oxy*R-*ahp*C region were predominantly restricted to INH-resistant isolates, not all alterations detectably increased the AhpC levels. The apparent rarity of AhpC upregulation among INH-resistant and *kat*G mutant isolates could be attributed partially to the rare occurrence of MTB strains with complete *kat*G deletion (38-41,44). Alternatively, among *kat*G mutant isolates, selection of AhpC upregulatory mutations may be subject to the selective pressure exerted by residual catalase-peroxidase activity (36). However, AhpC upregulation was not observed among MTB isolates with *kat*G315 codon mutations, which reportedly lead to more than a 20-fold decrease in KatG activity and confer high MICs against INH (>90 µg/ml) (42, 43). This inconsistency and rarity of AhpC upregulation among *kat*G mutant INH-resistant isolates indicates a more complex relationship between the two and underlines the need for in-depth studies to determine precisely the conditions regulating AhpC expression.

Clinical studies to validate the paradigm of *kat*G deletions and INH resistance showed that complete deletion rarely occurred (38-41). We constructed a 35-mer oligonucleotide probe specific for *kat*G gene. Southern hybridization demonstrated the presence of *kat*G in all INH-resistant isolates, precluding complete deletion of *kat*G gene as a dominant mechanism for INH resistance (44). Previous studies using polymerase chain reaction (PCR) amplification had also established these findings; sequence analysis of *kat*G from INH-resistant strains showed randomly distributed mutations, including point mutations and deletions and insertions of up to 1 to 3 bases (38-41). These mutations could disrupt the *kat*G gene, leading to the production of an inactive gene product or a gene product with compromised peroxidative activity. PCR amplification of the *kat*G gene followed by single strand conformational polymorphism (SSCP) detected mobility shifts supporting the presence of these mutations and thereby INH resistance.

Our analysis of the *kat*G gene by PCR-SSCP resulted in the amplification of the 237 bp fragment of the *kat*G gene and demonstrated a 67.3% (n = 19) correlation between mutations in the *kat*G gene and INH resistance (45). The results were consistent with those from earlier studies indicating that *kat*G gene mutations had a correlation rate of less than 60% to 70% with INH resistance (46-48). Sequence analysis of INH-resistant strains demonstrating altered SSCP patterns showed that the most common mutation was G>T transversion in codon 463 (42). In this G>T change, Leu is substituted for Arg, and the restriction site for *Nci*I and *Msp*I is lost (40). Polymorphism in the *kat*G locus can then be easily detected by restriction digestion. Recent kinetic and spectroscopic studies have demonstrated striking similarities between KatG from wild-type strains and the R463L mutant isolates (49). Both enzymes had similar visible and electron-paramagnetic-resonance spectra and similar ability to oxidize INH and inactivate InhA. Further, when the INH-resistant *kat*G-defective strains of *M. smegmatis* with wild-type *kat*G or the R463L *kat*G were transformed, INH susceptibility was restored to about the same extent (50). These similarities do not support the contention that the R463L mutation of *kat*G allows discrimination against INH as a substrate and thereby confers resistance to INH. Although the exact role of the R463L mutation of *kat*G requires further scrutiny, this mutation may be a frequent polymorphism and may not affect INH susceptibility.

Other common mutations resulting in an attenuated KatG have been identified primarily as missense mutations that result in single amino acid substitutions (46-48). While the data point towards mutations in the *kat*G gene as the dominant mechanism for INH resistance, they also point to other factors that could mediate MTB acquisition of resistance to INH.

Mutations in the *oxy*R regulon, from which *Ahp*C is divergently transcribed, could explain the acquisition of INH resistance in the remaining INH-resistant isolates (33,51). OxyR confers high-level intrinsic resistance to INH in *Escherichia coli* and *Salmonella* Typhimurium; mutations in the *oxy*R or *Ahp*C restore INH susceptibility in these species (51). The MTB *oxy*R regulon is much smaller than in *M. leprae* and other mycobacteria—because of two important deletions of 29 bp and 372 bp (32,52). In addition to these deletions, the *oxy*R regulon carries many frame shift mutations, which result in low expression of this regulon and eventually lead to low-level expression of AhpC (consistent

with the finding of low-level expression of AhpC in INH-sensitive strains vs. INH-resistant strains) (33). A related member of the genus resistant to INH, *M. leprae*, however, has a complete *oxy*R-*ahp*C region that is transcriptionally fully active and may play a role in the detoxification of active INH intermediates (52). By analogy, therefore, the loss of the OxyR function, in conjunction with its putative effects on *ahp*C expression, could explain the exquisite specificity of INH for the MTB complex. However, evidence from recent studies does not indicate a direct role for *oxy*R or the *ahp*C genes in determining susceptibility to INH (36,37). Polymorphisms in *oxy*R do not have any preferential predisposition and exist among both INH-resistant and -susceptible isolates with about the same frequency (36). The relationship of AhpC overexpression to INH resistance is more complex. Earlier observations based on transformation of *M. smegmatis* strains suggested a possible involvement of AhpC overexpression in acquiring INH resistance (53). Transformation of *M. smegmatis* isolates with multicopy constructs of *ahp*C led to almost a fivefold increase in the MIC for INH. However, an increasing body of evidence precludes any direct role of AhpC in determining INH susceptibility among MTB isolates. MTB transformants bearing multicopy constructs of *ahp*C did not demonstrate significant increase in the MIC for INH, thus any direct role for AhpC in acquisition of INH resistance was ruled out (37).

Efforts to determine the factors involved in resistance to INH led to the discovery of the *inh*A locus, which was proposed as the primary target for coresistance to INH and ethionamide (54). This locus is composed of two open reading frames (ORFs), designated *orf*1 and *inh*A, separated by a 21-bp noncoding region. InhA, an enoyl-ACP reductase (55), more than 40% homologous to the EnvM protein, catalyzes an early step in fatty acid synthesis among enterobacteria. Like EnvM, InhA activity is also thought to use NAD(H) as cofactor. INH susceptibility could result from incorporation of iso-NAD, which is formed as a consequence of the action of KatG on INH, and thus hinders the enzymatic activity of InhA and blocking fatty acid synthesis (56). A T>G transversion, observed in few of the resistant strains, at position 280 in the *inh*A gene, results in the ser94 to ala94 replacement (54). This replacement, thought to alter the binding affinity of InhA to NAD(H), ultimately results in INH resistance (57). Alternatively, because of mutations in the putative promoter region, hyperexpression of InhA could result in INH resistance.

Studies conducted in clinical settings to provide corroborating evidence of mutations in the *inh*A locus and INH resistance have shown approximately 10% correlation (46-48). Analysis of 37 INH-resistant isolates by Kapur et al. demonstrated no ser94-ala94 substitution in the resistant isolates. Only one isolate had a missense mutation: ATC>ACC at position 47, resulting in substitution of Ile16 by Thr16. Morris et al. also demonstrated the lack of mutations in the *inh*A gene among 42 INH-resistant MTB isolates. However, five of the INH-resistant isolates showed single nucleotide mutations in the putative *inh*A regulatory region upstream of *orf*1.

Subsequent biochemical characterization of InhA function demonstrated that it catalyzed the reduction of 2-trans-octenoyl-acyl carrier protein and also that protein of enoyl CoA esters (58-590, thereby acting at the final step in chain elongation in fatty acid synthesis (58). This observation contradicted earlier biochemical evidence suggesting that an enzyme involved in the synthesis of an unsaturated 24-carbon fatty acid was the target for activated INH (60,61). Thus, the targets identified biochemically and by complementation of *M. smegmatis* are different. Lipid pulse labeling experiments demonstrated that the lipid biosynthetic response of *M. smegmatis* and MTB after exposure with INH were different (62), indicating a different mechanism of action for the INH intermediate in the two species. Transformation of *M. smegmatis* with single-copy alleles of mutant *inh*A loci did not result in significant resistance to INH, indicating the presence of a different promoter in *M. smegmatis*. Further, the inability of multicopy vector constructs bearing the *inhA* gene to significantly increase the MIC for INH provided substantiating evidence for the limited involvement of this locus in mediating INH resistance among MTB isolates. These data, along with clinical evidence, preclude the likelihood that *inh*A is the primary target for the activated form of INH.

Functional characterization of *inh*A mutations, occurring with *kat*G mutations (as observed in isolates with very high MICs) (46) in relation to lipid metabolism of INH-resistant isolates, could perhaps resolve this discrepancy

and delineate the roles of the respective loci in the mechanism of action of INH and subsequent acquisition of drug resistance.

In summary, mutations in the *kat*G and the *inh*A genes are associated with approximately 70% to 80% of INH-resistant MTB isolates; molecular mechanisms operating in the remaining isolates are still unknown. The role of the MTB cell wall as an important permeability barrier needs to be explored in greater detail, particularly with reference to INH resistance (56).

Resistance to RIF

RIF, first introduced in 1972 as an antitubercular drug, is extremely effective against MTB. It has MICs of 0.1 µg to 0.2 µg (16,63). Because of its high bactericidal action, RIF, along with INH, forms the backbone of short-course chemotherapy (5). Although rare, resistance to RIF is increasing because of widespread application and results in selection of mutants resistant to other components of short-course chemotherapy. In this context, resistance to RIF can be assumed to be a surrogate marker for MDRTB (19). RIF had long been believed to target the mycobacterial RNA polymerase and thereby kill the organism by interfering in the transcription process (64). Using purified RNA polymerase from *M. smegmatis*, strain mc^{2}155, Levin and Hatfull demonstrated that RIF specifically inhibited the elongation of full-length transcripts and had virtually no effect on the initiation of transcription (65).

RNA polymerase, a complex oligomer composed of four different subunits (α,ß,ß'and σ, encoded by *rpo*A, *rpo*B, *rpo*C, and *rpo*D, respectively), is highly conserved among bacterial species (66). Characterization of the *rpoB* gene in *E. coli* demonstrated that RIF specifically interacted with the ß subunit of RNA polymerase, thereby hindering transcription, and that mutations in the *rpo*B locus conferred conformational changes leading to defective binding of the drug and consequently resistance (67). Subsequently, the *rpo*B locus from MTB was characterized and mutations conferring the resistant trait were identified (Figure 3; 68-71). Most mutations were determined to be restricted to an 81-bp core region and are dominated by single nucleotide changes, resulting in single amino acid substitutions, although inframe deletions and insertions also occur at lower frequencies. Changes in the codons Ser531 and His526 have been documented in more than 70% of the RIF-resistant isolates. A very small number of mutations in RIF-resistant isolates do not map in this 81-bp core region; it is speculated that additional mechanisms, including RIF permeability and mutations in alternate subunits of RNA polymerase, may also be involved in conferring the resistance phenotype.

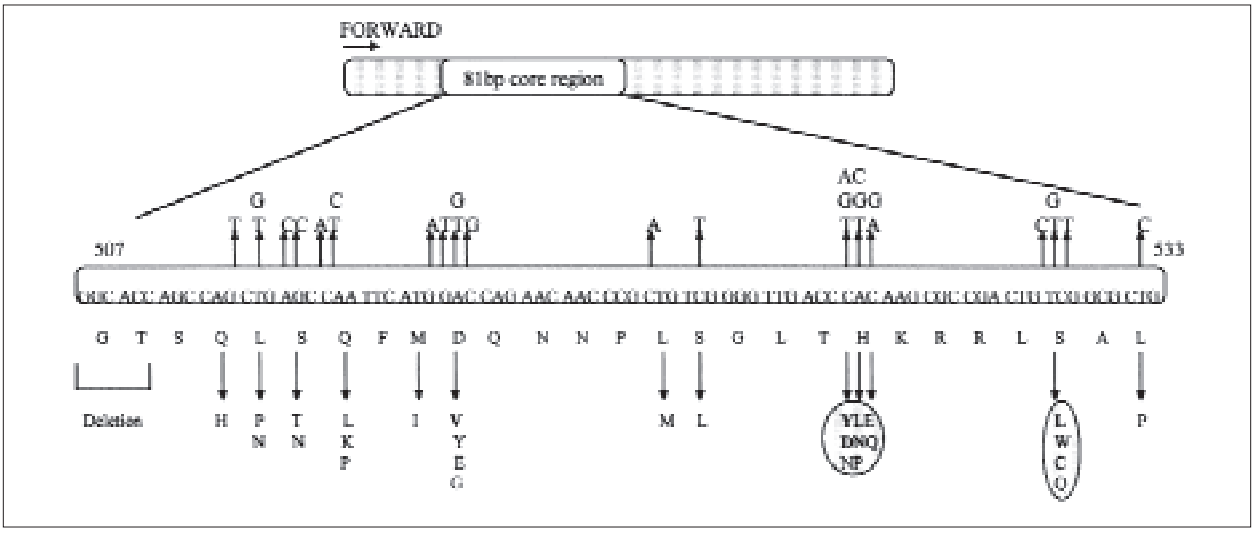

Figure 3. Single amino acid substitutions in the 81 bp core-region of the *rpo*B gene responsible for conferring rifampicin (RIF) resistance (Insertions and deletions that confer the RIF-resistance phenotype are not depicted). Amino acids are represented with single letter abbreviations. Changes in codon Ser531 and His526 account for more than 70% of the mutations with RIF resistance (depicted in shaded ellipses).

The consistency of mutations in the *rpo*B locus and the RIF- resistant phenotype (>95%) has marked clinical implications. Because it may act as a surrogate marker for MDRTB, RIF resistance has prompted development of various diagnostic tests to improve the sensitivity of mutation detection. Although automated sequencing has been unambiguously applied to characterize mutations associated with RIF resistance, a number of other techniques such as PCR-SSCP (41,45-48,121), dideoxy fingerprinting (72), heminested PCR (73), PCR heteroduplex analysis (70), and line probe hybridization (74,75) have been successfully applied to detecting these mutations. Such novel strategies to detect drug-resistant MTB isolates have been described elsewhere (76). PCR-SSCP analysis for detection of mutations responsible for conferring drug-resistance is increasingly useful. In particular, the development of nonisotopic PCR-SSCP analysis has simplified the procedure, enhancing its utility in routine laboratories (41,45). However, results obtained with SSCP analysis should be interpreted with caution as the technique only detects mutations and gives no information on the nature of associated mutation. For example, silent mutations in the *rpo*B gene have been identified that give altered mobility patterns on SSCP analysis but have no association with RIF resistance, which underlines the need for caution in interpreting results and phenotypic or genotypic correlation (77).

Resistance to EMB

EMB [dextro-2,2'-(ethyldiimino)-di-1onol], synthetic compound with profound antimycobacterial effects (78), is a first-line anti-MTB drug with a broad spectrum of activity, unlike INH. EMB is also advocated in disseminated *M. avium* complex infections, particularly in HIV-infected persons (79). Until recently, EMB's mechanism of action and the genetic basis for resistance to it were largely obscure. Specificity of EMB for mycobacterial species, however, indicated that its target may have been involved in the construction of the outer cell wall. Synergy resulting from coadministration of EMB and other drugs gave further evidence for the involvement of EMB in obstructing the formation of cell wall. The synergistic effect was explained as a consequence of increased permeability of the mycobacterial cell wall leading to increased drug uptake (80,81). Indeed, earlier studies of Takayama and colleagues demonstrated that administration of EMB led to rapid cessation of mycolic acid transfer to the cell wall and equally rapid accumulation of trehalose mono- and di-mycolates (82,83). Mycolic acids attach to the 5'-hydroxyl groups of D-arabinose residues of arabinogalactan and form mycolyl-arabinogalactan-peptidoglycan complex in the cell wall. Disruption of the arabinogalactan synthesis inhibits the formation of this complex and may lead to increased permeability of the cell wall. Subsequently, it was demonstrated that EMB specifically inhibited arabinogalactan synthesis (84).

A breakthrough was achieved in defining the precise cellular target for EMB with the isolation and identification of ß-D-arabinofuronosyl-1-monophosphoryl decaprenol (DPA), which accumulates rapidly (less than 2 minutes) on exposure of EMB- sensitive cells to EMB (86). DPA is an arabinosyl donor; cell-free assay systems developed for DPA established that it was one of the major intermediates of arabinan synthesis. It was later shown that EMB specifically inhibited arabinosyl transfer, suggesting that arabinosyl transferase was the primary cellular target for EMB (Figure 4).

Identification of arabinosyl transferase as the primary target for EMB helped unravel the

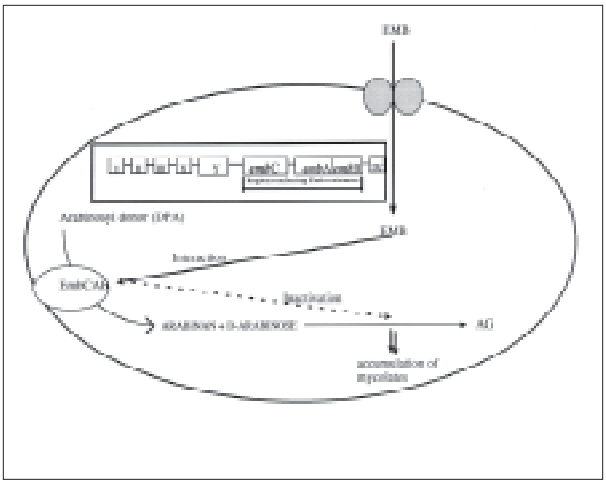

Figure 4. Mechanism of action of ethambutol (adapted from 84-88). EMB interacts with the EmbCAB proteins encoded by the *emb*C, *emb*A, and *emb*B genes, leading to inactivation of arabinogalactan synthesis. Mutations in the *emb*B locus cause alterations in EmbB, possibly leading to an altered target for EMB. Alternatively, hyperexpression of the EmbCAB proteins could lead to EMB resistance. *Inlet box*: Organization of the *emb* operon in *Mycobacterium tuberculosis* (MTB).

genetic basis for EMB resistance. Using target overexpression by a plasmid vector, Belanger et al. cloned the *emb* locus from an EMB-resistant strain of *M. avium* (86). Transformation of this *emb* locus conferred resistance to *M. smegmatis* mc^2155 strain and also demonstrated that the level of resistance conferred depended on the copy number of the gene, which was consistent with the notion of drug resistance due to target overexpression. Site-directed mutagenesis and overlapping clone analysis localized a 9.8-kb EMB resistance locus, subsequently shown to be ubiquitous among mycobacteria. Sequence analysis of this locus revealed three complete ORFs—designated *emb*R, *emb*A, and *emb*B. The *emb*R ORF is separated by a 178 bp divergent promoter region from the *emb*A and *emb*B ORFs. Characterization of the *emb*R ORF showed that the region was strongly homologous with a family of transcriptional activators of *Streptomyces* and thus could play a role in modulating the expression of *emb*A and *emb*B. Importantly, the *emb*B ORF lacks a potential ribosome binding site and is thus translationally coupled to *emb*A, which suggests that a heterodimeric enzyme complex may be the target for EMB. Mapping studies further demonstrated that both *emb*A and *emb*B, along with the divergent promoter region, were essential to EMB resistance.

In contrast to the organization of the emb locus in *M. avium*, molecular genetic approaches applied to MTB revealed a highly conserved 14-kb region comprising three homologous ORFs designated embC, embA, and embB preceded by a predicted coding region and by orfX (which encodes a putative protein belonging to the short chain alcohol dehydrogenase family) (87). Primer extension analysis of the emb region supported the notion of its organization as an operon and further indicated the polycistronic nature of its transcripts. The emb genes are translationally coupled the absence of any untranslated intercistronic region between the emb genes so indicated). However, the presence of a secondary stem loop structure between the embA and the embB genes indicates that the embB gene in MTB could be differentially regulated. The embCAB proteins are believed to be integral membrane proteins, consistent with their role in the synthesis of various arabinan-linkage motifs of the arabinogalactan and lipoarabinomannan (86,87).

Identification of the *emb*CAB genes prompted a detailed analysis of the molecular mechanisms responsible for conferring resistance to EMB in MTB isolates. Preliminary studies documented among EMB-resistant isolates missense substitutions in the conserved *emb*B codon 306 that coded for methionine; their role in conferring resistance to EMB was confirmed by gene tranfer assays (87). Recent analysis of the *emb*CAB region has confirmed the predominance of *emb*B Met306 substitutions among EMB-resistant clinical isolates of MTB (approximately 89% among EMB-resistant isolates with single amino acid substitutions) (88). Sequence analysis of 118 clinical isolates of MTB showed five mutants of the *emb*B codon 306, all leading to substitution of Met with Val, Leu, or Ile. MTB strains with Met306Leu and Met306Val substitutions demonstrated a higher MIC for EMB (40 µg/ml) than those for organisms with Met306Ile substitutions (20 µg/ml). The *emb*B codon 306 may contain important structure-function information; structural alterations in this codon may have a detrimental effect on the interaction of EMB and EmbB, thereby resulting in a EMB-resistant phenotype.

Sequence alterations in the *emb*CAB region correlate with approximately 70% of EMB-resistant strains. Overexpression of the EmbB protein has been documented to mediate resistance in *M. smegmatis* (87), and a homologous mechanism may operate in MTB, perhaps accounting for the remaining 30% of the EMB-resistant isolates. A full understanding of the mechanisms for acquisition of EMB resistance among these isolates requires further studies.

Resistance to PZA

PZA, a structural analog of nicotinamide, was shown to have considerable anti-MTB activity in 1952, but it became an important component of short-course chemotherapy only in the mid-1980s. PZA, active against semidormant bacilli not affected by any other drug, has strong synergy with INH and RIF and shortens the chemotherapeutic schedule for antitubercular treatment from 9 to 12 months to 6 months (15). Depending on the assay system and conditions applied, MICs of PZA vary from 8 µg/ml to 60 µg/ml. However, even at very high MICs, PZA has no significant bactericidal effect and is primarily considered a “sterilizing drug” (18). Activity of PZA is highly specific for MTB; PZA has scant or no effect on other mycobacteria, including *M. bovis*, which demonstrate high-level intrinsic

resistance to PZA (89). Naturally resistant strains of *M. bovis* lack the enzyme Pzase, which hydrolyzes PZA to pyrizinoic acid, the presumed active form of PZA (90,91). PZA in this context is similar to INH; it is transported as a neutral species into the cell, where it is converted into its active form. This notion was strengthened by evidence provided by in vitro studies that demonstrated the susceptibility of PZA-resistant MTB and *M. bovis* to pyrizinoic acid. MTB Pzase has both pyrazinamidase and nicotinamidase activities (90). Using sequence information of *E. coli* nicotinamidase, Scorpio and Zhang isolated the mycobacterial *pnc*A gene, which codes for the amidase (92). Characterization of the *pnc*A gene from *M. bovis* isolates identified a single point mutation that results in the substitution of His to Asp at position 57. This substitution results in the production of an ineffective Pzase in *M. bovis* strains. Point mutations in the *pnc*A gene of PZA-resistant MTB strains were also identified. Substitution of Cys138 with Ser, Gln141 with Pro, and Asp63 with His and deletion G nucleotide at positions 162 and 288 resulted in a defective Pzase. Transformation of Pzase-resistant strains with functional construct of MTB *pnc*A gene restored susceptibility to PZA, providing further evidence that mutations in the *pnc*A gene were responsible in conferring the resistant phenotype. Subsequent characterization of the *pnc*A gene from clinical isolates of MTB confirmed these findings (93,94). Mutations including missense alterations, nucleotide insertions or deletions, and termination mutations have been found in the *pnc*A gene from PZA-resistant MTB isolates. These sequence alterations are interspersed along the entire length of the *pnc*A gene, demonstrate limited degree of clustering, and vary in frequency from 70% to 100% (93,94). The absence of correlating mutations in the *pnc*A gene from PZA- resistant MTB isolates indicates that perhaps at least one additional mechanism mediates resistance to PZA.

The cellular target for PZA, however, has not been identified, although the apparent similarity of PZA to nicotinamide suggests that enzymes involved in pyridine nucleotide biosynthesis are probable targets. Implication of the *pnc*A gene in conferring PZA-resistant phenotype has profound clinical applications. Application of PCR-SSCP for detection of mutations in the *pnc*A gene could help circumvent the difficulties in determining PZA susceptibilities (96) and rapidly discriminate between MTB and *M. bovis* (96).

Resistance to Fluoroquinolones (FQ)

FQs as antimycobacterial agents were first described in 1984 and have primarily been used as therapeutic alternatives in MDRTB cases (97). DNA gyrase (Gyr), a member of the type II DNA topoisomerases (98), is the primary target for FQ action. Gyr introduces negative supercoils in closed circular DNA molecules and is a heterotetramer (A_2B_2), coded by *gyr*A and *gyr*B respectively (99,100). Quinolone sensitivity is determined by the GyrA protein, which contains the cleavage/ religation activity (100), while GyrB contains the intrinsic coumarin-sensitive ATPase activity (101).

FQs, synthetic derivatives of nalidixic acid, act by inhibiting DNA supercoiling and relaxation activity of Gyr without affecting the ATPase activity (102) and enhance the rate of DNA cleavage by Gyr. Quinolone-mediated cleavage of double-stranded DNA results in a 4 bp 5' overhangs on either strand, to which GyrA subunits become attached covalently by O^4 phosphotyrosine bond (103). Gyr catalyzes the cutting of DNA, denaturation of the overhang, and strand separation. The exact mechanism of inhibition of Gyr activity with respect to quinolones remains unknown. However, quinolone drugs bind with a greater affinity to single-stranded DNA than double-stranded DNA and possibly do not bind to Gyr at all (104). Consequently, by binding to the single-stranded DNA, the quinolones may inhibit religation, thereby imposing an effective transcriptional block (105), culminating in cellular death. However, questions about the specific interaction of quinolones and the Gyr/ DNA complex remain unsolved (106).

Cloning and expression of the MTB *gyr*A and *gyr*B genes allowed mapping of mutations that confer resistance to FQs (107). Mutations were found to be clustered in a small region in GyrA that is close, approximately 40 residues amino-terminal, in the linear amino acid sequence to the active site tyrosine, Tyr122 (*E. coli* numbering) (108). Other single amino substitutions, for residues 88 to 94, were also identified in ciprofloxacin-resistant MTB isolates (Figure 5). Because polymorphism encountered at codon 95 (Ser95>Thr95) occurred in both resistant and susceptible isolates, it may not be involved in

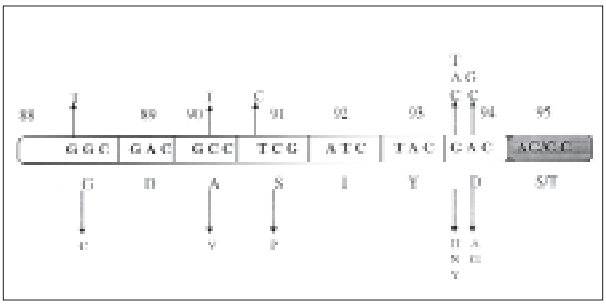

Figure 5. Single-amino acid substitutions responsible for conferring resistance to fluoroquinolones (FQ). Mutation in the Ser95 codon (shown in stippled box), observed in both FQ-sensitive and FQ-resistant isolates, rules out its role in acquisition of resistance.

acquiring the FQ-resistant phenotype. Alternative mechanisms to *gyr*A mutations, including changes in cell wall permeability and active quinolone efflux pumping, have also been proposed and could account for the low-level resistance among MTB isolates.

Newer FQ derivatives such as sparfloxacin have shown greater anti-MTB potency (MIC = 0.2 µg/ml) than ciprofloxacin and ofloxacin, giving hope for better therapeutic alternatives for MDRTB. However, FQ susceptibility in the treated patient population must be continuously monitored to prevent low-level FQ-resistant strains from acquiring additional mutations that lead to high-level resistance (109).

Resistance to Streptomycin and Other Inhibitors Of Protein Synthesis

Various drugs exert their antibacterial effects by inhibiting the protein transitional machinery. Among these, aminoglycosides, macrolides, tetracyclines, and basic peptides like viomycin and capreomycin are active against mycobacteria (110). SM, one of the oldest drugs known to be active against MTB, disrupts the decoding of aminoacyl-tRNA and thus inhibits mRNA translation or causes inefficient translation (111). One of the most common mechanisms for acquisition of resistance to SM is acetylation of the drug by aminoglycoside-modifying enzymes (111,112). However, this mechanism is not found in MTB. Instead, resistance to SM is attributed, at least partially, to two distinct classes of mutations including point mutations in S12 ribosomal protein, encoded by *rps*L gene (113), and mutations in the *rrs* operon encoding the 16S rRNA (114).

Point mutations in the *rps*L gene result in single amino acid substitutions (114-117) that affect higher order structures of 16S rRNA and thereby confer SM resistance. Mapping of the mutations in the *rps*L gene demonstrated that they primarily affected one of the two critical lysine residues at positions 43 and 88 and led to the substitution with either arginine at 88 or arginine and threonine at position 43 (115). An SM-resistant isolate (>60 µg/ml) showed an A>G transversion at position 904 in the 16S rRNA with an additional single A>C transversion in the *rps*L gene, which resulted in the substitution of Lys-Gln at position 88 (115). Because each of the corresponding mutations in the small subunit rRNA or the ribosomal protein S12 confer the resistant phenotype in *E. coli*, these mutations mediated ribosomal drug resistance and were responsible for conferring high-level SM resistance. Mutations in the *rps*L gene accounted for more than two thirds of SM-resistant cases.

The genesis of SM resistance in some of the SM-resistant isolates is due to point mutations in the 16S rRNA. Mutations in the *rrs* locus have been mapped to two regions, the 530 loop and the 915 region. Within the 530 loop, C>T transitions at 491, 512, and 516, in addition to the A>C transversion at position 513, are consistent with the SM-resistant phenotype (114) pseudoknot formation within the MTB 16S rRNA. Base pairing between residue 524-526 (of the 530 region of the hairpin loop) and residue 504-507 (of the adjacent 510 region bulge loop) (118) results in SM resistance in clinical isolates of MTB (114). Further, G-U wobble base pairing between residues 522-501 stabilizes the pseudoknot formation and thereby confers resistance to SM. It can thus be concluded that SM resistance in MTB stems from alterations of the drug target and not by drug modification.

However, no mutations in the *rps*L and the *rrs* genes are detected in a significant number of SM-resistant isolates (46,48). Curiously, intrinsically SM-resistant strains of *M. gordonae*, *M. szulgae*, and *M. avium* do not show any alterations in the *rps*L or the *rrs* genes, suggesting a probable third factor in conferring SM resistance. Earlier studies have documented the inhibitory effect of SM on protein synthesis in vitro to the same extent as observed in wild-type MTB strains. The same inhibitory effect was not observed on whole cells,

suggesting the probable role of cell wall permeability barrier in conferring SM resistance (119). More recently, it has been demonstrated that membrane-active substances augmented the MIC for SM in strains with alterations in the *rrs* genes, thus providing further evidence for a probable role of the MTB-permeability barrier in mediating resistance to SM (120).

Resistance to Other Drugs

Related aminoglycosides such as kanamycin, amikacin, and paromomycin demonstrate no obvious cross-resistance to SM and thus are alternatives in cases of SM resistance. Viomycin and capreomycins are bacteriostatic agents that act by binding to the 50S ribosomal subunit and inhibit the translocation reaction (111). Although cross-resistance between viomycin and capreomycin does occur, the exact mechanism for acquisition of drug resistance is not known.

Conclusions

Molecular insights suggest that accumulation of mutations in the individual drug target genes is the primary mechanism of MDRTB. Morris and colleagues' investigation of the molecular mechanisms of drug resistance in MDR strains found that 25 of 44 SM-resistant strains had mutations in the *rps*L gene, while five others had *rrs* gene perturbations (48). The *rpo*B gene had mutations in 28 of 29 RIF-resistant strains. Mutation in the *kat*G gene was seen in 20 of the 42 INH-resistant stains, while five had *inh*A gene mutations. Of the 20 MDRTB strains, 11 had mutations in genetic markers associated with resistance to each of these three drugs.

Similarly, Heym et al. reported that resistance to antitubercular agents in their collection of strains resulted from alterations to chromosomal genes encoding the drug targets; they excluded the possibility that MDRTB stemmed from acquisition of genes for novel resistance determinants (46). MDR appeared to result from the stepwise acquisition of new mutations in the genes for different drug targets. In all cases exactly the same mutations or combination of mutations were observed, regardless of the patient's HIV status.

Thus, the origin of MDRTB is due more to treatment difficulties, including noncompliance and administration of inadequate treatment regime, and not to the emergence of novel resistance mechanisms; this is reassuring for the future of short-course chemotherapy. Administration of directly observed combination chemotherapy (or Directly Observed Treatment Short-Course [DOTS]) appears to be the most effective way to ensure a decrease in primary resistance, acquired resistance, and relapses (3). DOTS has been successfully implemented in diverse geographic areas including Tanzania, Guinea, China, Bangladesh, New York City, and Peru, which reported more than a 90% cure rate (3). Nearly 70 countries have adapted DOTS as a part of their national TB control programs and achieve good cure rates. Successful implementation of DOTS in the coming decades requires not only a concerted effort from various funding agencies but also a strong social and political commitment. Apart from strategic interventions based on strong political will, grass-roots action will have to be strengthened mainly at the primary health-care level to check the unlimited upsurge of this preventable fatal disease. Basic research will have to be continually updated to prevent the drug-resistant strains from becoming an unmanageable clinical paradigm. DOTS currently is our only option to reverse the global TB epidemic and prevent MDRTB.

The inability to detect resistance early, however, is one of the major factors involved in the genesis and control of MDRTB; this invariably results in prolonged exposure to drugs that are virtually ineffective. One of the major consequences of unraveling the genetic basis of drug resistance in MTB is the development of various molecular strategies to rapidly detect MDRTB (76). However, the sheer multiplicity of gene loci to be investigated for diagnosis of MDRTB renders most of the approaches mentioned above as tedious and resource-intensive for a routine laboratory service program, particularly in developing countries like India, with limited resources and high disease incidence. Resistance to most anti-MTB drugs, with the exception of RIF, cannot be attributed to a single locus in substantial percentage (>90%), which is perhaps the greatest deterrent in the development of single amplification–based methods for rapid detection of resistance.

Working out the exact biochemical details of drug-drug target interaction acquires considerable attention in the era of MDRTB, because only then will more rational structure- and mechanism-based approaches to inhibitor design be

possible. Clearly, a concerted global effort is required to defeat TB resurgence.

Acknowledgments

We are indebted to Dr. Ellen Jo Baron and Prof. Eric C. Bottger for their suggestions and encouragement; Dr. James M. Musser for providing recent data on ethambutol resistance; and Swathi Arur, Nadeem Hasan, and Koninika Ray for help in preparing this manuscript.

Work in our laboratory is supported by financial grants from the Department of Biotechnology, Government of India.

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Hospitalizations for Unexplained Illnesses among U.S. Veterans of the Persian Gulf War

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Persian Gulf War veterans have reported a variety of symptoms, many of which have not led to conventional diagnoses. We ascertained all active-duty U.S. military personnel deployed to the Persian Gulf War (552,111) and all Gulf War era military personnel not deployed (1,479,751) and compared their postwar hospitalization records (until 1 April 1996) for one or more of 77 diagnoses under the International Classification of Diseases (ICD-9) system. The diagnoses were assembled by the Emerging Infections Program, Centers for Disease Control and Prevention, and are here termed "unexplained illnesses." Deployed veterans were found to have a slightly higher risk of hospitalization for unexplained illness than the nondeployed. Most of the excess hospitalizations for the deployed were due to the diagnosis "illness of unknown cause" (ICD-9 code 799.9), and most occurred in participants of the Comprehensive Clinical Evaluation Program who were admitted for evaluation only. When the effect of participation in this program was removed, the deployed had a slightly lower risk than the nondeployed. These findings suggest that active-duty Gulf War veterans did not have excess unexplained illnesses resulting in hospitalization in the 4.67-year period following deployment.

The Persian Gulf War was one of the briefest full-scale conflicts in U.S. history. For a 2-month period of fighting ending in March 1991, nearly 700,000 U.S. service members were deployed to the Persian Gulf region. Since returning from the war, many veterans have reported unexplained symptoms (1-5), prompting allegations of a new disease or diseases (2,3,5,6). Numerous expert panels and research projects have examined illness and death among Gulf War veterans (4,7,8). However, with the exception of self-reported symptoms (8-14), no consistent pattern of increased illness or death has been reported (10,14-18).

The U.S. Department of Defense (DoD) conducted an epidemiologic comparison of the postwar DoD hospitalizations of service members deployed to the Gulf War and service members of the same era not deployed (15). In the 2-year period after the war, no consistent increase in the overall risk for hospitalization (or specific risk for hospitalization for various broad diagnostic categories) was found for those deployed. This study relied upon diagnoses based on the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9) system (19). Medical providers may not consistently classify a new or poorly recognized syndrome, and consequently a true difference in hospitalization risk could be spread across numerous diagnostic categories and remain undetected.

The present study compares the postwar DoD hospitalizations for diagnoses consistent with an unexplained illness of Persian Gulf War veterans and their nondeployed peers.

Analytic Approach

Study Population

The study population consisted of active-duty service members (Army, Air Force, Navy, Marine Corps, and Coast Guard) who were either deployed to the Persian Gulf War for 1 or more days during the Gulf War deployment period (8 August 1990 through 31 July 1991) or were not deployed but were on active duty for at least part of the Gulf War deployment period. All deployed (n = 552,111) and nondeployed (n = 1,479,751) service members who remained on active duty at the end of the period were included in the study population.

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The study was restricted to active-duty personnel because they are rarely hospitalized outside DoD facilities. Members of the U.S. Reserve and National Guard forces were not studied because only a fraction of their postdeployment hospitalizations were in DoD facilities.

Study Outcome

The study outcome was determined by 77 ICD-9 diagnoses assembled by the Emerging Infections Program, Centers for Disease Control and Prevention, to monitor death certificates for unexplained deaths (20). These diagnoses span several of the 17 major categories delineated in ICD-9 and include selected diagnoses from diseases of the blood, nervous system, circulatory system, respiratory system, and digestive system; infectious and parasitic diseases; and symptoms, signs, and ill-defined conditions. These diagnoses primarily relate to nonspecific infections and other ill-defined conditions, and for convenience they are termed "unexplained illnesses" in this study.

All study population admissions to U.S. military hospitals worldwide (reported to the DoD computerized hospitalizations database by 1 October 1996), subsequent to the Gulf War deployment period and before 1 April 1996, were evaluated for unexplained illnesses among as many as eight ICD-9 diagnoses coded for each admission. For another analysis, only the first coded diagnosis (the principal diagnosis) was ascertained. The DoD ICD-9 coding guidelines define the principal diagnosis as "the condition established, after study, to be responsible for the admission." Hospitalizations prior to the conclusion of the deployment period were not evaluated because access to care for the deployed differed markedly from that for the nondeployed during this time. Outpatient visits were not studied because they are not computerized centrally by DoD.

Data

Demographic variables available for use as covariates included age, race/ethnicity, occupation, rank, salary, branch of service, length of service, marital status, and gender. Time-dependent variables were evaluated as of 31 July 1990. A previous study (15) found hospitalization for any reason in the 12 months preceding the Gulf War deployment period to be an important predictor of postwar hospitalization. This indicator variable may be a surrogate for baseline health status and was also used as a covariate. Demographic data, including deployment status, were obtained from the Defense Manpower Data Center, Seaside, California. Hospitalization information was obtained from the Data Processing Center, Fort Detrick, Frederick, Maryland. Comprehensive Clinical Evaluation Program data were obtained from the Deployment Surveillance Team, Falls Church, Virginia.

Statistical Analysis

Frequencies of selected diagnoses and causes of death were calculated. The Cox proportional hazards survival analysis model (21) was used to obtain the risk ratio (RR) and 95% confidence interval (CI) of deployment status (deployed relative to nondeployed) for an event consisting of hospitalization with an unexplained illness, adjusting for the covariates. Follow-up time was computed from 1 August 1991 until hospitalization in any DoD hospital worldwide with at least one unexplained illness, separation from the service, or until 31 March 1996, whichever occurred first. All data management and statistical calculations were performed with the Statistical Analysis System (22).

Findings

Frequent Unexplained Illnesses

Our study population consisted of 25,495 first hospitalizations (with at least one unexplained illness among the eight possible diagnoses), 6,672 in the deployed and 18,823 in the nondeployed. For these hospitalizations, the 10 most frequent first unexplained illnesses among all eight possible diagnoses were tabulated (Table 1). Eight of these diagnoses occurred with similar proportional distributions between the two groups. The diagnosis "nonspecific abnormal findings in the amniotic fluid" accounted for a higher proportion of hospitalizations among the nondeployed than among the deployed. This is consistent with a higher proportion of women among the nondeployed (12.7%) than among the deployed (6.1%). The tenth most frequent diagnosis, "illness of unknown cause," accounted for a considerably greater proportion of hospitalizations among the deployed (8.3%) than among the nondeployed (1.8%).

An unexplained illness was the principal diagnosis in 13,490 first hospitalizations, 3,525

Table 1. Frequencies of the 10 most common diagnoses for first hospitalizations with an unexplained illness, considering all eight coded diagnoses[a]

ICD-9 Code	Description	Frequency			Percentage of all diagnoses	
		Total	Deployed	Non-deployed	Deployed	Non-deployed
008.8	Unspecified intestinal infections	2286	581	1705	8.7	9.1
047.9	Unspecified viral meningitis	946	236	710	3.5	3.8
079.9	Unspecified viral infections	3540	896	2644	13.4	14.1
465.9	Acute URI[b], unspecified site	1999	527	1472	7.9	7.8
486	Pneumonia, organism unspecified	2840	719	2121	10.8	11.3
780.6	Pyrexia of unknown origin	961	233	728	3.5	3.9
785.6	Enlarged lymph nodes	1587	423	1164	6.3	6.2
786.0	Dyspnea	2736	703	2033	10.5	10.8
792.3	Nonspecific abnormal amniotic fluid	1317	188	1129	2.8	6.0
799.9	Illness of unknown cause	892	555	337	8.3	1.8
Subtotal		19,104	5061	14,043	75.9	74.6
Other		6391	1611	4780	24.1	25.4
Total		25,495	6672	18,823	100.0	100.0

[a]Only the first unexplained illness coded for each hospitalization was tabulated.
[b]URI, upper respiratory infection.

from the deployed and 9,965 from the nondeployed. The proportional distributions of the 10 diagnoses reported in Table 1 showed some rearrangements when only the principal diagnosis was considered (Table 2). The percentages of "acute upper respiratory infection, unspecified site"; "nonspecific abnormal amniotic fluid"; and "illness of unknown cause" were dramatically lower as the principal diagnosis than they were as any diagnosis. However, "illness of unknown cause" remained the only diagnosis that accounted for an appreciably larger proportion of admissions among the deployed (2.0%) than among the nondeployed (0.3%).

Screening of Covariates

The demographic characteristics of the deployed and nondeployed groups have been reported (15). All available covariates were included in a preliminary Cox proportional hazards model to assess their effect on the risk for hospitalization for an unexplained illness (Table 3). All available covariates (one variable at a time) were also included in a series of preliminary models for this same purpose. These two approaches to screening covariates gave somewhat different results, probably because of multicolinearity among the covariates. The second approach was less useful than the first, so it is not further reported here.

Given the other covariates, age, marital status, and length of service were only minimally related to risk (possibly because of colinearity) and were not included in subsequent model analyses. The covariates retained included race (coded as white; black; or other, including unknown); rank (coded as enlisted or warrant or commissioned officer, including unknown); salary (coded as less than $1,000; $1,000 to $1,399; or at least $1,400 a month, including unknown); and branch of service (coded as Army or other). The military system has a large number of occupational categories; however, only "health-care worker" appeared to have an appreciably higher risk for hospitalization for an unexplained illness than other occupational categories. Consequently, occupation was simply coded as health-care worker or other (including unknown). Prewar hospitalization status was coded as "yes" if one was hospitalized for any reason during the 12 months before 1 August 1990 and as "no" otherwise. All of these other covariates, as well as gender (with unknowns included with men), had a highly statistically significant effect on the risk for hospitalization for an unexplained illness and were included in all subsequent model analyses. Other possible covariates, whose effect on risk was either less highly significant or nonsignificant, were not included so as to minimize unnecessary computation and variance inflation.

Survival Analysis, Using All Eight Diagnoses

A model analysis including deployment status and the selected covariates showed that deployment status was significantly associated with hospitalization for an unexplained illness

Table 2. Frequencies of the 10 most common diagnoses for first hospitalization with an unexplained illness, considering only the principal diagnosis

ICD-9 Code	Description	Frequency			Percentage of all diagnoses	
		Total	Deployed	Non-deployed	Deployed	Non-deployed
008.8	Unspecified intestinal infections	1807	467	1340	13.2	13.5
047.9	Unspecified viral meningitis	912	228	684	6.5	6.9
079.9	Unspecified viral infections	2620	661	1959	18.7	19.8
465.9	Acute URI, unspecified site	487	151	336	4.3	3.4
486	Pneumonia, organism unspecified	2350	615	1735	17.5	17.4
780.6	Pyrexia of unknown origin	382	93	289	2.6	2.9
785.6	Enlarged lymph nodes	1000	274	726	7.8	7.3
786.0	Dyspnea	1176	302	874	8.6	8.8
792.3	Nonspecific abnormal amniotic fluid	5	1	4	0.0	0.0
799.9	Illness of unknown cause	101	72	29	2.0	0.3
Subtotal		10,840	2864	7976	81.2	80.0
Other		2650	661	1989	18.8	20.0
Total		13,490	3525	9965	100.0	100.0

when all eight possible diagnoses were used (Table 4). Separate model analyses for the two deployment status groups indicated that the parameter estimates were similar, and the effect of the covariates on the probability of hospitalization was essentially independent of deployment status.

The probabilities under the separate models of hospitalization with an unexplained illness, at the mean values of the included covariates, are presented as a function of follow-up time in Figure 1. These probabilities for the two groups were virtually coincidental and linear over time, until late 1994 when the deployed group's probability increased. This shift in the historical track for the deployed group suggests that the hazards for the two groups were not proportional over time and that additional analyses may be indicated.

The Comprehensive Clinical Evaluation Program

In May 1994, DoD announced a Comprehensive Clinical Evaluation Program (CCEP), offering thorough clinical examinations and evaluations to Gulf War veterans who sought them. This program was implemented in June 1994. The divergence in probability curves (Figure 1) in the last quarter of 1994 prompted us to investigate whether the introduction of CCEP may have affected the probability of hospitalization.

Referring to any hospitalization for unexplained illness after 1 June 1994 of a CCEP participant as a CCEP hospitalization, we found that 837 first unexplained illness hospitalizations in the deployed were CCEP hospitalizations, as were 55 in the nondeployed. (For the purpose of CCEP participation, deployment status was self-determined.) Many of the nondeployed (according to Defense Manpower Data Center data) CCEP participants may have been in the Persian Gulf region after 1 August 1991. Of these 892 hospitalizations, 59% of the first diagnoses for unexplained illnesses were "illness of unknown cause," the most nonspecific diagnosis in ICD-9. Furthermore, with 128 DoD hospitals operating worldwide in 1995, 60% of these hospitalizations were in six facilities—three Army hospitals and three Air Force hospitals—and fewer than 1% were in any Navy facility.

These unusual results prompted us to make inquiries at some of the DoD hospitals reporting large numbers of hospitalizations for "illness of unknown cause." We learned that several of the larger Army and Air Force hospitals established special wards for CCEP participants reaching phase 2 of the evaluation process, admitted them for several days, and performed extensive evaluations (including invasive procedures and sleep studies) during hospitalization. These facilities also gave at least some of these participants a diagnosis of "illness of unknown cause." This coding practice for evaluation admissions may have resulted from a memo dated 25 August 1994 from the Headquarters,

Table 3. Frequencies and risk ratios under the Cox proportional hazards model for all hospitalizations with at least one unexplained illness, all covariates, 1 August 1991 to 1 April 1996

Variable		Deployed	Nondeployed	Risk ratio[a]	95% Confidence interval
Not deployed			1,479,751		
Deployed		552,111		1.08	1.05 - 1.11
Not hospitalized before war[b]		513,486	1,375,169		
Hospitalized before war		38,625	104,582	1.73	1.66 - 1.79
Not married		258,821	602,553		
Married		291,009	875,309	1.05	1.02 - 1.08
Unknown marital status		2282	1889	0.64	0.41 - 1.00
Male		517,223	1,291,323		
Female		33,690	188,273	2.13	2.06 - 2.20
Unknown gender		1198	155	0.60	0.20 - 1.75
White		383,704	1,110,949		
Black		130,249	286,427	1.05	1.02 - 1.08
Hispanic		12,900	26,696	0.91	0.83 - 1.00
Other		23,592	54,128	0.91	0.85 - 0.97
Unknown race/ethnicity		1666	1551	1.08	0.69 - 1.68
Age in years:	17-21	142,547	343,535		
	22-25	146,652	306,272	0.94	0.90 - 0.97
	26-31	138,173	373,698	0.95	0.90 - 1.00
	≥ 32	123,353	455,302	1.05	0.99 - 1.12
	Unknown	1386	944	1.04	0.57 - 1.88
Infantry, gun crews, seamanship		140,742	273,079		
Electronic equipment repair		45,263	159,195	0.99	0.93 - 1.04
Communications/intelligence		55,665	120,108	1.00	0.95 - 1.06
Health care		28,398	107,591	1.46	1.39 - 1.53
Other technical		11,633	33,246	1.02	0.93 - 1.11
Administration		62,194	255,576	0.96	0.92 - 1.00
Electrical/mechanical repair		110,258	224,527	0.98	0.93 - 1.02
Construction/related trades		19,424	47,800	1.11	1.03 - 1.20
Supply handlers		53,966	113,093	1.11	1.06 - 1.17
Trainees, undesignated		6421	64,625	1.02	0.95 - 1.10
Unknown job category		18,147	80,911	1.18	1.11 - 1.25
Enlisted		489,034	1,238,790		
Warrant Officer		7760	13,555	0.79	0.69 - 0.89
Commissioned Officer		53,121	227,381	0.70	0.65 - 0.75
Unknown status		2196	25	0.43	0.20 - 0.93
Salary/month:	< $1000	122,161	328,187		
	$1000-$1399	269,500	573,995	0.81	0.77 - 0.85
	$1400-$2099	75,523	252,923	0.68	0.63 - 0.72
	$2100-$3199	60,528	200,295	0.73	0.68 - 0.79
	≥$3200	22,203	124,326	0.74	0.66 - 0.83
	Unknown	2196	25	–	– –
Army		258,858	459,644		
Navy		141,604	423,138	0.61	0.59 - 0.64
Marine Corps		83,377	113,467	0.66	0.63 - 0.70
Air Force		67,942	447,125	0.76	0.73 - 0.78
Coast Guard		330	36,377	0.42	0.37 - 0.48
Service in months:	< 22	124,629	262,260		
	22-54	162,123	269,727	1.05	1.00 - 1.11
	55-126	133,608	363,138	0.97	0.92 - 1.02
	≥ 127	131,751	584,626	1.12	1.07 - 1.18

[a]Risk ratios are relative to the first group in each variable category. When the algorithm failed to converge, because of lack of cases in the category, a dash (–) is given.

[b]In the 12 months preceding 1 August 1990.

Table 4. Frequencies and risk ratios under the Cox proportional hazards model for all hospitalizations with at least one unexplained illness, selected demographic variables, 1 August 1991 to 1 April 1996

Variable		Deployed	Nondeployed	Risk ratio[a]	95% Confidence interval
Not deployed		—	1,479,751		
Deployed		552,111	—	1.06	1.03 - 1.09
Not hospitalized before war[b,c]		513,486	1,375,169		
Hospitalized before war		38,625	104,582	1.71	1.65 - 1.78
Male[c]		518,421	1,291,478		
Female		33,690	188,273	2.11	2.04 - 2.18
White		383,704	1,110,949		
Black		130,249	286,427	1.04	1.01 - 1.08
Other race/ethnicity[c]		38,158	82,375	0.91	0.86 - 0.96
All other occupations[c]		523,713	1,372,160		
Health-care worker		28,398	107,591	1.44	1.38 - 1.50
Enlisted		489,034	1,238,790		
Officer[c]		63,077	240,961	0.66	0.62 - 0.70
Salary/month:	< $1,000	122,161	328,187		
	$1,000-$1,399	269,500	573,995	0.77	0.74 - 0.79
	≥ $1,400[c]	160,450	577,569	0.82	0.78 - 0.87
Army		258,858	459,644		
Other branches		293,253	1,202,107	0.60	0.58 - 0.62

[a]Risk ratios are relative to the first category for each variable.
[b]In the 12 months preceding 1 August 1990.
[c]Includes unknown.

U.S. Army Medical Command at Fort Sam Houston, San Antonio, Texas, which directed Army facilities to code CCEP participants with "unexplained complaints with no confirmed diagnosis" as 799.9. The practice of admitting CCEP participants was discontinued by mid-1995. The probability of hospitalization curves tended to become parallel again about mid-1995 (Figure 1). Thus, we inferred that a substantial majority of the CCEP hospitalizations were primarily for evaluation.

Independent evaluation supported this inference. Since DoD computerized hospital records do not include cause of hospitalization or any other indication of whether a patient may have been hospitalized primarily for evaluation, we randomly selected for chart review 50 CCEP hospitalizations from each of the Army and Air Force facilities with the greatest numbers of CCEP hospitalizations. Both of these facilities were located in the same metropolitan area, San Antonio, Texas. The selected CCEP hospitalizations were independently evaluated by two clinicians, one from each facility. Seventy-nine charts were reviewed, 44 from the Air Force facility and 35 from the Army facility. The other 21 records were located in satellite facilities or otherwise not readily accessible. The two clinicians agreed that 77 of these hospitalizations were for evaluation only and would not have been considered hospitalizations had the CCEP not been in effect. One of the clinicians thought that two of the hospitalizations were for clinical management and would have occurred regardless of CCEP.

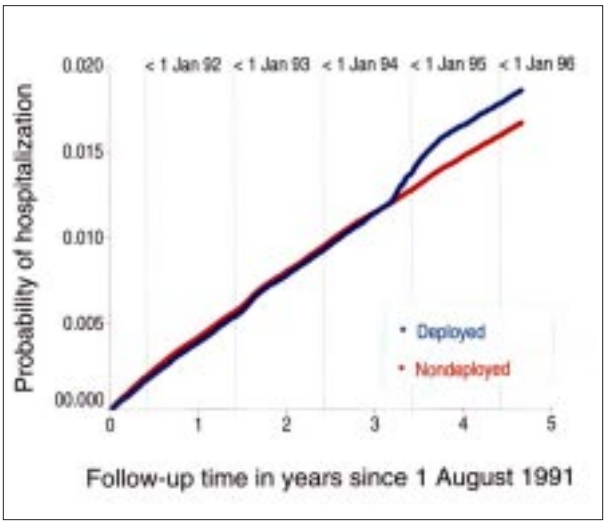

Figure 1. Probability of hospitalization for unexplained illness, deployed and nondeployed veterans.

Survival Analysis, Using All Eight Diagnoses, Censoring CCEP Participants

We repeated the survival analyses (reported in Table 4; Figure 1), censoring all CCEP participants on 1 June 1994. This change resulted in 5,835 first hospitalizations for an unexplained illness in the deployed group and 18,768 in the nondeployed. No important differences were observed in the effects of the covariates between the models censoring CCEP participants on 1 June 1994 and those reported in Table 4. However, when CCEP participants were censored, the risk for hospitalization for an unexplained illness was lower in the deployed than in the nondeployed (RR = 0.93, CI = 0.91 to 0.96), and the probability of hospitalization for an unexplained illness was generally lower over time for the deployed than for the nondeployed (Figure 2). Also, the proportional hazards assumption now appeared reasonable.

CCEP participants may truly have been at increased risk for hospitalization for an unexplained illness, in spite of many CCEP hospitalizations having been for evaluation only. Consequently, the true risk for the deployed is likely to be intermediate to that depicted in Figures 1 and 2. However, our record review, which concluded that the preponderance of CCEP hospitalizations were for evaluation only, suggested that the results are more closely depicted in Figure 2 than Figure 1.

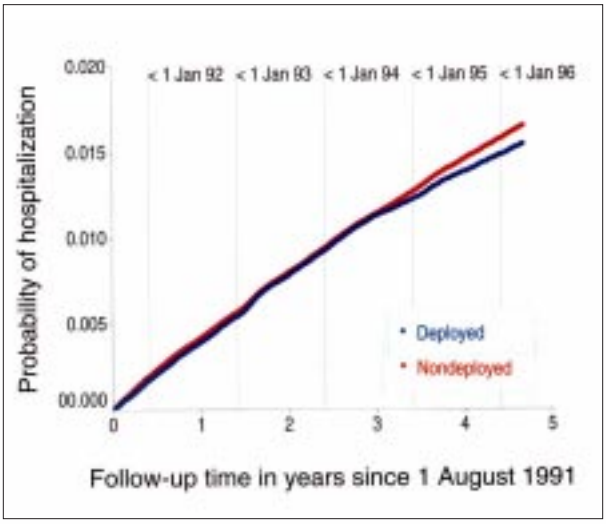

Figure 2. Probability of hospitalization for unexplained illness, deployed and nondeployed veterans, censoring comprehensive clinical evaluation program participants on 1 June 1994.

Survival Analyses, Using Principal Diagnosis Only

Model analysis of hospitalizations for which the principal diagnosis was an unexplained illness showed that deployment status was not a statistically significant predictor (RR = 0.99, CI = 0.95 to 1.03). When CCEP participants were censored on 1 June 1994, model analysis results were similar and showed that the deployed were slightly less likely than the nondeployed to be hospitalized (RR = 0.93, CI = 0.89 to 0.97). Model analysis results for hospitalization for an unexplained illness using only the principal diagnosis thus were similar to the results when all eight diagnoses were employed, except that there were many fewer admissions with "illness of unknown cause" as a principal diagnosis than as a secondary diagnosis.

Deaths

During hospitalization with an unexplained illness 348 veterans died. Of those who died, 86 had been deployed, and 262 had not. Of the 348 deaths, only 62 had an unexplained illness indicated as the underlying cause. Of these 62, 17 had been deployed and 45 had not. Deployed veterans with an unexplained illness as the underlying cause of death included four with "unspecified septicemia"; six with "pneumonia, organism unspecified"; three with "other diseases of the lung"; and one each with "other primary cardiomyopathies," "thrombotic microangiopathy," "unexplained death," and "respiratory failure."

Conclusions

Because they live in close quarters and are required to travel, military personnel commonly acquire diseases endemic to the regions they visit and may serve as reservoirs and vectors for disease transmission when they return home. A number of military deployment-related epidemics have occurred in recent years. Persian Gulf War veterans have been diagnosed with a new manifestation of leishmaniasis (23). Veterans of the peace-keeping effort in Somalia have had increased hospitalization rates for malaria (24), deployed Navy personnel have brought nonendemic strains of HIV virus into the United States (25), and Russian soldiers have been implicated in the mass epidemics of diphtheria in Eastern Europe (26). These and other observations make surveillance for unexplained illnesses among U.S. military personnel an important federal public health issue.

We designed this study to screen for unexplained illnesses using a collection of ICD-9 diagnoses (20). Since unexplained illnesses generally have no specific ICD-9 diagnoses, a nonspecific diagnosis from this collection is likely to be used by hospital coders when an unexplained illness is encountered. The DoD worldwide hospitalization data subsequent to the Persian Gulf War were analyzed using the Cox proportional hazards model for evidence of unexplained illnesses. Analyses were performed both with all eight coded diagnoses and with only the principal diagnosis.

Model analysis showed that Gulf War veterans were more likely than their peers to be hospitalized for unexplained illness. After 4.67 years of follow-up, the cumulative probability of hospitalization for unexplained illness was 0.0185 in the deployed and 0.0166 in the nondeployed (Figure 1). This increased hospitalization risk of 11% for the deployed was a consequence of the recruiting for free clinical evaluations beginning in June 1994, with most of the resulting CCEP hospitalizations being for medical evaluation and not for clinical management. When CCEP participants were censored on 1 June 1994, deployed Gulf War veterans were not at greater risk than those not deployed. The slightly lower hospitalization risk for the deployed than for the nondeployed (Figure 2) is consistent with a healthy service member effect; that is, those selected for deployment are, on average, slightly healthier than those not selected.

This study had a number of limitations. Some miscoding of Gulf War deployment status was suggested by the finding that some nondeployed veterans were evaluated under the CCEP as being Gulf War veterans. However, veterans who did not serve in the Gulf region until after 31 July 1991 were eligible for the CCEP, and this apparent discrepancy was less than 6%. Also, many personnel separated from the service during the period of follow-up; 59.8% of the deployed and 54.3% of the nondeployed had separated by 1 April 1996. However, the fact that separating veterans receive thorough medical screening and have the potential of receiving lifelong disability benefits motivates them to be thorough in their reporting of illness before separation. The broad categorization of deployment status may have masked illness due to time- and geography-specific exposures, and illnesses not serious enough to require hospitalization have not been captured by our analyses. Finally, the collection of diagnoses used as the outcome measure was not designed specifically for our purposes, but it does have the advantage of prior development by other researchers (20).

This study also had its strengths. The large group sizes, along with the availability of numerous important covariates (demographic variables and prewar hospitalization), offer unusually high statistical power for the detection of differences in the hospitalization risk between groups. Additionally, discharge diagnoses are thoroughly recorded and edited by DoD hospitals, and active-duty personnel have few opportunities to be hospitalized outside the DoD system, which ensures high-quality data with few missing values.

In summary, these analyses show a slightly greater hospitalization risk for unexplained illness among deployed Gulf War veterans than among those not deployed. However, the excess hospitalizations for the deployed are attributable to participation in CCEP; most of these hospitalizations were for medical evaluation, not clinical management. Consequently, before initiation of CCEP, active-duty Gulf War veterans were not at increased risk for hospitalization for unexplained illness.

Acknowledgments

We thank Drs. Edwin C. Matthews and Gregg T. Anders for completing the record review of the 79 randomly selected CCEP hospitalizations. We also thank Michael A. Dove and Wayne F. Woo for their assistance in obtaining the deployment status and covariate data.

This is Naval Health Research Center report no. 96-35, supported by the Department of Defense (Health Affairs) and the Naval Medical Research and Development Command, Department of the Navy, Bethesda, Maryland, under work unit no. 63738DP4464.001-6423.

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Agricultural Use of *Burkholderia (Pseudomonas) cepacia*: A Threat to Human Health?

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In the past 2 decades, *Burkholderia cepacia* has emerged as a human pathogen causing numerous outbreaks, particularly among cystic fibrosis (CF) patients. One highly transmissible strain has spread across North America and Britain, and another between hospitalized CF and non-CF patients. Meanwhile, the organism has been developed as a biopesticide for protecting crops against fungal diseases and has potential as a bioremediation agent for breaking down recalcitrant herbicides and pesticides. However, *B. cepacia* is inherently resistant to multiple antibiotics; selection of strains "safe" for environmental application is not at present possible phenotypically or genotypically; molecular epidemiology and phylogenetic studies demonstrate that highly transmissible strains emerge randomly; and the organism has a capacity for rapid mutation and adaptation (facilitated by numerous insertion sequences), and a large, complex genome divided into separate chromosomes. Therefore, the widespread agricultural use of *B. cepacia* should be approached with caution.

Burkholderia (previously known as *Pseudomonas) cepacia*, a nutritionally versatile, gram-negative organism, was first described in 1949 by Walter Burkholder of Cornell University, as the phytopathogen responsible for a bacterial rot of onions (1) (Figure 1). Ironically, *B. cepacia* is now being considered by agricultural microbiologists as an agent to promote crop growth.

B. cepacia is inherently resistant to multiple antibiotics, can metabolize diverse substrates, and is found in soil and in moist environments. The organism has a particular predilection for the lung in patients with cystic fibrosis (CF) and has emerged as an important opportunistic human pathogen in hospitalized and immunocompromised patients (2,3).

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Figure 1. *B. cepacia* causes an onion rot known as slippery skin (1). The onions shown were inoculated with three strains of *B. cepacia*. Rot occurred in onion1 (left), which was inoculated with strain originally isolated from onions. Rot did not occur with environmental isolates tested or with strains from CF lung.

B. cepacia as a Human Pathogen

In CF Patients

CF is a recessively transmitted genetic disease that occurs in approximately 1 in 2,500 Caucasians (carrier frequency of 1 in 25). The condition is characterized by a generalized dysfunction of the exocrine glands, giving rise to a broad spectrum of clinical syndromes, especially malabsorption due to pancreatic insufficiency and chronic progressive lung disease giving rise to bronchiectasis. *B. cepacia* is associated with increased illness and death among CF patients. In the early 1980s, the organism emerged as a major threat, causing superinfection in as many as 40% of patients in some CF centers (4-6). While in some patients indolent pulmonary infection occurs with only gradual deterioration in lung function similar to that associated with *Pseudomonas aeruginosa*, approximately 35% of *B. cepacia*–infected patients contract accelerated pulmonary deterioration or fulminant, necrotizing pneumonia with rapidly fatal bacteremia (3,7-10), sometimes referred to as "*cepacia* syndrome" (7). Unlike infection with *P. aeruginosa, B. cepacia* infection significantly increases death rates among CF patients (11) at all levels of lung function.

Over the last 20 years, CF survival rates have increased as a result of improved treatment, the median length of survival increasing from early childhood to more than 29 years. As a result, approximately one third of CF patients are adults. Pulmonary infection causes the most illness and ultimately more than 90% of CF-related deaths. *B. cepacia*'s emergence as a pathogen coincided with social and medical grouping of CF patients in specialized units, clinics, and social groups. Studies subsequently demonstrated that social contact between CF patients was an important factor in transmission of *B. cepacia* (12).

The threat of *B. cepacia* infection led to severe control measures, affecting not only the treatment but also the social and family lives of CF patients. CF centers adopted stringent infection control policies, assuming that all *B. cepacia* isolates were highly transmissible (13,14). CF summer camps in North America were closed, and many lung transplant centers ceased to accept *B. cepacia*–infected CF patients as transplant candidates. Newly formed national and international associations for CF adults and CF families (providing conferences, holidays, and support groups) addressed the issue of *B. cepacia* transmission by abandoning many activities and adopting exclusion and segregation measures (13).

Numerous CF-associated *B. cepacia* epidemics have now been described, and the epidemic strains have been characterized (15-18). One particular highly transmissible strain, epidemically spread within and between CF centers on both sides of the Atlantic, carries the *cblA* gene (18). This gene encodes for the major structural subunit of unique mucin binding cable pili (4). These enormously long pili (radiating 2 to 4 microns) are peritrichously arranged and are intertwined to form cablelike structures that adhere to CF mucin (4,18) (Figure 2). This *cblA*+ strain has spread across Canada and has now been isolated in 50% of CF centers in the United Kingdom (19). Another strain of *B. cepacia* has spread among CF centers in four regions of France (20).

However, it has become clear that transmissibility varies markedly from strain to strain, and that most strains are not involved in epidemics but appear to be independently acquired and show no evidence of transmission. For example, in 8 years no cases of transmission were detected at the University of North Carolina CF center, despite clinical and social contact between patients and the absence of stringent infection control measures (21). Independent acquisition of *B. cepacia* with no evidence of transmission between CF patients was also reported from Denmark (22). Lack of transmission of some strains has also been observed between siblings with CF (23).

In Patients Without CF

B. cepacia was first reported as a human pathogen causing endocarditis in the 1950s. Since then the organism has caused numerous catheter-associated urinary tract infections, wound infections, and intravenous catheter–associated bacteremias. In 1971, it was reported as the causative organism of "foot rot" in U.S. troops on swamp training exercises in northern Florida; it was also isolated from troops serving in the Mekong Delta, Vietnam (24).

In the 1980s the number of nosocomial infections with *B. cepacia* increased markedly, with deaths particularly associated with lung infections (25). Numerous small focal hospital outbreaks involving non-CF patients have usually been due to a contaminated common source, such as disinfectant preparations or

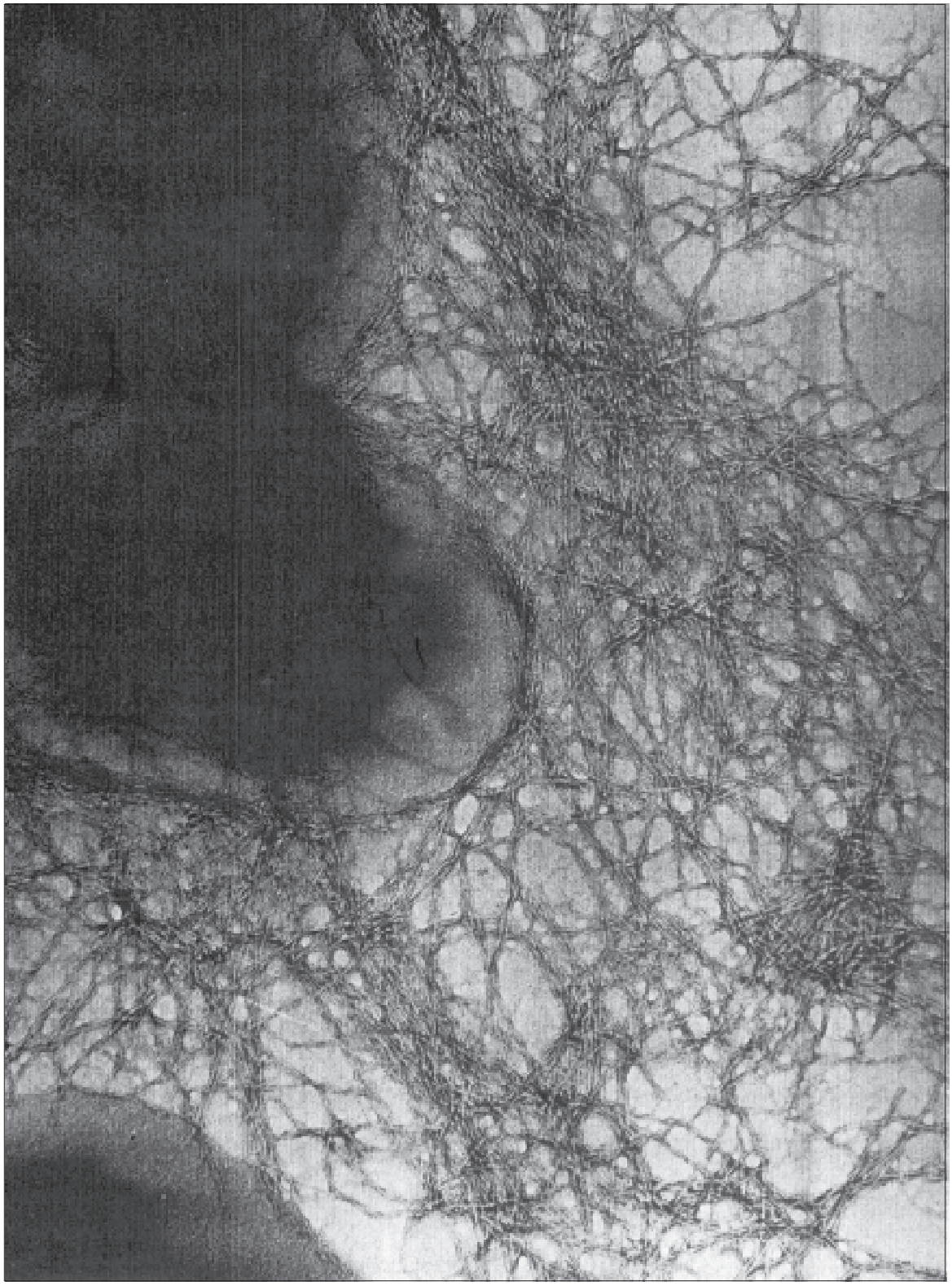

Figure 2. Transmission electron micrograph of Toronto/Edinburgh epidemic clone of *B. cepacia* expressing CF mucous-binding Cbi adhesin pili. High resolution was achieved by using a JEOL 100CX electron microscope and negative staining. Reprinted with permission from Richard Goldstein and Journal of Bacteriology (J Bacteriol 1995;177:1039-52).

intravenous solutions (2,26,27). The organism's unusually broad metabolic capabilities, which enable it to survive and proliferate in water-based environments, probably account for its propensity to cause nosocomial outbreaks (2,27-29). Because of its resistance to multiple antibiotics, once acquired, the organism can be difficult to treat.

B. cepacia is rarely found in the non-CF patient (30); however, when it is found the organism can spread to and from CF patients. Transmission between CF and non-CF patients has been associated with serious illness and death (31;Holmes et al., unpub. data) and presents a greater nosocomial threat than previously recognized. In addition, although *B. cepacia* is not a common commensal organism, hospitalized patients without CF may harbor it and pose an infection threat to vulnerable CF patients. This possible source of infection may account for the known association between hospitalization and of *B. cepacia* infection (32). *B. cepacia* may be underreported because selective media for *B. cepacia*, which greatly increase the yield (33,34), are rarely used except in specimens from CF patients.

B. cepacia as an Agricultural Agent

While emerging as a human pathogen, *B. cepacia* has attracted intense interest from the agricultural industry as a possible biologic control agent. The organism, which has been shown to have remarkable potential as an agent for both biodegradation and biocontrol, is being considered as a plant-growth-promoting rhizobacterium (23,35-43).

B. cepacia has extraordinary metabolic versatility and can degrade chlorinated aromatic substrates (toxic compounds found in complex pesticides and herbicides, some with carcinogenic potential) for use as carbon energy sources. One important toxic compound degraded by *B. cepacia* is 2,4,5 chlorophenoxy acetic acid (2,4,5-T), a potent herbicide that is not easily biodegradable and persists for long periods in the environment (37).

B. cepacia can also antagonize and repress many soilborne plant pathogens. It can prevent leaf and stem blight caused by the fungus *Alternaria* by inhibiting spore germination. Economically important crop diseases such as blight due to *A. solani* and the blight caused by *A. brassicae* and *A. brassicola*, which affects the oil-producing plants rape and canola, can be controlled by *B. cepacia*. The organism is also being used to prevent the blight of ginseng plants due to *A. panax* (41) and is effective against the fungus *Aphanomyces euteiches*, which causes root rot in peas, alfalfa, and snap beans (39,40). It can prevent *Pythium* diseases of cucumber and peas, and *Rhizoctonia solani* stem rot of poinsettia (42). To prevent these plant diseases, *B. cepacia* provides a seemingly environmentally friendly alternative to potent and toxic fungicides, which cannot be broken down in the environment.

The forestry industry also sustains large economic losses from the pathogenic effects of fungi such as *Fusarium*, *Pythium*, *Rhizoctonia*, *Cylindrocarpum*, and *Botrytis*. These widespread fungal pathogens cause seedling loss in nurseries and may kill or stunt transplanted seedlings. A strain of *B. cepacia* has been developed as a successful seed and root inoculant, which can suppress these fungi on a variety of conifers (43).

Numerous patents are being sought for specific agricultural applications of different strains of *B. cepacia*. The ecologic and economic benefits could be enormous if the organism's antifungal activity is used to enhance crop yields and reduce the need for pesticides and its ability to degrade complex herbicides and pesticides is harnessed for bioremediation.

Molecular Epidemiology of *B. cepacia*

Evolution of Highly Transmissible Strains

B. cepacia isolates are closely related (a panmictic population structure) (18,31,44) and epidemic isolates are scattered throughout (18,31), as demonstrated by *rrn*-based phylogenic trees, which include large numbers of environmental and clinical isolates. Such a phylogeny indicates that in *B. cepacia* strains, genetic changes conferring high transmissibility are occurring at random, or, given the right epidemiologic circumstances, random environmental strains are innately highly transmissible. In addition, the widespread geographic distribution of different epidemic strains of *B. cepacia* would also suggest that highly transmissible strains are emerging independently and randomly.

Taxonomy

Isolates presumptively identified as *B. cepacia* belong to at least five genomovars

(23,45,46). This group of phenotypically similar subpopulations is referred to as the *B. cepacia* complex (6,46). After multiphasic taxonomic analysis, the species names *B. multivorans* and *B. vietnamiensis* have been proposed for genomovars II and V, respectively (45). The pathogenic and epidemic potential of each of these subpopulations of the *B. cepacia* complex requires further examination. Although it appears that strains associated with *cepacia* syndrome belong to genomovar III (46), isolates belonging to each of these five genomovars have been cultured from CF patients.

The Unusual Genome

B. cepacia's capacity to propagate as an environmental microbe and as an opportunistic pathogen may be due to its possession of an unusually large (more than four times that of *H. influenzae*, two times that of *E. coli*, and half again as large as *P. aeruginosa*), complex, and variable genome. The genome contains numerous insertion sequences and is divided into one to four circular replicons (47,48). A few other bacterial species of agricultural and medical importance also have multiple chromosomes: *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *Rhodobacter sphaeroides*, and *Agrobacterium tumefaciens* (49-51).

This unusual genomic arrangement may account for *B. cepacia's* nutritional versatility and adaptability. Such a division of genomic content would allow for high levels of homologous and illegitimate recombination. The resultant chromosomal rearrangements and associated mutations could provide a basis for spontaneous "pulsed" evolutionary spurts, such as that seen from soil to the CF lung, suited for rapid adaptation to radical changes in environmental growth conditions.

Selection of Strains for Agricultural Use

Because evolution of highly transmissible strains occurs randomly and transmission of *B. cepacia* between CF and non-CF populations can cause severe illness and death, the deliberate widespread environmental application of strains of this organism should be considered carefully. Although *B. cepacia* does not appear to survive on dry surfaces for more than 1 week, it can survive for many months in water. The agricultural application of *B. cepacia* will lead to environmental and water contamination and increased human exposure. In addition to medical, metabolic, and taxonomic issues, increasing knowledge of *B. cepacia* raises important ecological issues, including the evolution of pathogenicity and multiresistant environmental bacteria through horizontal gene transfer. The agricultural use of *B. cepacia* risks this hazard of horizontal gene transfer between the strains applied and existing soil organisms and the subsequent emergence of pathogenic, highly resistant organisms.

Recently, in an attempt to assess the human risk associated with the use of rhizophere isolates as field inoculants, two clinical isolates of different strain types from two CF patients and two agricultural isolates from the rhizopheres of rice and maize were examined (38). The clinical isolates had identical 16S ribosomal rDNA sequences, but differences were seen between the soil isolates and clinical isolates. The results were presented as evidence of evolutionary divergence of the rhizophere isolates from their clinical counterparts. Alternatively, in the light of the multiple replicon model, this difference in 16S *rrn* is most likely due to the multiple replicons carrying varying sequences of the 16s gene. Furthermore, evidence based on four isolates alone is inadequate to support the safe application of rhizophere isolates. We have seen diversity among 16s *rrn* that is unrelated to source in a large collection of *B. cepacia* isolates (Holmes, Truong, Geisselsoder, Goldstein, unpub. data).

The possibility of highly virulent strains of *B. cepacia* with the potential to survive intracellularly exists if the organism acquires virulence genes from *B. pseudomallei*, a very closely related pseudomonad. *B. pseudomallei* is a soil saprophyte that gives rise to melioidosis, a life-threatening tropical disease seen mostly in Southeast Asia. The disease has a broad clinical spectrum and can remain dormant for years before giving rise to sepsis and death. The pattern of disease produced by *B. pseudomallei* may warn of a spectrum of clinical consequences from *B. cepacia* acquisition. The transfer of genetic material between these two closely related organisms in the environment is highly probable, with the subsequent emergence of a hybrid pathogen. This speculation is supported by recent detection of insertion sequences within *B. pseudomallei* that have been identified in *B. cepacia*, including an isolate belonging to the highly transmissible transatlantic epidemic lineage (18,52).

With the potential emergence of diseases related to new and developing practices in the food and agricultural world, it seems prudent that communication and information sharing between medical microbiologists, agricultural microbiologists, and public health professionals be optimized and promoted. Meanwhile, it is impossible to identify, phenotypically or genotypically, strains of *B. cepacia* that could safely be used in agriculture. Even if environmental strains incapable of human infection could be identified, their potential to evolve into human pathogens remains. Current molecular genetic evidence indicates that the deliberate environmental distribution of the organism might pose a hazard to human health, regardless of which particular strains are selected. Until more is known about the organism and the risks from environmental application, a moratorium should be called on the widespread use of *B. cepacia* in agriculture.

This work was supported in part by a research grant award to RG from the National Institutes of Health (NIDDK RO1 DK50838).

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Haemophilus influenzae Invasive Disease in the United States, 1994–1995: Near Disappearance of a Vaccine-Preventable Childhood Disease

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We analyzed national *Haemophilus influenzae* (Hi) surveillance data from 1994 and 1995 to describe the epidemiology of Hi invasive disease among persons of all ages. Serotype data were available for 376 (56%) of 669 reported Hi cases among children aged 4 years or younger; 184 (49%) were *H. influenzae* type b (Hib). Among children aged 4 or younger, incidence (per 100,000) of all Hi invasive disease was 1.8 in 1994 and 1.6 ($p < 0.05$) in 1995. Children aged 5 months or younger had the highest average annual incidence rate of Hib invasive disease (2.2 per 100,000); children aged 6 to 11 months had the next highest rate (1.2 per 100,000) ($p < 0.05$). Of 181 children with Hib invasive disease whose age in months was known, 85 (47%) were too young (aged 5 months or younger) to have completed a primary series with an Hib-containing vaccine. Of the 83 children with known vaccination status who were eligible to receive a primary series (aged 6 months or older), 52 (63%) were undervaccinated, and the remaining 31 (37%) had completed a primary series in which vaccine failed. Among persons aged 5 years or older with Hi invasive disease, the lowest average annual incidence was among those 20 to 39 years of age (0.15 per 100,000), and the highest was among those aged 80 years or older (2.26 per 100,000). Among persons aged 5 years or older, serotype data were available for 1,372 (71%) of the 1,940 Hi invasive disease cases; 159 (28%) of the 568 Hi cases with known serotype were due to Hib.

Before the first *Haemophilus influenzae* type b (Hib) polysaccharide vaccine was introduced in 1985, Hib was the most common cause of bacterial meningitis in children under 5 years of age (approximately 12,000 cases per year, most in children younger than 18 months [1]); approximately 5% of affected children died. Neurologic sequelae developed in 15% to 30% of the surviving children (1). An additional estimated 7,500 cases of other invasive Hib infections also occurred annually in young children (1). The cumulative risk for Hib invasive disease before the age of 5 was one in 200 children, similar to the risk for poliomyelitis during the 1950s (1,2).

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The incidence of Hib invasive disease among children aged 4 years or younger has declined by 98% since the introduction of Hib conjugate vaccines, initially licensed in 1989 for use in children aged 15 months or older, then for infants beginning at 2 months of age in 1990 (3-6). One goal of the Childhood Immunization Initiative (7) was to eliminate invasive Hib disease among children aged 4 years or younger by 1996; however, approximately 300 cases of *H. influenzae* (Hi) invasive disease per year continue to be reported (3).

Moreover, Hi invasive disease is an important cause of illness and death in immunocompromised persons and adults with certain underlying medical conditions (8-11). The average incidence of Hi invasive disease from 1988 to 1990 was estimated at 1.7 cases per 100,000; 50% of cases examined in this study were due to Hib (8).

Prior studies of Hi invasive disease among adults found case-fatality rates of 26% to 36% (8-11).

In this article, we summarize the epidemiology of Hi and Hib invasive disease among children aged 4 years or younger and persons aged 5 years or older in the United States during 1994 and 1995.

Data Collection

We used three sources of surveillance data to document the number of reported Hi invasive disease cases with onset in 1994 and 1995. Cases were reported to the Centers for Disease Control and Prevention (CDC). Two sources were passive surveillance systems: the National Notifiable Diseases Surveillance System (NNDSS) and the National Bacterial Meningitis and Bacteremia Reporting System (NBMBRS); the third source was the active, laboratory-based surveillance sites.

The NNDSS case reports of Hi invasive disease are transmitted electronically from state health departments to CDC as part of the weekly notifiable disease reporting system. These reports contain only demographic information. Supplemental information, including Hi serotype, infection site, outcome, and Hib vaccination status and specific vaccine received among children aged 4 years or younger is transmitted electronically or mailed to CDC on NBMBRS reporting forms. During 1994, 10.5 million persons were under active, laboratory-based surveillance for Hi invasive disease: these included residents of the state of Oklahoma; three counties in the San Francisco Bay area of California; eight counties in the metropolitan area of Atlanta, Georgia; and four counties in Tennessee (3). In 1995, 12.8 million persons were under surveillance; an additional county in Tennessee was added, and Maryland was included in place of Oklahoma. Each site was contacted every 2 weeks to identify Hi invasive disease cases; surveillance personnel then collected detailed data on these cases. Data on cases from the active surveillance sites were also mailed to CDC on a form similar to the NBMBRS report form.

Information from the three data sources was combined, and duplicates (cases with identical date of birth, onset, county of residence, and demographic data) were eliminated. After the end of each calendar year, each state health department was contacted by telephone to send information on Hi invasive disease cases among children aged 4 years or younger not previously reported to CDC and to provide missing critical supplementary information, such as vaccinations received.

Our analysis included confirmed and probable cases. A confirmed case of Hi invasive disease was defined as an illness clinically compatible with invasive disease (such as meningitis, pneumonia, cellulitis, epiglottitis, peritonitis, pericarditis, septic arthritis, empyema, and abscesses) with isolation of Hi from a normally sterile site (e.g., cerebrospinal fluid or blood). A probable case was defined as an illness clinically compatible with detection of Hib antigen in cerebrospinal fluid (12). Up to three infection sites could be recorded from a list including primary bacteremia, meningitis, pneumonia, cellulitis, epiglottitis, peritonitis, pericarditis, septic arthritis, or another specified site.

Completion of a primary vaccination series was defined according to recommendations from the Advisory Committee on Immunization Practices (4). Hib vaccine doses administered fewer than 14 days before the onset of disease were excluded.

Data Analysis

U.S. census population estimates for 1994 and 1995 were used to calculate age- and race-specific average annual incidence rates (calculated by [events/2] ÷ [population/2]) for all Hi invasive disease and for documented Hib disease (13). The difference between two rates was regarded statistically significant at the 0.05 level if it exceeded the following: $2 \times (R1^2/N1 + R2^2/N2)^{1/2}$, where R1 is the rate corresponding to N1 events and R2 is the rate corresponding to N2 events (14). County average annual incidence rates of Hi invasive disease were calculated by using 1990 county population estimates and were mapped with Atlas Mapmaker (15). For Hi invasive disease cases among children aged 4 years or younger, we looked at the proportion due to Hib, the Hib vaccination history, and the age-specific and geographic distribution of residual Hib cases. A chi-square test of independence was used to test the association between age category and outcome.

Findings

In 1994 and 1995, 1,277 and 1,332 cases of invasive Hi disease were reported, respectively. Although the overall incidence of invasive Hi disease remained constant, the incidence of invasive Hi disease among children aged 4 years or younger was lower in 1995 (305 cases; 1.56 per 100,000) than in 1994 (364 cases; 1.84 per

100,000) ($p < 0.05$). During 1994 and 1995, children 5 months or younger had the highest reported disease incidence of Hi invasive disease (8.02 per 100,000 children), Hib invasive disease (2.20 per 100,000), and Hi invasive disease of unknown serotype (3.57 per 100,000) (Table 1). The incidence of Hi invasive disease, Hib invasive disease, and Hi invasive disease of unknown serotype was significantly greater ($p < 0.05$) among children aged 5 months or younger than among children 6 to 11 months of age, who had the next highest rates; a high rate of Hi invasive disease was also observed among adults aged 80 years or older. The lowest disease rates were found in persons 5 to 19 and 20 to 39 years of age.

Hi isolates were serotyped for only 36% of 2,609 reported cases. A higher percentage of isolates were serotyped among children aged 4 years or younger (56%) than among older persons (29%) (Table 2). Among Hi cases in children aged 4 years or younger with reported serotype information, Hib was the most common single serotype (184 type b of 376 serotyped isolates); Hib represented 49% of invasive disease and 65% of meningitis. The average annual incidence for Hib invasive disease was 0.47 per 100,000 children aged 4 years or younger. Among persons aged 5 years or older, Hib accounted for 28% of serotyped isolates.

Among the 433 Hi case-patients younger than 1 year of age for whom age in months was reported during the study period, most (72%) were aged 5 months or younger; 29% were younger than 1 month of age (Figure 1). Similarly, of 184 children aged 4 years or younger with Hib invasive disease, most (133, 72%) cases were in children younger than 1 year of age.

The highest average annual incidence rates (5.1 per 100,000) of Hi invasive disease among case-patients aged 4 years or younger were in American Indians; comparisons with other race or ethnic groups were statistically significant ($p < 0.05$) (Table 3). Similar race and ethnic differences were noted for Hib invasive disease incidence but not for Hi invasive disease of unknown serotypes. Among case-patients aged 5 years or older, differences between race or ethnic groups were less marked. The incidence rates among male and female case-patients aged 5 years or older and among case-patients aged 4 years or younger were equivalent.

Infection site data were available for 1,556 (60%) case-patients (Table 4). Bacteremia was the most frequently reported site of Hi invasive disease for both children aged 4 years or younger (62%) and persons aged 5 years or older (51%). The second most frequently reported site of

Table 1. Number of cases and average annual incidence rates of Hi and Hib invasive disease and Hi invasive disease of unknown serotype, by age,[a] United States, 1994–1995

	Hi		Hib		Hi serotype unknown	
Age group	No.[b]	Incid.[c]	No.[b]	Incid.[c]	No.[b]	Incid.[c]
0-5 mo	310	8.02	85	2.20	138	3.57
6-11 mo	123	3.18	45	1.16	45	0.16
1-4 yr	219	0.69	51	0.16	99	0.31
5-19 yr	179	0.16	23	0.02	108	0.10
20-39 yr	249	0.15	25	0.02	166	0.10
40-59 yr	405	0.33	42	0.03	280	0.23
60-79 yr	715	1.01	51	0.07	539	0.76
80 + yr	361	2.26	18	0.11	152	0.95

[a]Does not include 31 cases missing age and 17 patients aged <1 year but missing age in months.
[b]Number of cases over the 2-year period.
[c]Incidence per 100,000 population.

Table 2. Serotype information for reported Hi invasive disease (n=2609) and meningitis cases (n=380), stratified by age, United States, 1994–1995

	No. (%) of children aged 0–4 years		No. (%) of persons aged ≥ 5 years	
Serotype	Invasive disease	Meningitis	Invasive disease	Meningitis
Serotype b	184 (27)	101 (47)	159 (8)	41 (25)
Other[a]	114 (17)	39 (18)	171 (9)	26 (15)
Non-typeable	78 (12)	16 (8)	238 (12)	25 (15)
Unknown	293 (44)	57 (27)	1,372 (71)	75 (45)
Total	669 (100)	213 (100)	1,940 (100)	167 (100)

[a]Includes serotypes a, c, d, e, f.

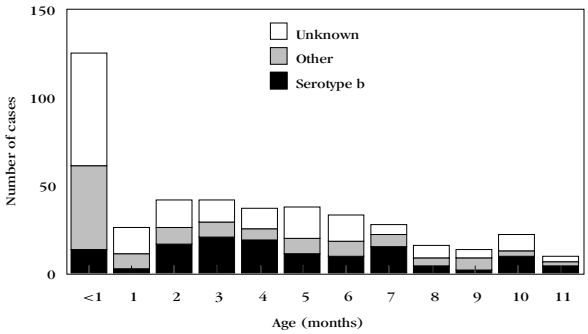

Figure 1. Reported Hi invasive disease cases by age and serotype, children aged <1 year,[a] United States, 1994–1995.

[a]N=433; excluding 17 children aged <1 year missing age in months.

Table 3. Number of cases and average annual incidence rates of Hi and Hib invasive disease and Hi invasive disease of unknown serotype, by race and ethnicity,[a] United States, 1994–1995

	Children aged 0–4 years						Persons aged ≥5 years	
	All Hi		Hib		Hi: unknown serotypes		All Hi	
Race/ethnicity	No.[b]	Incidence[c]	No.[b]	Incidence[c]	No.[b]	Incidence[c]	No.[b]	Incidence[c]
Non-Hispanic								
White	338	1.34	102	0.40	150	0.59	1,269	0.35
Black	122	2.08	32	0.55	52	0.89	258	0.45
American Indian	18	5.10	10	2.83	3	0.84	12	0.37
Asian or Pacific Islander	13	0.86	3	0.20	6	0.40	21	0.13
Hispanic	94	1.49	7	0.11	31	0.49	70	0.15

[a]Does not include 84 (13%) of children aged ≤ 4 years, and 310 (16%) of persons aged ≥ 5 years for whom race and ethnicity data were missing.
[b]Number of cases over the 2-year period.
[c]Per 100,000 population.

invasive disease among children aged 4 years or younger was meningitis (44%), and among persons aged 5 years or older it was pneumonia (36%).

Outcome data were available for 1,380 (53%) of 2,609 case-patients with Hi invasive disease. Among case-patients with known outcome, the overall case-fatality ratio (CFR) was 14%. The CFR differed by age; the CFRs among case-patients aged 0 to 1 year, 1 to 29 years, 30 to 59 years, and 60 years of age or older were 10%, 6%, 13%, and 24%, respectively ($p < 0.001$).

During 1994 and 1995, a moderate seasonal pattern of Hi and Hib invasive disease was observed, with more cases occurring during the winter months (Figure 2); this pattern was similar for children 4 years of age or younger (data not shown).

To determine the remaining cases of Hib invasive disease, data on the number of Hib vaccine doses received were reviewed for the 184 reported cases among children aged 4 years or younger by the age (in months) of the case-patient (Table 5). Eighty-five (47%) of the 181 children aged 4 years or younger with known age in months were too young (5 months or younger) to have completed a primary series with the most commonly used Hib vaccines (three-dose primary series). Of the 149 children aged 4 years or younger with known vaccination status, 83 were eligible (aged 6 months or older) to receive a primary series; of these, 52 (63%) were undervaccinated. Thirty-one (37%) of the 83 children had completed a primary series with either three doses of HbOC (Wyeth-Lederle Laboratories, Pearl River, NY) or PRP-T (Pasteur Mérieux Connaught, Lyon, France), or with two doses of PRP-OMP (Merck, Inc., West Point, PA), or one dose with any Hib-containing vaccine on or after 15 months of age. Manufacturer and vaccine lot information was available for 22 of the

Table 4. Reported infection site[a] among Hi invasive disease cases,[b] United States, 1994–1995

	Number (%)	
Site	Children aged 0-4years[c]	Persons aged ≥5 years[d]
Bacteremia[e]	299 (62)	551 (51)
Meningitis	213 (44)	167 (16)
Pneumonia	42 (9)	390 (36)
Cellulitis	19 (4)	7 (1)
Epiglottitis	8 (2)	19 (2)

[a]Includes case-patients with multiple infection sites, such as meningitis and bacteremia.
[b]Case-patients with known infection site (n=1,556).
[c]N=485.
[d]N=1,071.
[e]Bacteremia is reported following positive blood culture results, with or without foci.

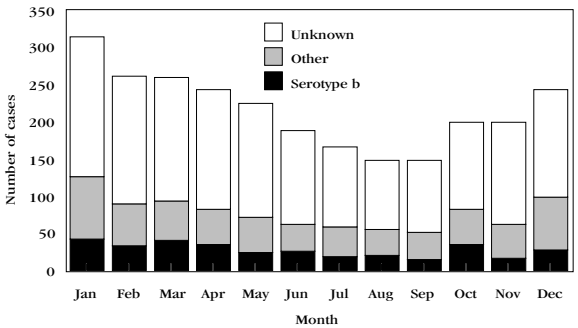

Figure 2. Reported Hi invasive disease cases by serotype and month of onset,[a] United States, 1994–1995.
[a]N=2,609; excluding 10 cases with unknown month of onset.

Table 5. Children aged ≤4 years with invasive Hib disease,[a] by number of Hib vaccine doses received and age group, United States, 1994–1995

Age (mos.)	No. of children receiving vaccine[b] Rec.[c]	0	1	2	3	4	Vaccination status unknown	Total
0-1	0	15					2	17
2-3	1	17	12				9	38
4-5	2	7	13	2			8	30
6-11	3	13	8	11	6		7	45
12-18	4	5	1	2	7	2	0	17
19-59	4	11	5	1	7	4	6	34
Total	4	68	39	16	20	6	32	181

[a]Excludes three children aged <1 year but missing age in months; vaccination status was unknown for two of the children, and one child was unvaccinated.
[b]Five children completed a primary series with 2 doses of PedvaxHIB (at ages 7, 11, 13, 14, and 43 months); two children completed a primary series with 1 dose at ≥15 months of age.
[c]Cumulative recommended doses for PRP-T and HbOC vaccines.

31 children who had received a primary series. No single lot predominated, and no child received vaccine from the same lot for all doses.

Overall, 49 states and 673 (21%) of the 3,137 U.S. counties reported at least one Hi invasive disease case with disease onset during the 2-year period; of these, 327 (49%) counties (from 47 states) reported two or more Hi cases (Figure 3A). Forty-seven states and 339 (11%) of counties reported Hi invasive disease cases among children aged 4 years or younger, and 42 states and 137 (4%) counties reported Hib cases during the 2-year period; 25 (18%) of these counties reported two or more Hib cases (range 2 to 8 cases). In these 25 counties, the incidence for Hib invasive disease was 0.41 to 12.19 per 100,000 children aged 4 years or younger (Figure 3B). Among the 14 counties with two Hib cases, the median time between cases was 139 days (range 14 to 334 days). In three of the 11 counties with three or more Hib invasive disease cases, children were too young (aged 5 months or younger) to have completed a primary series with an Hib vaccine; in the remaining eight counties, 20 (67%) of 30 children had been eligible for vaccination (aged 6 months or older) and only one child had completed a primary series. In these 11 counties, the incidence for Hib invasive disease was 0.41 to 4.31 per 100,000 children aged 4 years or younger.

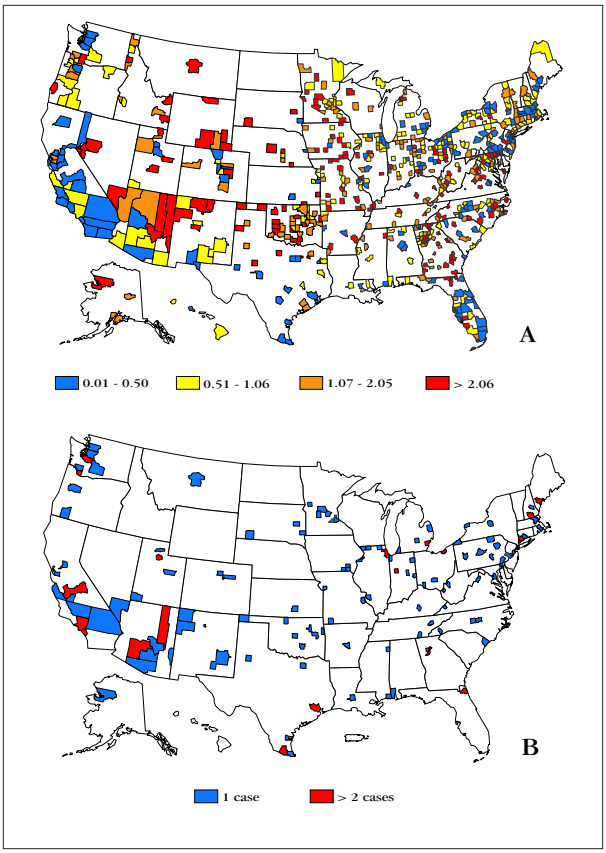

Figure 3. A. Average annual incidence of Hi invasive disease among persons of all ages, by county, United States, 1994–1995. B. Reported number of Hib invasive disease cases among children aged ≤ 4 years, by county, United States, 1994–1995.

Conclusions

Invasive Hib disease among children aged 4 years or younger in the United States has become rare because of the introduction and widespread use of conjugate Hib vaccines. Although national vaccination coverage of children 19 to 35 months of age with three or more doses of Hib conjugate vaccines only reached 90% in 1995 and some metropolitan areas had lower coverage rates (16,17), all Hi invasive disease incidence in 1994 and 1995 was 98% lower than during the prevaccine era. During that era, Hib invasive disease was the cause of more than 95% of Hi invasive disease (18-22). While our results were not directly comparable to prevaccine Hib invasive disease estimates because of reliance on passive reporting and lack of serotype data (23), in the active surveillance sites, the race-adjusted

incidence of Hib invasive disease declined 99% from 1989 to 1995 (3). Before Hib conjugate vaccine availability in 1988, the highest Hib invasive disease incidence had been in children 6 to 17 months of age (estimated annual incidence of 275 and 223 per 100,000 for children 6 to 11 and 12 to 17 months of age, respectively), compared with children 5 months of age or younger (estimated annual incidence of 148 per 100,000) (18-22). However, during 1994 and 1995, children aged 5 months or younger had the highest incidence of Hib invasive disease of all age groups (2.2 per 100,000) (Table 1); these children were too young to have completed a primary vaccination series.

Consistent with previous studies of Hib invasive disease in children aged 4 years or younger in the prevaccine era, we found higher Hi and Hib disease incidence among American Indian (the highest incidence rates), black, and Hispanic children than among Asian or Pacific islander and white children (18,20). In the prevaccine era, Hib invasive disease rates among American Indian children were approximately five times higher than among non-American Indian children in Alaska (annual incidence of 601 per 100,000 children) (20). Although important progress has been made in reducing Hib invasive disease in this population, American Indian children continue to have higher disease incidence than other racial or ethnic groups. The reasons for the higher Hib invasive disease incidence among some ethnic or racial groups or some regions are unknown but could be associated with one or more of the following: low Hib vaccine coverage levels in an area (e.g., an inner-city "pocket of need"), low socioeconomic status, cofactors, host susceptibility, and high levels of carriage in the population. In 1996, a study of Alaskan native children aged 6 years or younger with high vaccination coverage (>90%) found a carriage level of 8%, a rate similar to that in the prevaccine era (24). However, a 1992 study in the Atlanta, Georgia, metropolitan area documented a low (0.2%) carriage level among children with a 75% Hib vaccination coverage (25). In the United States, the reduction in the number of reported cases has been greater than expected when the efficacy of Hib conjugate vaccines and the coverage of a primary series alone are taken into account; this is likely due to the reduction of pharyngeal carriage in vaccinated children, which protects nonvaccinated persons in the community (i.e., herd immunity) (18,26). High carriage levels are unlikely in most areas of the United States; the difference in the results of the two studies (24,25) may be due to the use of different Hib conjugate vaccines, as well as to different host and environmental factors. In European countries that use Hib conjugate vaccines, increasing vaccine use has been associated with diminishing rates of both Hib pharyngeal carriage and invasive Hib disease (26).

With the marked decline among children, the occurrence of Hib invasive disease among older persons (aged 5 years or older) has achieved greater prominence. Although serotype data were missing from most cases, reported cases among both children (n = 184) and adults (n = 159) indicate persistent circulation of Hib. Reported Hib invasive disease cases and nasopharyngeal carriage among children have decreased; however, because these data were from passive reporting sources it is not clear if a similar decrease in invasive Hib disease in older children and adults has occurred nationally. Because of underreporting of adult Hib invasive disease cases nationally, 1994 to 1995 incidence rates cannot be directly compared with rates found in prior studies (8-11). In our study, 28% of serotyped isolates were Hib; however, among adults in the Atlanta, Georgia, metropolitan active surveillance site, Hi invasive disease cases due to Hib declined in 1990 to 1991 from 50% to 0% in 1995 to 1996 (8,27). Nevertheless, because previous studies have found that most Hi and Hib invasive disease occurred among immunocompromised patients or patients with underlying conditions (e.g., chronic lung disease, splenectomy, leukemia, HIV infection, sickle-cell disease), health-care providers should consider Hib vaccination for patients at high risk (6). Because of continued occurrence of Hib invasive disease among older persons and infants too young to be vaccinated, additional strategies may be needed to eliminate Hib invasive disease among children aged 4 years or younger. In addition, ongoing surveillance for Hi invasive disease is needed to monitor incidence among older persons; case investigations may include assessments for underlying immunosuppressive conditions or ascertainment of other cofactors such as prior or concomitant viral infections.

Continued decline in incidence of Hib invasive disease in the United States requires that children aged 4 years or younger be

appropriately vaccinated to protect them and other children and infants in the community. Intensive efforts to increase vaccination levels among children in areas with coverage rates lower than 90% are needed to reach the elimination goal. Children should receive a primary Hib vaccination series at ages 2, 4, and 6 months with either HbOC or PRP-T, or at ages 2 and 4 months with PRP-OMP (6). A booster dose with any of the four licensed Hib conjugate vaccines should be given at 12 to 15 months of age (6). Among children who were eligible for vaccination, timely completion of a primary series might have prevented 63% of reported Hib invasive disease cases in 1994 and 1995. Few (28) Hib cases among children 4 years or younger were reported in those who had completed a primary series with Hib vaccine, which suggests that vaccine failure was uncommon. However, because of passive reporting, vaccination status and vaccine failure may be underestimated. Hib invasive disease in children too young to have completed a primary series will continue to be a barrier for elimination unless Hib nasopharyngeal carriage rates in the population (including adults) approach zero.

Because the highest Hib invasive disease incidence occurs among infants, all infants should be appropriately vaccinated, beginning at 2 months of age. In a case-control study for undervaccination among Hib invasive disease cases among children aged 2 to 18 months in the United States, risk factors for undervaccination included low maternal education and having a single mother (29); special outreach efforts may be needed to vaccinate children at risk. Other programmatic strategies to increase vaccination rates include linkage of the Special Supplemental Nutrition Program for Women, Infants and Children (WIC) to vaccination assessment and referral (30), implementation of immunization registries and reminder/recall systems to encourage parents to vaccinate their children in a timely fashion (28,31,32), and monitoring clinic coverage levels and providing feedback to clinic staff (33). In addition to high vaccination coverage of other children in the community to help reduce carriage, breast-feeding of infants should be promoted because it is protective against invasive Hi disease in this age group (21). For children aged 4 years or younger with Hib invasive disease who have completed a primary Hib vaccination series, case investigations may include examining reasons for vaccine failure, such as measuring antibodies to the polyribosylribitol phosphate (PRP) capsule of Hib; undetectable levels suggest lack of immune response (18).

To monitor the changing epidemiology of Hib invasive disease and look for an increase or decline in incidence among children and adults, serotype information for all Hi invasive disease cases is essential and will generate data needed to examine new strategies for elimination. Surveillance is necessary for detecting pockets of continuing transmission and identifying areas in need of improved vaccine coverage (3). Each case of Hib invasive disease is a sentinel for circulation of Hib in a community. In addition, Hib invasive disease cases among children aged 4 years or younger may be a marker for low community coverage. Therefore, community immunization policies, practices, and coverage levels should be reviewed, and recommendations should be made, if needed, to improve Hib vaccination coverage levels.

Acknowledgments

The authors thank Wendy Wattigney for her review of the manuscript and assistance with statistical analysis and Shalani Desai for her meticulous assistance in data cleaning.

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Multiple-Drug Resistant Enterococci: The Nature of the Problem and an Agenda for the Future

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Enterococci, leading causes of nosocomial bacteremia, surgical wound infection, and urinary tract infection, are becoming resistant to many and sometimes all standard therapies. New rapid surveillance methods are highlighting the importance of examining enterococcal isolates at the species level. Most enterococcal infections are caused by *Enterococcus faecalis*, which are more likely to express traits related to overt virulence but—for the moment—also more likely to retain sensitivity to at least one effective antibiotic. The remaining infections are mostly caused by *E. faecium*, a species virtually devoid of known overt pathogenic traits but more likely to be resistant to even antibiotics of last resort. Effective control of multiple-drug resistant enterococci will require 1) better understanding of the interaction between enterococci, the hospital environment, and humans, 2) prudent antibiotic use, 3) better contact isolation in hospitals and other patient care environments, and 4) improved surveillance. Equally important is renewed vigor in the search for additional drugs, accompanied by the evolution of new therapeutic paradigms less vulnerable to the cycle of drug introduction and drug resistance.

The past few years have witnessed increasing interest in enterococci. Until recently, these ordinary bowel commensals languished as misclassified streptococci, commonly perceived "with the exception of endocarditis and rare cases of meningitis … [as] not … a major cause of serious infection" (1). In the last decade, however, enterococci have become recognized as leading causes of nosocomial bacteremia, surgical wound infection, and urinary tract infection (2,3). Two types of enterococci cause infections: 1) those originating from patients' native flora, which are unlikely to possess resistance beyond that intrinsic to the genus and are unlikely to be spread from bed to bed, and 2) isolates that possess multiple antibiotic resistance traits and are capable of nosocomial transmission. The therapeutic challenge of multiple-drug resistant (MDR) enterococci—those strains with significant resistance to two or more antibiotics, often including, but not limited to, vancomycin—has brought their role as important nosocomial pathogens into sharper focus.

The accretion and spread of antibiotic resistance determinants among enterococci, to the point where some clinical isolates are resistant to all standard therapies, highlight both the vulnerability of our present armament as well as the looming prospect of a "postantibiotic era" (4). This review focuses on the magnitude and nature of the problem posed by enterococci in general, and MDR enterococci in particular. For many points, only representative citations are provided.

Habitat and Microbiology

Enterococci normally inhabit the bowel. They are found in the intestine of nearly all animals, from cockroaches to humans. Enterococci are readily recovered outdoors from vegetation and surface water, probably because of contamination by animal excrement or untreated sewage (5). In humans, typical concentrations of enterococci in stool are up to 10^8 CFU per gram (6). Although the oral cavity and vaginal tract can become colonized, enterococci are recovered from these sites in fewer than 20% of cases. The

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predominant species inhabiting the intestine varies. In Europe, the United States, and the Far East, *Enterococcus faecalis* predominates in some instances and *E. faecium* in others (6). Ecologic or microbial factors promoting intestinal colonization are obscure. Of 14 or more enterococcal species (7), only *E. faecalis* and *E. faecium* commonly colonize and infect humans in detectable numbers. *E. faecalis* is isolated from approximately 80% of human infections, and *E. faecium* from most of the rest. Infections to other enterococcal species are rare.

Enterococci are exceedingly hardy. They tolerate a wide variety of growth conditions, including temperatures of 10°C to 45°C, and hypotonic, hypertonic, acidic, or alkaline environments. Sodium azide and concentrated bile salts, which inhibit or kill most microorganisms, are tolerated by enterococci and used as selective agents in agar-based media. As facultative organisms, enterococci grow under reduced or oxygenated conditions. Enterococci are usually considered strict fermenters because they lack a Kreb's cycle and respiratory chain (8). *E. faecalis* is an exception since exogenous hemin can be used to produce d, b, and o type cytochromes (9,10). In a survey of 134 enterococci and related streptococci, only *E. faecalis* and *Lactococcus lactis* expressed cytochrome-like respiration (11). Cytochromes provide a growth advantage to *E. faecalis* during aerobic growth (9). *E. faecalis* cytochromes are only expressed under aerobic conditions in the presence of exogenous hemin (9,10,12) and, therefore, may promote the colonization of inappropriate sites.

Enterococci are intrinsically resistant to many antibiotics. Unlike acquired resistance and virulence traits, which are usually transposon or plasmid encoded, intrinsic resistance is based in chromosomal genes, which typically are nontransferrable. Penicillin, ampicillin, piperacillin, imipenem, and vancomycin are among the few antibiotics that show consistent inhibitory, but not bactericidal, activity against *E. faecalis*. *E. faecium* are less susceptible to ß-lactam antibiotics than *E. faecalis* because the penicillin-binding proteins of the former have markedly lower affinities for the antibiotics (13). The first reports of strains highly resistant to penicillin began to appear in the 1980s (14,15).

Enterococci often acquire antibiotic resistance through exchange of resistance-encoding genes carried on conjugative transposons, pheromone-responsive plasmids, and other broad-host-range plasmids (6). The past two decades have witnessed the rapid emergence of MDR enterococci. High-level gentamicin resistance occurred in 1979 (16) and was quickly followed by numerous reports of nosocomial infection in the 1980s (17). Simultaneously, sporadic outbreaks of nosocomial *E. faecalis* and *E. faecium* infection appeared with penicillin resistance due to ß-lactamase production (18); however, such isolates remain rare. Finally, MDR enterococci that had lost susceptibility to vancomycin were reported in Europe (19,20) and the United States (21).

Among several phenotypes for vancomycin-resistant enterococci, VanA (resistance to vancomycin and teicoplanin) and VanB (resistance to vancomycin alone) are most common (22). In the United States, VanA and VanB account for approximately 60% and 40% of vancomycin-resistant enterococci (VRE) isolates, respectively (23). Inducible genes encoding these phenotypes alter cell wall synthesis and render strains resistant to glycopeptides (22).

VanA and VanB types of resistance are primarily found among enterococci isolated from clinical, veterinary, and food specimens (24), but not other common intestinal or environmental bacteria. In the laboratory, however, these genes can be transferred from enterococci to other bacteria (22). For example, *Staphylococcus aureus* has been rendered vancomycin-resistant through apparent transfer of resistance from *E. faecalis* on the surface of membrane filters and on the skin of hairless obese mice (25), which indicates that there is no biologic barrier to the emergence of vancomycin-resistant *S. aureus*. Clinical isolates of highly vancomycin-resistant *S. aureus* have yet to be identified, although strains with reduced susceptibility to vancomycin have appeared (26). The mechanism of resistance for these strains remains undetermined but does not appear to involve genes associated with VanA or VanB phenotypes.

Epidemiology

Enterococci account for approximately 110,000 urinary tract infections, 25,000 cases of bacteremia, 40,000 wound infections, and 1,100 cases of endocarditis annually in the United States (2,27,28). Most infections occur in hospitals. Although several studies have suggested an increase in nosocomial infection rates for enterococci in recent years, National Nosocomial

Infections Surveillance system data show little change in the percentage of enterococcal bloodstream (12% vs. 7%), surgical site (15% vs. 11%), and urinary tract (14% vs. 14%) infections over the past 2 decades (3,29). Adequate surveillance data prior to 1980 are not available. Enterococcal infection deaths have also been difficult to ascertain because severe comorbid illnesses are common; however, enterococcal sepsis is implicated in 7% to 50% of fatal cases (6). Several case-control and historical cohort studies show that death risk associated with antibiotic-resistant enterococcal bacteremia is severalfold higher than death risk associated with susceptible enterococcal bacteremia (30). This trend will likely increase as MDR isolates become more prevalent.

Colonization and infection with MDR enterococci occur worldwide. Early reports showed that in the United States, the percentage of nosocomial infections caused by VRE increased more than 20-fold (from 0.3% to 7.9%) between 1989 and 1993, indicating rapid dissemination. New database technologies, such as The Surveillance Network (TSN) Database-USA, now permit the assessment of resistance profiles according to species. TSN Database electronically collects and compiles data daily from more than 100 U.S. clinical laboratories, identifies potential laboratory testing errors, and detects emergence of resistance profiles and mechanisms that pose a public health threat (e.g., vancomycin-resistant staphylococci).

Data collected by the TSN Database between 1995 and September 1, 1997 were analyzed to determine whether the earlier increase in vancomycin resistance was unique to vancomycin, whether it represented a continuing trend, and whether speciation is quantifiably important in analyzing this trend. *E. faecalis* resistance to ampicillin and vancomycin is uncommon (Figure 1); little change in resistance prevalence occurred from 1995 to 1997. In contrast, *E. faecium* vancomycin and ampicillin resistance increased alarmingly. In 1997, 771 (52%) of 1,482 of *E. faecium* isolates exhibited vancomycin resistance, and 1,220 (83%) of 1,474 isolates exhibited ampicillin resistance (Figure 1). *E. faecium* resistance notwithstanding, *E. faecalis* remained by far the most commonly encountered of the two enterococcal species in TSN Database. *E. faecalis* to *E. faecium* total isolates were approximately 4:1 (Figure 1), blood isolates 3:1, and urine isolates 5:1. This observation underscores

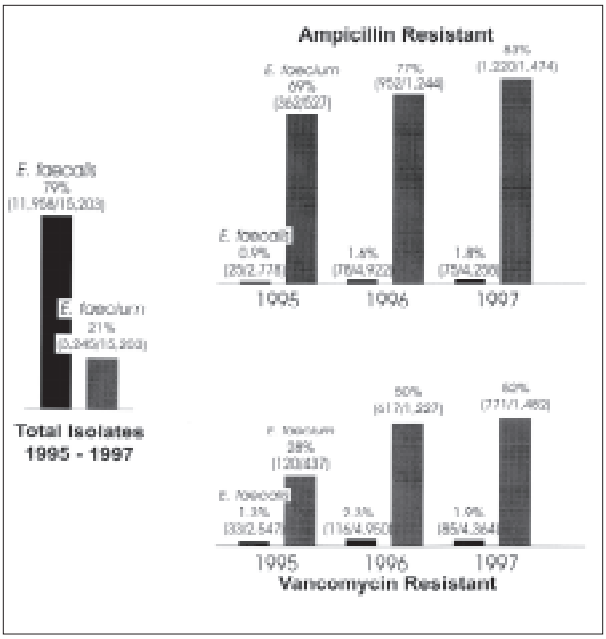

Figure 1. Epidemiology of enterococcal infection based on 15,203 susceptibility results obtained by The Surveillance Network (TSN) Database-USA, 1995 to Sep 1, 1997. The increase in total numbers between 1995 and 1996 represents additional reporting centers coming on line. Numbers for 1997 represent total collected for the partial year to Sep 1, 1997.

important differences in the survival strategies and likelihood of therapeutic success, critical factors usually obscured by lumping the organisms together as *Enterococcus* species or enterococci. Widespread emergence and dissemination of ampicillin and vancomycin resistance in *E. faecalis* would significantly confound the current therapeutic dilemma. There is little reason to suspect that vancomycin and ampicillin resistances only provide selective advantage for the species *faecium* and not *faecalis*. The relative absence of these resistances in *E. faecalis* may simply reflect a momentary lack of penetrance and equilibration of the traits. Because of these important differences between the two species, meaningful surveillance of enterococcal resistance must include species identification.

Although exact modes of nosocomial transmission for MDR enterococci are difficult to prove, molecular microbiologic and epidemiologic evidence strongly suggest spread between patients, probably on the hands of health-care providers or medical devices, and between hospitals by patients with prolonged intestinal

colonization. At least 16 outbreaks of MDR enterococci have been reported since 1989 (31); all but two were due to *E. faecium*. This disparity, particularly in view of the higher numbers of clinical *E. faecalis* isolates, may reflect a reporting bias due to the novelty of the combinations of resistance that occur in *E. faecium*. When isolates from outbreaks of MDR enterococci have been analyzed by genetic fingerprints, more than half involve clonally related isolates (18,32).

Prior treatment with antibiotics is common in nearly all patients colonized or infected with MDR enterococci (33-35). Clindamycin, cephalosporin, aztreonam, ciprofloxacin, aminoglycoside, and metronidazole use is equally or more often associated with colonization or infection with MDR enterococci than vancomycin use. Other risk factors include prolonged hospitalization; high severity of illness score; intraabdominal surgery; renal insufficiency; enteral tube feedings; and exposure to specific hospital units, nurses, or contaminated objects and surfaces within patient-care areas.

Infection Control

Controlling the spread of MDR enterococci among inpatients is difficult. We know relatively little about the biology of enterococcal transmission or the specific microbial factors favoring colonization by exogenous enterococcal strains. Nevertheless, VRE infection control policies, which could apply to MDR enterococci, were recently published by the Hospital Infection Control Practices Advisory Committee (36). Control methods include routine screening for vancomycin resistance among clinical isolates, active surveillance for VRE in intensive care units, contact isolation to minimize person-to-person transmission, and vancomycin restriction.

These measures to limit VRE spread, however, have failed on occasion (35). Not all hospitals can or are willing to perform active surveillance. Because more patients are typically colonized with VRE (3% to 47%) than are infected (35,37,38), and because intestinal colonization can be prolonged, passive surveillance by routine cultures allows colonized inpatients to go unidentified and serve as point sources for continued spread of VRE. Even if all colonized inpatients are successfully identified, VRE may be spread by health-care workers through either inadequate hand washing (39) or through contact with items such as bedrails, sinks, faucets, and doorknobs (enterococci can persist for weeks on environmental surfaces) (40). Decontamination efforts must be rigorous.

The Hospital Infection Control Practices Advisory Committee strongly recommended restricting oral and parenteral vancomycin to control VRE (36). However, limiting use of vancomycin while ignoring widespread use of other broad spectrum antibiotics likely will not lead to maximal control of VRE or of MDR enterococci.

Antibiotics may promote colonization and infection with MDR enterococci by at least two mechanisms. First, many broad spectrum antibiotics have little or no anti-enterococcal activity, and administration commonly leads to overgrowth of susceptible (or resistant) enterococci at sites at risk for infection. Second, most antibiotics substantially reduce the normal resistance of the intestinal tract to colonization by exogenous organisms (41). Colonization resistance results primarily from the "limiting action" of the normal anaerobic flora, and to a lesser extent from an intact mucosa, gastric acid secretion, intestinal motility, and intestinal-associated immunity (41). Antibiotic-induced alterations in the protective flora of the intestine provide large footholds for colonization with exogenous pathogens such as MDR enterococci (41). Antibiotic restriction programs would be more effective if they included prudent prescribing of all antibiotics, not just single agents such as vancomycin. This approach substantially decreased intestinal colonization with VRE in one hospital pharmacy that restricted vancomycin, cefotaxime, and clindamycin (42).

At a minimum, a successful program for control of MDR enterococci requires effective passive and active surveillance to identify colonized and infected patients, absolute adherence to contact isolation by health-care workers, rigorous decontamination of patient-contact areas and judicious use or restriction of vancomycin and other broad spectrum antibiotics.

Therapeutic Approaches

Suitable antibiotics often are not available to treat MDR enterococcal infections, e.g., endocarditis or bacteremia, in the presence of neutropenia. Combinations of penicillin with vancomycin, ciprofloxacin with ampicillin, or novobiocin with doxycycline, among others, have been used (43) but can be unpredictable and

remain clinically unproven. In one report chloramphenicol successfully treated chloramphenicol-susceptible infections (44), but these findings await confirmation in controlled trials.

Promising new antibiotics for MDR enterococcal infection under investigation include fluoroquinolones, streptogramins, oxazolidinones, semisynthetic glycopeptides, and glycylcyclines. Clinafloxacin, a fluoroquinolone with improved potency against enterococci compared with ciprofloxacin, has excellent activity against VRE, appears bactericidal in vitro, and has been effective in treatment of enterococcal infections in a murine model (45). Although single-step resistance to clinafloxacin could not be detected in vitro, multistep resistance is readily achieved. Should this agent gain approval for treatment of enterococcal infections, selection for resistance may limit its effectiveness.

Quinupristin/dalfopristin (Synercid) is a combination of streptogramins A and B that inhibits protein synthesis and has a narrower spectrum of activity against enterococci than clinafloxacin (46). Many, but not all, *E. faecium* isolates with VanA and VanB phenotypes are susceptible (47); however, *E. faecalis* is uniformly resistant, and superinfection has been reported during therapy (48). In addition, quinupristin/dalfopristin is bacteriostatic only, potentially allowing emergence of resistance (49). For these reasons the drug may have only a limited role in treating MDR enterococcal infections. Novel oxazolidinones and glycylcyclines have also shown potent activity against enterococci, including MDR enterococci (50,51), but await further testing.

The substantial drawback of the broad spectrum approach is that the more organisms affected (both protective commensals as well as pathogens), the more opportunities for resistance to evolve. Broad spectrum antibiotics permit empiric therapy in the absence of a specific diagnosis and generate a more substantial return on investment in the short term. However, broad spectrum antibiotics affect not only disease-causing organisms but also commensals present in numbers large enough to generate resistance by otherwise rare mutations or genetic exchange events. As long as market forces favor development of broad spectrum therapeutics, a cycle of drug introduction followed by emergence of resistance undoubtedly will continue.

Targeted Therapeutics

In contrast to the historical reliance on broad spectrum antibiotic therapy, the continuing development and introduction of rapid diagnostic techniques (52) may allow a more focused approach to infectious disease therapy. Any of the myriad microbial-host interactions that subvert the host response or damage tissues during an infection represent potential therapeutic targets. However, many key interactions in disease pathogenesis are specific to the organism involved—a characteristic that is both a strength and a weakness. Because of the specificity of these interactions, rapid and accurate diagnosis is required. However, therapeutics aimed only at interaction between host and a specific pathogen should leave the diverse commensal flora essentially unaffected. As a result, the targeted population would be restricted to the relatively small numbers of disease-producing bacteria and would not likely reach the numbers or diversity required to make development of resistance a statistical probability.

The current spectrum of approaches to identify new antiinfective compounds has two extremes: 1) screening vast libraries of compounds to identify substances that by chance inhibit a microbial property and 2) detailed study of interactions between host and parasite to identify critical events leading to host tissue damage or compromise (53).

With a long-term view toward new therapeutic approaches as well as optimal use of existing therapies, we and others have begun examining in detail the interactions between enterococci and host (6). A major obstacle is that enterococci also form part of the commensal or autochthonous flora; as such, they are nearly devoid of traits traditionally associated with overt pathogens and have subtle interactions with the host. Using inocula with as few as 10 organisms, we have developed sensitive biologic systems for examining the host-parasite interactions (54).

Although *E. faecium* strains are resistant to vancomycin and ampicillin more often than *E. faecalis strains*, the relative proportion of infections caused by these species has not dramatically changed in recent years (Figure 1). Since both organisms are frequently isolated from the commensal flora, this bias suggests that *E. faecalis* traits confer a greater degree of

intrinsic virulence, for example, cytolysin production, pheromone-responsive plasmid transfer (and accompanying production of aggregation substance), extracellular superoxide production, and a newly identified surface protein tentatively termed Esp (5,56,57) (Figure 2). These properties provide logical points of departure for developing new targeted therapeutic approaches to enterococcal disease; examination of more subtle interactions between *E. faecium* and host will follow as an understanding of enterococcal biology evolves.

Targeting the *E. faecalis* Cytolysin

Cytolysin is disproportionately expressed by *E. faecalis* strains associated with disease (5,55,56). This cytolysin causes rupture of a variety of target membranes, including bacterial cells, erythrocytes, and other mammalian cells, with activity observed as a hemolytic zone on some types of blood agar. Cytolysin contributes to the toxicity or lethality of infection in several infection models and is associated with a fivefold increased risk of sudden death from nosocomial bacteremia (54,56-59). Cytolysin also contributes to the appearance of enterococci in a murine bacteremia model (Figure 3; 45,60), an observation consistent with the disproportionate representation of cytolytic strains among human blood isolates (56,62).

Beginning with E.W. Todd in 1934 (63) and culminating in a recent study (64), the *E. faecalis* cytolysin has now been characterized as a unique, extensively modified bacterial toxin (Figure 4).

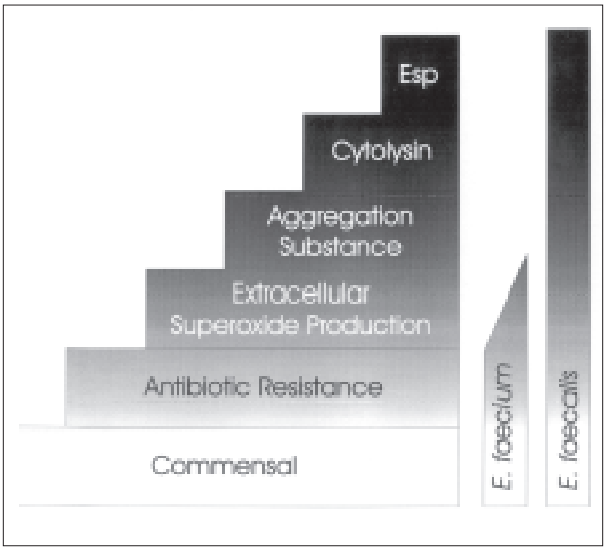

Figure 2. Virulence traits and their association with enterococcal species.

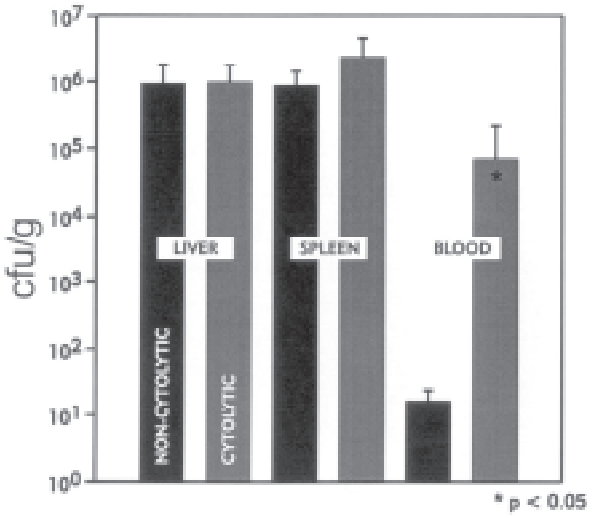

Figure 3. Cytolysin favors the appearance of circulating enterococci. In this experiment, 10^7 CFU of *E. faecalis*, either cytolytic FA2-2(pAM714) (60) or noncytolytic FA2-2(pAM771) (64), were intraperitoneally injected (45) into groups of five BalbC mice. Viable bacteria in liver, spleen, and the bloodstream were enumerated 48 hrs following injection, and significance assessed by Student's t-test. (P. Coburn, L.E. Hancock, and M.S. Gilmore, in preparation).

The cytolysin maturation pathway is ideally designed for therapeutic targeting because the two structural subunits are activated by an extracellular protease, an event that is accessible and potentially inhibitable by a novel therapeutic. Moreover, the activator protease, CylA, belongs to the subtilisin class of serine proteases (64), whose structure-function relationships and inhibitor design we are beginning to understand. Investigations are in progress to design and test inhibitors of extracellular cytolysin activation to determine whether a reduction by several logs in the levels of circulating enterococci can be attained as would be predicted by the observed behavior of cytolysin mutants (Figure 3).

An inhibitor of cytolysin activation, accompanied by appropriate rapid diagnostics, would be of potential value in treating bacteremia caused by cytolytic strains of *E. faecalis* without affecting commensal flora. Development of resistance should be exceedingly improbable because of the small number of bacteria targeted and because unlike antibiotics, cytolysin inhibitors would not act directly on the organism itself.

Other Enterococcal Targets

Several laboratories are using information on the *E. faecalis* genome and genomes of other pathogens to identify therapeutic targets (66) and facilitate studies on pathogenesis for the remaining 60% of noncytolytic enterococcal infections. The genome of an *E. faecalis* strain that caused multiple hospital infections (56) was sampled at high frequency by sequence analysis. Several sequences appeared to have a role in host-parasite interaction. The gene specifying Esp encodes an apparent surface protein of unusual repeating structure (67). Although a role for this protein in enterococcal infection has yet to be determined, its distribution among clinical and commensal strains is tantalizing: 29 of 30 strains with this gene were recovered from patients with bacteremia or endocarditis; one of 34 isolates obtained from healthy volunteers possessed Esp. The core of this large protein (inferred mass of 202 kDa) consists of a series of 82 amino acid repeats encoded by highly conserved tandem 246 base pair repeats. Lack of divergence in repeat sequences suggests that recombination occurs at high frequency, perhaps during infection. Moreover, the number of repeats observed in homologous genes from different *E. faecalis* isolates is 3 to 9 (67). This gene is flanked by a sequence similar to the transposase of IS*905*. None of 24 clinical or laboratory *E. faecium* isolates had this gene (67; V. Shankar, G. Lindahl, and M. Gilmore, unpub. data).

A second promising lead involves a series of genes encoding products highly related to enzymes involved in O-antigen synthesis in gram-negative bacteria (68). Preliminary evidence suggests that in *E. faecalis* these genes contribute to cell wall carbohydrate synthesis and that this carbohydrate relates to persistence in vivo. A knockout in one of these genes results in a strain with normal in vitro growth, but after subcutaneous injection, the mutant was more readily cleared than the wild type parental strain (68). One of the genes studied was present in all *E. faecalis* strains examined, whereas another occurs only in *E. faecalis* strains that share a periodate-susceptible epitope (68). Collectively, these data indicate that enzymes for synthesis of *E. faecalis* surface carbohydrates are important for persistence in vivo and may represent a useful therapeutic target. Taking a different approach,

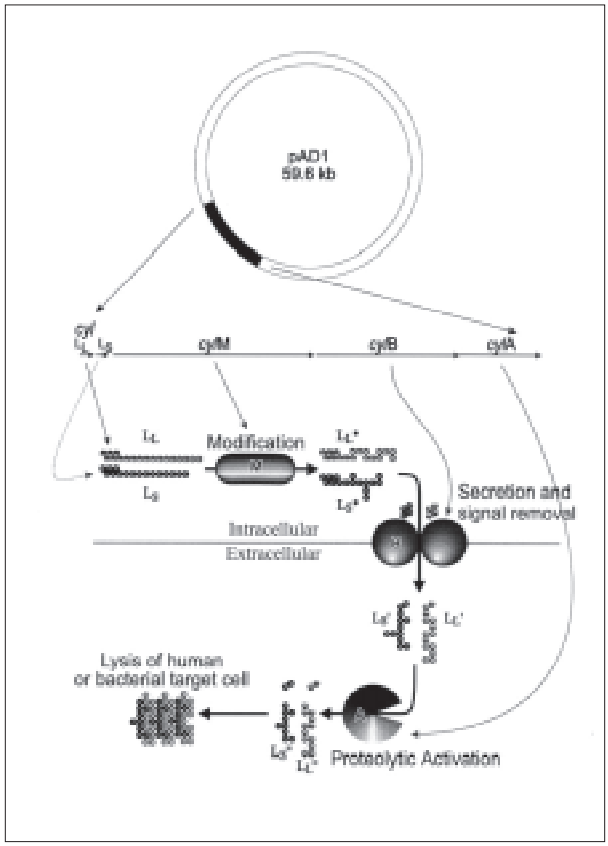

Figure 4. Cytolysin is expressed and processed through a complex maturation pathway (64). The cytolysin precursors, $CylL_L$ and $CylL_S$, are ribosomally synthesized. The putative modification protein, CylM, is required for the expression of $CylL_L$ and $CylL_S$ in an activatable form, and the secreted forms, $CylL_L$ and $CylL_S$ were recently shown to possess the amino acid lanthionine as the result of posttranslational modification (64). $CylL_L$ and $CylL_S$ both are secreted by CylB (65), which is accompanied by an initial proteolytic trimming event (64) converting each to $CylL_L$' and $CylL_S$', respectively. Once secreted, $CylL_L$' and $CylL_S$' are both functionally inactive until six amino acids are removed from each amino terminus. This final step in maturation is catalyzed by CylA (64), a subtilisin-type serine protease. Since this final catalytic event is essential, occurs extracellularly, and is catalyzed by a class of enzyme for which a substantial body of structural information exists, it represents an ideal therapeutic target. As shown in Figure 3, inhibition of cytolysin by mutation (or potentially by therapeutic intervention) results in a reduction by several orders of magnitude in the number of circulating organisms.

Arduino et al. (69,70) identified a protease-resistant, periodate susceptible substance associated with some strains of *E. faecium*, but not *E. faecalis*, which conferred resistane to phagocytosis in vitro. The relationship between the putative carbohydrate of *E. faecalis* under study above and the inhibitory substance of *E. faecium* remains to be determined. It may be found that many enterococci produce such carbohydrates at biologically significant levels in vivo, but only some strains of *E. faecium* do so in vitro.

Finally, recent observations indicate that nearly all *E. faecalis* strains, and only a few *E. faecium* strains, generate substantial extracellular superoxide. When *E. faecalis* isolates from patients with endocarditis and bacteremia were compared with isolates from healthy volunteers (71), on average, extracellular superoxide production was 60% higher among blood isolates than commensal strains. These data raised several questions: Do *E. faecalis* that produce larger amounts of extracellular superoxide possess greater metabolic flexibility, facilitating adaptation to nonintestinal infection sites? Does free radical production lead to host cell damage, allowing release of normally sequestered nutrients (e.g., hemin) that might promote enhanced *E. faecalis* growth through cytochrome formation? Might antioxidants modulate colonization or invasive infection? Answers to these questions may provide new insights into the transition from intestinal colonization to infection and may suggest new preventive strategies.

Obstacles to Further Development

Although important insights into enterococcal biology and pathogenesis are being gleaned from a reverse genetic approach, a paucity of information still exists on how enterococci colonize the intestinal tract and cause infection. For example, do *E. faecalis* or *E. faecium* colonize the colon through specific interactions with ligands on human epithelial cells or intestinal mucin? Do MDR enterococci possess alternate binding activities that enable them to colonize the intestinal tract at new sites without competing with the indigenous enterococci? Do probiotics have a role in restoring colonization resistance to an intestinal ecology altered by broad spectrum antibiotics?

Is enough being done to combat the emergence of highly resistant nosocomial pathogens? To effectively compete, industry remains highly responsive to market opportunities. Research in the public sector has been slow to respond, and as a result, our understanding of the biology of enterococcal infection is inadequate. Reasons for the modest public sector response include the following. 1) The emergence of resistant enterococci coincided with a reduction of public support for non-AIDS related infectious disease research. 2) The pathogenesis of nosocomial infection deviates from paradigms established for obligate pathogens. 3) The research infrastructure is relatively small because of the low importance traditionally attached to enterococci as etiologic agents of human disease and the deemphasis on antibiotic resistance research in the 1980s.

Conclusions

Historically, substantial resources have been invested in developing an in-depth understanding of the molecular biology of model organisms. During the 1960s and 1970s, when gram-negative organisms were leading causes of hospital- and community-acquired infections and gram-positive organisms were typically sensitive to existing antibiotics (72), a sizable investment in gram-negative model organisms was appropriate. However, with the emergence of gram-positive organisms as leading causes of both hospital- and community-acquired infection in the 1990s, a reevaluation of public research priorities is warranted.

Since antibiotic use became widespread 50 years ago, bacteria have steadily and routinely developed resistance. Control of the emergence of resistance will depend on new approaches to prudent antibiotic use in hospitals and clinics, based in part on improved surveillance for MDR enterococci and on better systems to encourage staff adherence to contact isolation procedures. Equally important will be development of new drugs with narrower spectra of activity aimed at known and potentially new targets and the evolution of market conditions that favor their use.

Portions of the work described were supported by Veterans Administration Merit Review Program, grants from the Public Health Service (EY08289 and AI41108), and an unrestricted award from Research to Prevent Blindness, Inc.

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Enteroaggregative *Escherichia coli*

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Enteroaggregative *Escherichia coli* (EAEC), an increasingly recognized cause of diarrhea in children in developing countries, has been particularly associated with persistent diarrhea (more than 14 days), a major cause of illness and death. Recent outbreaks implicate EAEC as a cause of foodborne illness in industrialized countries. The pathogenesis of EAEC infection is not well understood, but a model can be proposed in which EAEC adhere to the intestinal mucosa and elaborate enterotoxins and cytotoxins, which result in secretory diarrhea and mucosal damage. EAEC's ability to stimulate the release of inflammatory mediators may also play a role in intestinal illness.

Since first described in 1987, enteroaggregative *Escherichia coli* (EAEC) have been recognized increasingly as agents of diarrhea in developing countries and (more recently) in industrialized countries. We review the data supporting the pathogenicity of EAEC, discuss clinical and epidemiologic features, and summarize the current status of EAEC pathogenesis research.

History

E. coli have been implicated as agents of diarrheal disease since the 1920s (1). In 1979, Cravioto et al. reported that most enteropathogenic *E. coli* (EPEC) adhered to HEp-2 cells in culture and that the adherence phenotype could be used to differentiate EPEC. Subsequently, many *E. coli* not of EPEC serogroups were also found to adhere to semiconfluent HEp-2 cells, but the adherence phenotype was clearly different from that induced by EPEC (2,3). Whereas the adherence pattern of EPEC was localized, the non-EPEC pattern was diffuse. The diffuse adherent strains were later subdivided into two further phenotypic subcategories: aggregative and true "diffuse" (4). Only the aggregative were associated with pediatric diarrhea in Santiago, Chile (4), prompting the investigators to propose two independent categories: EAEC and diffusely adherent *E. coli*. At the same time, non-EPEC, HEp-2 adherent strains (termed enteroadherent *E. coli*) were associated with pediatric and traveler's diarrhea in Mexico (5,6). These enteroadherent strains were later found to belong to the EAEC or diffusely adherent *E. coli* categories (7).

Thus, EAEC are now defined as *E. coli* that do not secrete heat-labile or heat-stable enterotoxins and adhere to HEp-2 cells in an aggregative (AA) pattern (Figure 1). This definition may encompass both pathogenic and nonpathogenic clones that share a factor(s) conferring a common phenotype.

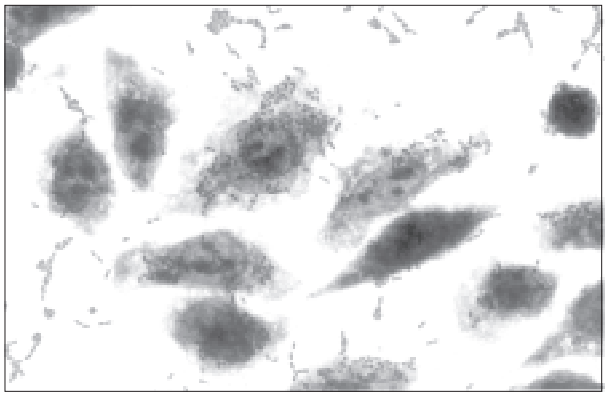

Figure 1. Aggregative pattern of adherence in the HEp-2 assay after 3 hours incubation of bacteria with HEp-2 cells. Note bacteria on the surface of the HEp-2 cells as well as on the glass substratum.

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Pathogenesis

Histopathology

EAEC strains adhere to the mucosal epithelium of gnotobiotic piglets in a thick mucus-containing blanket, irrespective of whether or not the strain induces diarrhea in this animal (8; Nataro, unpub. obs.; Figure 2). In these studies, the intestinal epithelium displayed pitting of goblet cells, suggesting stimulation of mucus secretion. EAEC strains adhered to sections of pediatric small bowel mucosa in an in vitro organ culture (IVOC) model (9); the bacteria were also embedded within a mucus-containing biofilm. Two further observations support a role of mucus in EAEC pathogenesis: EAEC bound rabbit mucus avidly in an in vitro model (10) and volunteers fed EAEC strains excrete mucoid stools (11). The formation of a heavy mucus biofilm may contribute to EAEC diarrheagenicity and, perhaps, to its ability to cause persistent colonization and diarrhea.

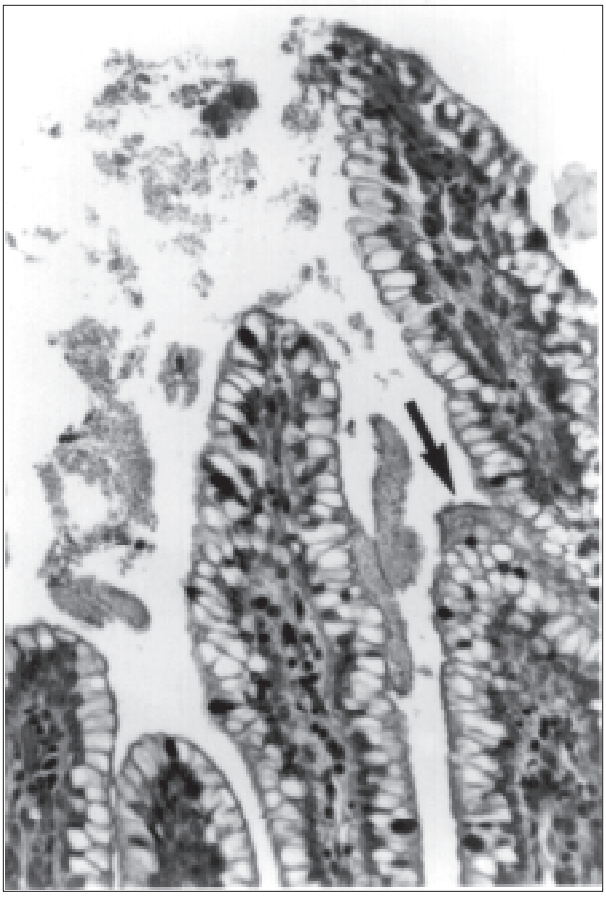

Figure 2. Biofilm (arrow), containing aggregating bacteria and mucus, adhering to the mucosa of a gnotobiotic piglet fed enteroaggregative *E. coli* strain 042 and sacrificed after 3 days. This piglet did not contract diarrhea. (Reprinted with permission of James Nataro and Clinical Microbiology Reviews. Clin Microbiol Rev 1998;11:142-201.)

In addition to forming the characteristic mucus biofilm, many EAEC strains induce cytotoxic effects on the intestinal mucosa. Infection with EAEC strains in rabbit and rat ileal loop models resulted in a destructive lesion demonstrable by light microscopy (7). EAEC induced shortening of the villi, hemorrhagic necrosis of the villous tips, and a mild inflammatory response with edema and mononuclear infiltration of the submucosa. Transmission electron microscopy showed normal microvillar architecture and no invasion of enterocytes; both light and electron microscopy showed adherent bacteria without the attaching and effacing lesion, which is characteristic of EPEC.

Mucosal damage has also been demonstrated in ileum specimens taken after patients died of EAEC persistent diarrhea during an outbreak in the malnutrition ward of Mexico City Hospital (12). This effect was reproduced in the IVOC model; EAEC induced exfoliation of enterocytes from the mucosal surface of pediatric intestinal biopsies (9). Indeed, EAEC strains elicit cytotoxic effects on T84 cells (human intestinal carcinoma) in vitro (13; Figure 3). In the in vitro model, EAEC induced the microvillar membrane to vesiculate and the cells from the monolayer to exfoliate. This effect was accompanied by increased vacuole formation and separation of the nucleus from the surrounding cytoplasm. Damage was most prominent in areas where EAEC were adhering to the cells. In both the T84 and IVOC systems, the toxic effects required genes encoding the adherence fimbriae (14), as well as other genes on the 65MDa plasmid.

Adherence

Adherence of EAEC to the intestinal mucosa has been well studied. A flexible, bundle-forming EAEC fimbrial structure 2 nm to 3 nm in diameter, designated aggregative adherence fimbriae I (AAF/I) (15), mediates HEp-2 adherence and human erythrocyte hemagglutination in EAEC strain 17-2. The genes for AAF/I are organized as two separate gene clusters on the 60 MDa plasmid separated by 9 kb of intervening DNA (16-18). Region 1 genes encode the fimbrial structure itself; nucleotide sequence analysis of the Region 1 cluster suggests that

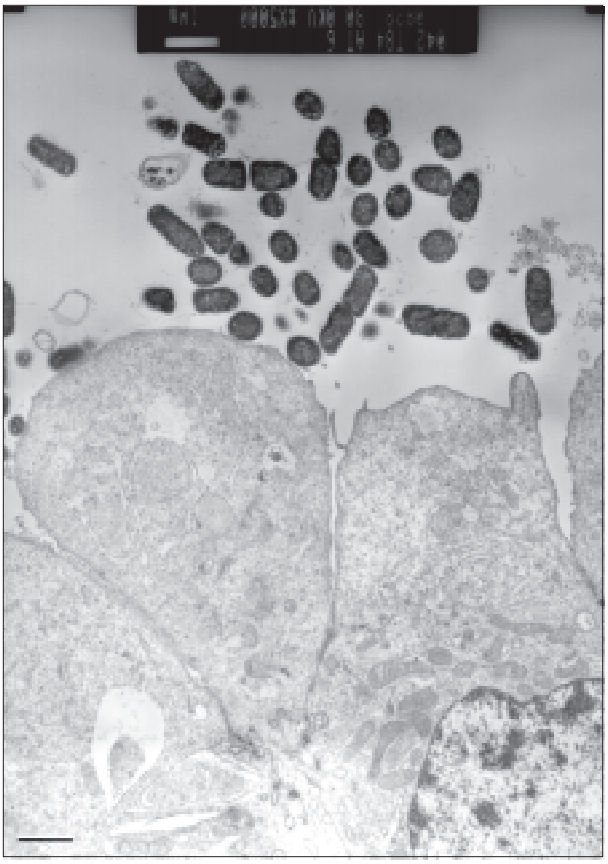

Figure 3. Effects of enteroaggregative *E. coli* strain 042 on T84 cells in culture. Polarized T84 monolayers were washed and inoculated with 10^6 CFU of 042 and allowed to coincubate at 37°C for 6 hrs. Transmission electron microscopy reveals adherence of bacteria to the apical surface of the T84 cells without internalization. The apical brush border is effaced; cells are ballooning and will eventually be extruded from the monolayer. (J.P. Nataro and S. Sears, unpub. data). Bar, 1 mm.

AAF/I is related to the Dr family of adhesins (18). Region 2 genes encode a transcriptional activator of AAF/I expression (AggR), which is a member of the AraC family of DNA binding proteins (17). The AAF/I fimbriae are bundle-forming fimbriae but do not show homology with members of the so-called type 4 class of fimbriae (19).

A second fimbrial structure, designated AAF/II, has been identified (14); it is distinct from but related to AAF/I. Insertional mutants in AAF/II genes no longer adhered to colonic mucosa in IVOC. DNA probe analysis suggested that AAF/I and AAF/II were each present in only a minority of EAEC strains and thus, as is the case with enterotoxigenic *E. coli*, intestinal colonization of EAEC appears to be mediated by one or more of several different fimbrial antigens.

In some EAEC strains, AA may be due to factors other than the AAF fimbriae (20,21). An afimbrial outer membrane protein or a 38 kDa outer membrane protein may be responsible for AA in some strains (20,21).

EAEC ST-Like Toxin

Savarino et al. have identified an open reading frame encoding a 4,100 Da homologue of the heat-stable enterotoxin designated EAST1 (22,23), which is a 38-amino acid protein featuring four cysteine residues, instead of six (which are characteristic of *E. coli* ST). A role for EAST1 in human disease has not been demonstrated, although EAST1 clones yield net increases in short circuit current in the rabbit mucosal Ussing chamber model (22). Of the four strains given to volunteers, one that induced diarrhea, as well as one that did not, secreted EAST1 (11).

EAST1 has been found in approximately 40% of EAEC strains and in a similar proportion of nonpathogenic *E. coli* strains (24). Other *E. coli* categories, most notably EHEC, elaborate EAST1 with a higher frequency than EAEC (24).

Invasiveness

Some EAEC strains may invade intestinal epithelial cells in vitro (25). However, human intestinal explants do not internalize EAEC, and clinical evidence for or against a role for invasiveness is lacking (9).

Other EAEC Toxins

In an outbreak of EAEC diarrhea in Mexico, Eslava et al. identified (in serum from patients in the outbreak) an approximately 108 kDa protein that induces exfoliation of enterocytes in the rat ileal loop model. DNA sequence analysis of the plasmid-borne gene (C. Eslava, J. Czeczulin, F. Navarro-Garcia, I. Henderson, A. Cravioto, J.P. Nataro, unpub. obs.) encoding this protein suggests that the toxin is a member of the family of proteins (26) called autotransporters because their secretion through the bacterial outer membrane is mediated by the carboxy terminus of the molecule. The 108 kDa toxin (termed Pet for plasmid-encoded toxin) also induces enterotoxic activity from rat intestinal tissue mounted in the Ussing chamber (F. Navarro-Garcia, C. Eslava,

J.P. Nataro, A. Cravioto, unpub. obs.). A 120 kDa EAEC supernatant protein had been described that elicited rises in intracellular calcium in HEp-2 cells (27). Whether or not this protein is related to the protein described by Eslava et al. remains to be determined.

Intestinal Inflammation

An ongoing study of infant diarrhea in Fortaleza, Brazil (28-30), has shown that children with EAEC infection have intestinal inflammation, as measured by the presence of inflammatory markers in the stool (31). These markers include lactoferrin, a stable neutrophil product and sensitive marker for fecal neutrophils; interleukin-8 (IL-8), a neutrophil chemokine; and interleukin-1β (IL-1β), a polyfunctional proinflammatory cytokine. Fecal lactoferrin, IL-8, and IL-1β were elevated in children with persistent diarrhea due to EAEC but not in controls (children in the same cohort without stool pathogens and free of diarrhea for 3 weeks before and after the time of the stool sample in prospective surveillance). Fecal lactoferrin and IL-1β were also elevated in children with EAEC but no diarrhea for 3 weeks. The origin of these inflammatory factors remains unknown, although they may be the result of direct stimulation of cytokine release from the epithelium.

While studying cytokine release in vitro, Steiner et al. observed IL-8 release from Caco-2 intestinal epithelial cells exposed to cell-free filtrates of EAEC strains 042 or 17-2. This release was apparently mediated by a heat-stable, high molecular weight, chromosomally encoded protein (31). Lipopolysaccharide does not appear responsible for this effect as it is not blocked by polymyxin B and is not elicited by normal *E. coli* flora.

The relationship between IL-8 release and the symptoms of EAEC infection is not known, especially since severe infiltration by neutrophils has not yet been reported in EAEC histopathologic specimens. However, a hypothetical role for IL-8 release in EAEC diarrhea can be envisioned. IL-8 released from epithelial cells in response to EAEC could act as the first step in a secretory cascade by recruiting neutrophils, since neutrophils in the intestinal epithelium release 5'-adenosine monophosphate, which is converted by a 5'-nucleotidase on the apical surface of enterocytes to adenosine, an agonist for chloride secretion (32). A similar mechanism of neutrophil chemotaxis and cytokine release may also be involved in the diarrhea produced by other microbial products, such as *Clostridium difficile* toxin A (33,34).

Strain Heterogeneity

EAEC strains exhibit considerable heterogeneity. Studies at the University of Maryland found that at a dose of 10^{10} CFU with bicarbonate, strain 042 (which produces AAF/II fimbriae, EAST1, and the 108 kDa Pet toxin) elicited loose stools in 4 of the 5 adult volunteers (11). Three other strains, all of which expressed AAF/I and one of which secreted EAST1, did not produce any intestinal symptoms. These data suggest that virulence is likely to be heterogeneous and that Pet may play an important role.

A Model of EAEC Pathogenesis

Extrapolating from the observations described above, we propose a three-stage model of EAEC pathogenesis. Stage I involves initial adherence to the intestinal mucosa and the mucus layer; this initial adherence is apparently mediated by the AAF fimbriae. Stage II comprises enhanced mucus production, apparently leading to the deposit of a thick mucus-containing biofilm encrusted with EAEC. The blanket may promote persistent colonization and nutrient malabsorption. Stage III, suggested from histopathologic and molecular evidence, includes the elaboration of toxins or inflammation, which result in damage to the mucosa and intestinal secretion. Malnourished hosts may be unable to repair the mucosal damage and may thus become prone to the persistent diarrhea syndrome.

The site of EAEC infection in the human intestine has yet to be clearly demonstrated. IVOC experiments have shown that EAEC strains can adhere to both small and large bowel mucosa (9). Tissue specimens in these studies were derived from pediatric patients and were not formalin-fixed before they were incubated with EAEC strains. These features may explain the discrepant results obtained by other investigators (35,36). The short incubation period observed in some humans challenged with strain 042 (as short as 8 hours) is also consistent with involvement of the small bowel in diarrheagenicity (11).

Clinical Features

The clinical features of EAEC diarrhea are increasingly well defined in outbreaks, sporadic

cases, and the volunteer model. Typical illness is manifested by a watery, mucoid, secretory diarrhea with low grade fever and little to no vomiting (37,38). However, up to one-third of patients with EAEC diarrhea have had grossly bloody stools (39); this outcome may be strain dependent. In volunteers infected with EAEC strain 042, the diarrhea was mucoid, of low volume, and without occult blood or fecal leukocytes; all patients remained afebrile. In such volunteers, the incubation period of the illness was 8 to 18 hours (11).

The duration of EAEC diarrhea is its most striking feature. In a community surveillance study in Anapur-Palla in northern India, the mean duration of EAEC-associated diarrhea cases in children under 3 years of age was 17 days, longer than that associated with any other pathogen (37). In this study, of the 41 cases of diarrhea, fever was found in 12%, gross blood in 12%, vomiting in 7%, and persistence of the episode more than 14 days in 44%.

A large percentage of patients excreting EAEC have detectable fecal lactoferrin and supranormal levels of IL-8 in the stool (31). Although this observation suggests that EAEC infection may be accompanied by mucosal inflammation, most patients lack overt clinical evidence of inflammation.

Diagnosis

EAEC colonization is detected by isolating *E. coli* from stool samples and demonstrating the AA pattern in the HEp-2 assay. Analysis of small bowel aspirates has not increased yield (40). Implication of EAEC as the cause of the patient's disease must be done cautiously, given the high rate of asymptomatic colonization in many populations (4,30,41-43). If no other organism is implicated in the patient's illness and EAEC is isolated repeatedly, it may be considered to be a probable cause of the patient's illness.

Despite various methods of performing the HEp-2 assay (4,44,45), comparative studies suggest that the technique first described by Cravioto et al. (45,46) (i.e., a single 3-hour incubation of bacteria with cells, without a change in medium during the assay) best discriminates among the three adherence patterns. Because AAF adhesins are expressed maximally in static L-broth cultures at 37°C (15), we incubate all HEp-2 assay inocula in this manner.

A DNA fragment probe has proven highly specific in detecting EAEC strains. This probe was developed by Baudry et al. (47), who tested fragments derived empirically from the plasmids of strains 17-2 and 042. A 1.0 kb plasmid-derived *Sau*3a fragment hybridized with 56 (89%) of 63 EAEC strains (defined by HEp-2 assay); of 376 strains representing normal flora and other diarrheagenic categories, only 2 hybridized with the probe. The correlation of the EAEC probe-positivity with AA varies by location. In some studies, the correlation achieves the 89% sensitivity reported by Baudry et al. (47,48), while in other studies, the sensitivity may be substantially lower (40). The epidemiologic significance of probe-positive versus probe-negative strains has not been determined. The nucleotide sequence of the AA probe represents a cryptic open reading frame adjacent to the plasmid replicon (J.P. Nataro, unpub. obs.). A polymerase chain reaction assay using primers derived from the AA probe sequence shows similar sensitivity and specificity (49).

Epidemiology

Volunteer studies and outbreak investigations have shown that at least some EAEC strains are human pathogens. A growing number of studies have supported the association of EAEC with diarrhea in developing countries; the increasing number of such reports and the rising proportion of diarrheal cases in which EAEC are implicated suggest that EAEC are important emerging agents of pediatric diarrhea.

EAEC in Sporadic Diarrhea

The earliest epidemiologic reports showed that EAEC were most prominently associated with persistent (lasting $\geq$14 days) cases of pediatric diarrhea (Table 1). In some of these studies, EAEC cultured from the stool during the first few days of diarrhea were predictive of a longer illness (37,43).

The importance of EAEC in diarrheal disease appears to vary geographically. On the Indian subcontinent, six studies (which included hospitalized patients with persistent diarrhea [50], outpatients visiting health clinics [51], and cases of sporadic diarrhea detected on household surveillance [37]) have demonstrated the importance of EAEC in pediatric diarrhea (37,38,48,50,51,53).

Table 1. Epidemiologic studies associating enteroaggregative *Escherichia coli* (EAEC) with diarrhea ($p < 0.05$)

Source or reference	Syndrome	Site	EAEC in cases vs controls	Comments
4	All diarrhea	Santiago, Chile	84(33%)/253 vs 20(15%)/134[a]	Community and hospital-based. First description of EAEC
37	Persistent diarrhea	Anapur-Palla, India	18(30%)/61 vs. 20(10%)/201[b]	Household surveillance of rural children ≤3 yrs; first association of EAEC with PD
50	Persistent diarrhea	New Delhi, India	18(20%)/92 vs 6(7%)/92[b]	Case-control study of nonbloody PD in children ≤ 2 yrs
28	Persistent diarrhea	Fortaleza, Brazil	8(20%)/40 vs. 2(5%)/38[b]	Household surveillance of children ≤ 5 yrs
39	All diarrhea; persistent diarrhea	Morales, Mexico	78(12%)/636[a] and 29(51%)/57[b] vs 5(5%)/100	Household surveillance of children ≤ 2 yrs
43	Persistent diarrhea	Mirzapoor, Bangladesh	17(27%)/62 vs 5(18%)/28[c]	Household surveillance of children ≤ 6 yrs. EAEC isolated in first 2 days of episode predicts persistence
29	Persistent diarrhea	Fortaleza, Brazil	8(20%)/40 vs 2(5%)/38[b]	Outpatients ≤ 29 months with PD
51	All diarrhea	New Delhi, India	9(21%%)/42 vs 4(4%)/107[b]	Children ≤ 4 yrs referred to hospital
38	Acute diarrhea	Calcutta, India	17(11%)/159 vs 3(2%)/174[d]	EAEC associated with acute secretory diarrhea in hospitalized pediatric patients
42	All diarrhea	Tehran, Iran	99(32%)/309 vs 17(17%)/100[a]	Pediatric outpatients. High rate of antibiotic resistance among EAEC
39	Persistent diarrhea	Fortaleza, Brazil	38(68%)/56 vs 13(31%)/42[b]	Outpatients ≤ 3 yrs; EAEC associated with more cases of PD than all other agents combined
48	Acute diarrhea	Vellore, India	60(8%)/794 vs 22(4%)/566[a]	Outpatients ≤ 3 yrs with diarrhea ≤ 3 days
52	All diarrhea	Wurzberg, Germany	16(2%)/798 vs 0/580[a]	Hospitalized children ≤ 16 yrs. First description of EAEC disease burden in industrialized countries
41	Acute diarrhea	Caracas, Venezuela	138(27%)/513 vs 36(15%)/241[a]	Inpatient and outpatient children ≤ 2 yrs with diarrhea ≤ 3 days. EAEC only associated with diarrhea in infants ≤ 2 mos of age
Jalla-luddin et al., 1997, pers. comm.	All diarrhea	Lwiro, Zaire	29(25%)/115 vs 3(8%)/34[a]	Outpatients ≤ 5 yrs. First description of EAEC in Africa

[a]All diarrhea vs. asymptomatic.
[b]Persistent diarrhea (PD) vs. asymptomatic.
[c]Persistent diarrhea vs. acute diarrhea.
[d]Secretory vs. invasive diarrhea.

EAEC and persistent diarrhea syndrome have been consistently associated (28,29,30,40). In Brazil, EAEC have been implicated in up to 68% of persistent diarrhea cases (40), which represent a disproportionate share of deaths due to diarrhea. EAEC have also been implicated as causes of sporadic diarrhea in Mexico, Chile, Bangladesh, and Iran (4,39,42,43).

The epidemiologic characteristics of EAEC (e.g., likely sources, reservoirs of infection, routes of transmission, seasonality, and age-distribution) are largely unknown. Most studies in which EAEC are implicated as causes of diarrhea have isolated the organism from infants and small children, yet volunteer studies and outbreak investigations suggest that school-age children and adults are also susceptible (54-56). Regarding sources of infection, EAEC strains have been isolated from the lacteal contents of infant feeding bottles in Brazil (57).

Recent studies have suggested that EAEC are found frequently in stool samples of patients in industrialized countries and may be important agents of diarrhea. EAEC were isolated from 2% of pediatric patients with diarrhea in Germany; the pathogen was associated with diarrheal illness (58). The patients from whom EAEC were isolated had characteristically prolonged illness, often followed by apparent abdominal pain mimicking infant colic.

EAEC Outbreaks

Several outbreaks of EAEC diarrhea have now been reported (Table 2). The first detailed description of an outbreak from which EAEC were clearly implicated involved 19 infants in a hospital nursery in Nis, Serbia, during 9 days in 1995 (52). Twelve of the 19 infants had the same multidrug-resistant EAEC strain of serogroup O4, while none of five well neonates had this organism ($p = 0.02$). The illness lasted 3 to 9 days (mean 5.2 days) in 16 babies, but in three infants, persistent diarrhea developed, lasting 18 to 20 days. Infants with diarrhea typically had liquid green stools. In three infants, mucus was visibly apparent; no infant had bloody stools. All but three infants required intravenous hydration but all survived. The source of infection was unclear.

In two outbreaks of severe lethal diarrhea in malnutrition wards of Mexico City hospitals (12), persistent diarrhea developed in affected infants, and five patients died despite aggressive support. Autopsy findings from infants who died in these outbreaks showed severe necrotic lesions of the ileal mucosa.

During a diarrhea epidemic in a village in southern India in January 1996, EAEC were identified in the stools of 11 of 20 persons who were thought to have outbreak-related diarrhea; 1 of 11 stools from asymptomatic controls from the same village ($p < 0.02$) contained EAEC (59). Intake of water from open wells was associated with diarrheal illness; however, EAEC were not identified from this source, and contaminated water was not definitively implicated as the source of the epidemic.

EAEC outbreaks also occur in industrialized countries. In a massive outbreak of EAEC diarrhea in Gifu Prefecture, Japan, in 1993, 2,697 children at 16 schools became ill after consuming contaminated school lunches (54). The illness was characterized by abdominal pain, nausea, and severe diarrhea, which was protracted in at least 30 cases. The incubation period for this illness averaged 40 to 50 hours. A single EAEC strain of serotype O:H10 was implicated, but the organism was not found in any of the foods served in the implicated lunch.

Four outbreaks of EAEC-related diarrhea occurred in the United Kingdom in 1994 (56), which involved 19, 10, 51, and 53 persons, respectively, most of them adults. Only in the last

Table 2. Outbreaks of enteroaggregative *Escherichia coli* diarrhea

Reference	Setting	Patients	Illness
52	Nursery; Nis, Serbia, 1995	19 infants	Watery diarrhea
56	Conference Center, UK, 1994	51 adults	Watery diarrhea for 3-7 days
56	Hotel, UK, 1994	53 adults	Watery diarrhea, mean duration 68 hr
59	Village in southern India, 1996	20 children and adults	Watery diarrhea, mean duration 11 days
12	Nursery, Mexico City	Not reported	Severe diarrhea; 5 deaths
54	16 schools, Gifu, Japan	2,697 school children	Diarrhea; some cases protracted

two outbreaks was a single EAEC strain implicated. Although each of these outbreaks was linked to consumption of a meal, no single vehicle has been implicated. The illnesses were characterized by vomiting and diarrhea, occasionally with fever. The mean duration of illness in one outbreak was 68 hours. Strains of *E. coli* O44:H18 implicated in outbreaks in the United Kingdom (and previously presumed to be EPEC) are in fact EAEC (55). This report mentions three EAEC outbreaks, two among children and one among adult patients.

EAEC and Malnutrition

Perhaps even more significant than the association of EAEC with diarrhea may be the recent data from Fortaleza, Brazil, that link EAEC with growth retardation in infants (31). In this study, EAEC isolated from the stools of infants was associated with a low *z*-score for height or weight, irrespective of the presence of diarrheal symptoms. Such an association has been suggested for *Cryptosporidium* (60). A study among an Australian aborigine population supports the association of EAEC with growth retardation in the absence of diarrhea (S. Elliott, J.P. Nataro, unpub. data). Given the high prevalence of asymptomatic EAEC excretion in many areas (4,30,41-43), if this observation holds up in further studies, the contribution of EAEC to human disease might be much greater than is currently thought.

Conclusions

EAEC is an emerging diarrheal pathogen linked to acute and persistent diarrhea in both developing and industrialized countries. Both sporadic diarrhea and outbreaks (possibly foodborne) have been described. The mechanisms of pathogenesis are being elucidated; likely not all EAEC strains are equally pathogenic. The mechanism of the association between EAEC and malnutrition is not entirely clear, although plausible pathogenetic models can be proposed. Malnutrition predisposes to persistent diarrhea (61,62); whether this is due to impaired host immunity (preventing normal killing of pathogens) or to altered intestinal physiology (predisposing to more severe infections) is not known. However, it seems also plausible that asymptomatic colonization with EAEC may lead to malnutrition caused by increased metabolic demand secondary to intestinal inflammation, persistent mucosal damage due to cytotoxins, and/or the presence of a barrier to the absorption of nutrients imposed by the mucus/bacteria biofilm. A vicious cycle may thus operate wherein malnutrition and infection perpetuate and enable each other (Figure 4). The pathophysiologic machinery that propels children through this circle is probably multifactorial and opens several possible avenues for intervention against this important global problem.

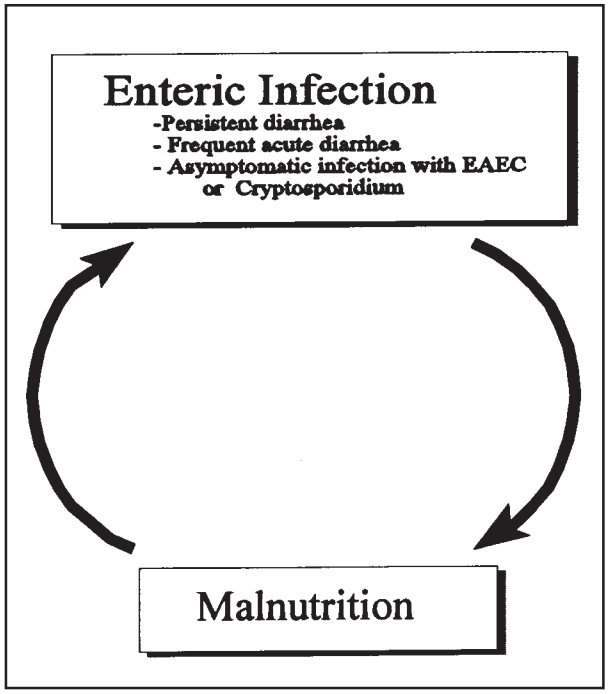

Figure 4. Relationship between diarrhea and malnutrition (31, 60–62).

Supported by Public Health Service grant AI 33096 to JPN.

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Campylobacter jejuni Strains from Patients with Guillain-Barré Syndrome

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Guillain-Barré syndrome (GBS), an acute demyelinating peripheral neuropathy, may be triggered by an acute infectious illness; infection with *Campylobacter jejuni* is the most frequently reported antecedent event. In Japan, O:19 is the most common serotype among GBS-associated *C. jejuni* strains. To determine whether serotype O:19 occurs among GBS-associated strains in the United States and Europe, we serotyped seven such strains and found that two (29%) of seven GBS-associated strains from patients in the United States and Germany were serotype O:19. To determine whether GBS-associated strains may be resistant to killing by normal human serum (NHS), we studied the serum susceptibility of 17 GBS- and 27 enteritis-associated strains (including many O:19 and non-O:19 strains) using *C. jejuni* antibody positive (pool 1) or negative (pool 2) human serum. Using pool 1 serum we found that one (6%) of 18 serotype O:19 strains compared with 11 (42%) of 26 non-O:19 strains were killed; results using pool 2 serum were nearly identical. Finally, 8 O:19 and 8 non-O:19 strains were not significantly different in their ability to bind complement component C3. Serotype O:19 *C. jejuni* strains were overrepresented among GBS-associated strains in the United States and Germany and were significantly more serum-resistant than non-O:19 strains. The mechanism of this resistance appears unrelated to C3 binding.

Guillain-Barré syndrome (GBS) is an acute demyelinating polyneuropathy characterized by an immunologic attack upon peripheral nerve myelin (1,2). The trigger for this immune attack is unknown; however, GBS is frequently preceded by an acute infectious illness (3). In recent years, infection with *Campylobacter jejuni* has emerged as one of the most common antecedent events associated with GBS. Up to 40% of patients with GBS have culture or serologic evidence of *C. jejuni* infection when neurologic symptoms begin (4-6). The many variants of GBS—including acute motor axonal neuropathy, acute inflammatory demyelinating neuropathy, and Miller Fisher syndrome—have also been associated with preceding *C. jejuni* infection (7-9).

C. jejuni infections are common in the United States, affecting approximately 1% of the population each year (10). Typically, they cause a self-limited gastrointestinal illness characterized by diarrhea, abdominal pain, and fever. However, approximately 1 in 2,000 *C. jejuni* infections may be complicated by GBS (11). Because *C. jejuni* infections occur far more commonly than GBS, either host (12) or strain (5,13) characteristics may determine which infected persons contract GBS. Several reports from Japan showed that a particular serotype, O:19, was overrepresented among *C. jejuni* strains isolated from GBS patients (5,13). Among 12 Japanese patients with GBS and culture-confirmed *C. jejuni* infection, 10 (83%) were infected with strains of O:19, a serotype that accounts for only 2% of randomly selected *C. jejuni* isolates in Japan (5). However, in England, none of four *C. jejuni* strains isolated from patients with GBS were serotype O:19 (6). Other *Campylobacter* serotypes predominate among GBS patients in other parts of the world. All nine *C. jejuni* strains isolated from GBS patients in South Africa were serotype O:41 (14);

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this serotype accounts for fewer than 2% of isolates from South African children with uncomplicated enteritis. With Lior typing (a serotyping system based upon heat-labile antigens), all four GBS-associated *C. jejuni* strains in Germany were serotype O:11 (15).

Whether the predominance of O:19 strains among GBS patients is limited to Japan is not clear. Furthermore, the reason for the O:19 overrepresentation among Japanese GBS strains is unknown, but their resistance to host bacterial clearance mechanisms is one possibility. *C. jejuni* strains vary in their susceptibility to the bactericidal activity in normal human serum (NHS) (16). Bacteria that are more resistant to nonspecific serum killing may elicit more extensive specific responses to eliminate the organism; such elicited immune responses may have the potential of greater tissue injury and myelin damage.

To determine whether *C. jejuni* serotype O:19 is an important cause of GBS outside Japan, we serotyped GBS-associated strains from the United States and Germany. To investigate the immunologic response to GBS-associated *C. jejuni* strains, we performed two additional sets of experiments. First, we examined the susceptibility of *C. jejuni* strains to NHS. Our hypothesis was that GBS-associated *Campylobacter* strains, more specifically O:19 strains, are more serum-resistant than those from patients with uncomplicated enteritis. To further characterize the mechanism of resistance, we determined whether *C. jejuni* strains isolated from GBS patients differ in their capacity to bind complement component C3.

Statistical Analysis

We determined mean values, standard deviations, odds ratios, p-values, paired T-tests, 95% confidence limits, and interquartile ranges. Software included Lotus 123 and EpiInfo (a statistical software package designed by the Centers for Disease Control and Prevention [CDC]).

Serotyping of Strains

Seven GBS-associated strains were serotyped with the O (formerly heat-stable) serotyping scheme of Penner and Hennessy (17) and the heat-labile serotyping scheme of Lior (18). Serotyping was conducted (19) at the CDC *Campylobacter* Reference Laboratory. A GBS-associated strain was defined as one isolated from a patient with GBS (n = 6) or from a patient infected during an outbreak in which at least one infected person contracted GBS (n = 1). Four strains were from patients in the United States, and three were from patients in Germany; four of these were isolated from women.

Serotype O:19 was present in one of four U.S. GBS-associated *C. jejuni* isolates and one of three German isolates. Thus, 2 (29%) of 7 GBS-associated *C. jejuni* isolates from patients in the United States and Germany were O:19. The O and heat-labile serotypes of the seven GBS-associated strains are shown in Table 1.

Serum Bactericidal Assays

We studied 44 GBS- and enteritis-associated strains from all over the world. We divided *C. jejuni* isolates into two major groups. Group 1 (17 GBS-associated strains) was divided into two subgroups. Subgroup 1A (GBS-associated/type O:19) consisted of seven *C. jejuni* O:19 isolates: three from the United States, three from Japan, and one from Germany. Subgroup 1B (GBS-associated/non-O:19 serotype) consisted of 10 *C. jejuni* isolates with serotypes other than O:19: three from the United States, two from Germany, and five from England. Group 2 (27 enteritis-associated strains from patients with uncomplicated enteritis but no known GBS association) was also divided into two subgroups. Subgroup 2A (not GBS-associated/type O:19) consisted of 11 *C. jejuni* O:19 isolates: 10 from the United States and one from Japan. Subgroup 2B (not GBS-associated/non-O:19 serotype) consisted of 16 *C. jejuni* isolates from U.S. patients with serotypes other than O:19. Serum-resistant *C. fetus* strain 23D and its spontaneous serum-susceptible mutant, 23B, were used as controls (20).

Strains were grown on trypticase soy blood agar plates (BBL Cockeysville, MD, USA) at 37°C for 48 hours in an incubator containing 10% CO_2, 5% O_2, and 85% N_2. The bacteria were harvested in 0.15M saline and centrifuged at 8,000 g for 15 minutes. The supernatant was discarded, the pellet was resuspended in 300 µl of saline, and 10-fold serial dilutions were performed in saline. From the 10^{-4}, 10^{-5}, and 10^{-6} dilutions, 400 µl aliquots of cells were added to 1.2 ml of Hanks balanced salt solution (HBSS).

Two sources of NHS were used in these assays. Pool 1 consisted of serum from five healthy adults that was pooled, aliquotted, and frozen at -70°C. The level of *C. jejuni* antibodies in pool 1 serum was determined by enzyme-linked immunosorbent assay (ELISA) (4). The optical

Table 1. Serotypes of Guillain-Barré syndrome–associated *Campylobacter jejuni* strains from the United States and Germany

Strain designation		(O)[a]-sero-type	(HL)[b] sero-type		Age/ sex
CDC[c]	Vanderbilt[d]			Location	
D459	93-002	19	77	Florida	79F
D2769	93-006	2	4	Maine	F
D4262	84-158	19	84	Germany	59F
D4266	84-196	2	4	Germany	24M
D4267	84-197	8,17	40	Germany	71F
D4271	86-381	2	4	Wisconsin	30M
D4808	93-001	1	4	Washington	57M

[a]O = heat-stable.
[b]HL = heat-labile.
[c]Designated strain number assigned by the Centers for Disease Control and Prevention *Campylobacter* Reference Laboratory.
[d]Designated strain number in the Vanderbilt *Campylobacter/Helicobacter* strain collection.

density ratio (ODR) for immunoglobulin G (IgG) was 5.14; the ODR for IgM was 0.66. Documentation of previous *C. jejuni* infection was not required to be included in this pool. In contrast, pool 2 consisted of serum from two healthy adults with only low-titer antibody. The ODR for pool 2 serum in the IgG assay was 0.37; and the ODR in the IgM assay was 0.30. As a control, the NHS was heated to 56°C for 30 minutes (heat inactivated NHS [HINHS]) to ablate all complement activity.

The assay to determine susceptibility to NHS was performed in sterile, disposable 96-well microtiter U-bottom plates (Falcon MICROTEST III, Becton Dickinson & Co., Franklin Lakes, NJ, USA). From each of the bacterial suspensions, a 150 µl aliquot was added to duplicate wells. In addition, 50 µl of NHS or HINHS (diluted to 40% with HBSS) was added; the final serum concentration in the suspension was 10%. After the assay plate was incubated at 37°C for 1 hour, 50 µl of the suspension from each well was poured onto blood agar plates and incubated for 48 hours; the number of CFUs was then calculated. The difference between the counts for cells incubated with NHS and HINHS was expressed as median $\log_{10}$ kill for each strain; greater than 1 $\log_{10}$ kill was considered a serum-susceptible strain (21). If less than 1 $\log_{10}$ kill occurred, the strain was considered serum-resistant. The identical procedure was performed using pool 1 and pool 2 serum.

The resistance of the 44 *C. jejuni* strains studied to killing by NHS is shown in Table 2. Of these strains, 12 (27%) were resistant to killing by *C. jejuni* antibody-positive pool 1 serum and 10 (23%) were resistant to killing by *C. jejuni* antibody-negative pool 2 serum; thus, as described previously (22), antibodies to *C. jejuni* had little impact on serum-killing. The GBS-associated strains were no more likely to be resistant to serum-killing than were the strains from patients with uncomplicated enteritis. However, O:19 strains were significantly more likely than other *C. jejuni* serotypes to resist serum-killing, regardless of GBS-association and serum pool used (Table 2). In serum pool 1, only 1 of 18 O:19 strains was serum-susceptible compared with 11 (42%) of 26 non-O:19 strains (odds ratio = 12.5, p = 0.008). Similarly, in serum pool 2, no O:19 strain was serum-susceptible compared with 10 (38%) of the non-O:19 isolates.

^{125}I-C3 Binding Assays

Eight strains from Group 1 (four randomly selected from each subgroup) and eight from Group 2 (four randomly selected from each subgroup) were grown on blood agar plates as described above. *C. fetus* strains 23D and 23B again served as controls and the assays were conducted (21). In brief, bacteria from each plate were harvested in 1.5 ml of HBSS and were centrifuged at 8,000 g for 15 minutes; the pellet was resuspended in 0.5 ml HBSS and adjusted to OD_{450} = 3.0. For each strain studied, a suspension of 4 µl ^{125}I-C3 (with 20,000 cpm to 65,000 cpm) and 2.5 µl of either NHS or HINHS from pool 2 and 100 µl of the bacteria-HBSS suspension were incubated at 37°C for 15 minutes. ^{125}I-C3 was prepared in the laboratory of one of the authors (RGW) (21). The suspensions were then centrifuged twice at 175xg for 5 minutes, the pellet was resuspended in HBSS, and the supernatant was discarded. The bottom 5 mm (containing the pellet) of each tube was clipped, and emissions were determined in a gamma counter. The counts in the negative control mixtures containing HINHS were subtracted from the NHS counts. An assay was considered valid only if the net pellet counts (NHS minus HINHS) for control strain 23B were at least four times higher than for 23D. To control for nonspecific binding, the counts for each strain studied were expressed as the ratio of net counts relative to the serum susceptible control (23B). Each strain was assayed two to four times.

Mean binding of serum-susceptible control strain 23B was 497 cpm, whereas mean binding of serum-resistant control strain 23D was 54. The

Table 2. Susceptibility of *Campylobacter jejuni* strains to killing by normal human serum (NHS)

Illness	O serotype	No. of strains studied	No. (%) with >1 log kill[a,b]		Median log kill (interquartile range)	
			Pool 1[c]	Pool 2[c]	Pool 1	Pool 2
GBS[d]	19	7	1 (14)	0 (0)	0.53 (0.18-0.63)	0.39 (0.08-0.52)
Enteritis	19	11	0 (0)	0 (0)	0.37 (0.09-0.47)	0.48 (0.29-0.50)
GBS	non-19	10	3 (30)	3 (30)	0.70 (0.44-1.18)	0.43 (0.03-1.32)
Enteritis	non-19	16	8 (50)	7 (44)	0.90 (0.24-2.00)	0.59 (0.35-2.53)
All	19	18	1 (6)	0 (0)	0.38 (0.17-0.58)	0.45 (0.23-0.52)
All	non-19	26	11 (42)	10 (38)	0.70 (0.26-1.86)	0.54 (0.17-2.27)

[a]Bacterial suspensions were incubated in either NHS or heat inactivated NHS at 37°C for 1 hr as described in text, and net CFU (NHS minus HINHS) determined. Values greater than 1 $\log_{10}$ kill were considered to denote a serum-susceptible strain.
[b]Comparison of O:19 strains with non-O:19 strains: Pool 1, odds ratio = 12.5, p = 0.008; Pool 2, odds ratio = undefined, p = 0.003.
[c]Pool 1 consists of *C. jejuni*-antibody positive serum from five healthy adults; Pool 2 consists of *C. jejuni*-antibody negative serum from two healthy adults.
[d]GBS = Guillain-Barré syndrome.

mean ratio of C3-binding for strain 23D to 23B of 0.114 was as expected (21). In contrast, the mean ratio for C3 binding to *C. jejuni* strains in comparison with strain 23B was 0.022 to 0.464 (mean 0.216). The eight O:19 strains were not significantly different from the eight non-O:19 strains in their ability to bind ^{125}I-C3 (Table 3).

Conclusion

This study of GBS patients in the United States and Germany confirms the observation made in Japan that serotype O:19 is overrepresented among patients with *C. jejuni*-induced GBS. Of 298 randomly collected *Campylobacter* isolates from patients with enteritis in the United States, only 3% were serotype O:19 (19). A similarly low prevalence of O:19 strains is found in all parts of the world, including North and South America, Asia, and Europe (5,6,19,23). Although specific serotyping surveys have not been done in Germany, it is unlikely that serotype O:19 is more frequent among German *C. jejuni* isolates. Thus, the GBS-associated strains in our study were more than 11 times as likely to belong to this serotype (p = 0.03). Although the association among GBS and *C. jejuni* serotype O:19 was not as marked as in Japan (where more than 80% of GBS-associated isolates are O:19), this serotype clearly is overrepresented among GBS-associated strains in other countries. We conclude that the association of O:19 strains with GBS is not just a local phenomenon in Japan but likely reflects a fundamental characteristic of O:19 strains. O-serotype 2 and heat-labile serotype 4, which were common among the GBS strains, are commonly represented among infected persons in the United States (23).

Despite the frequency of *Campylobacter* infections in GBS patients, such strains are difficult to obtain for several reasons. First, in most *C. jejuni*-infected patients, stools are clear before neurologic symptoms begin. Second, most neurologists do not culture stool samples when GBS is first diagnosed. And finally, even if a stool culture is ordered and *Campylobacter* is present, few microbiology laboratories save their isolates; by the time the case is reported, the strain has been discarded. Thus, these seven strains represent one of the largest collections of GBS-associated strains described. Additionally, these are likely to be representative of the population of GBS-associated *C. jejuni* strains. Unless serotype O:19 strains persist in stools longer, are more easily cultured, or are less likely to be discarded by microbiology laboratories (and no data support any of these possibilities), these strains probably are not different in any systematic way from other GBS-associated isolates.

Most *C. jejuni* strains are susceptible to killing by human serum (22), but because we studied highly selected strains (most either serotype O:19 or from GBS patients), a high proportion of strains in this investigation were resistant. In this context, the finding that *C. jejuni* strains from GBS patients were no more likely than the strains from patients with uncomplicated enteritis to be serum resistant is not surprising. Even when the analysis was limited only to serotype O:19 strains, no differences were found between GBS- and enteritis-associated strains. However, serotype O:19 strains were substantially less serum-

Table 3. Comparison of ^{125}I-C3 binding to *Campylobacter jejuni* strains

Measure	Serotype of *C. jejuni* strain	
	O:19 (n = 8)	non-O:19 (n = 8)
Counts per minute[a]	85 +/-51	98 +/- 48
Mean binding ratio[b]	0.187 +/- 0.116	0.245 +/- 0.130

[a]Bacterial cells were incubated with pool 2 normal human serum or heat inactivated normal human serum and ^{125}I-C3 (mean 40,416 [range 20,234-63,503]. cpm) at 37°C for 15 minutes. The cells were centrifuged and washed; net (NHS minus HINHS) ^{125}I binding was measured as counts per minute. p-value = 0.20 (paired T-test, 1-tailed).
[b]The mean binding ratio is an average of the ratio of the net counts for each study strain relative to the net counts of the serum-susceptible control (23B). p-value = 0.31 (paired T-test, 1-tailed).

susceptible than strains of other serotypes and were 12 times as likely to be serum-resistant. Although serotype O:19 represents only a small percentage of *C. jejuni* strains from patients with uncomplicated enteritis in the United States or Japan, it is the most common serotype identified in GBS patients in both locales. The relatively small variation in serum susceptibility of the O:19 strains is consistent with the close genetic relationship observed among these strains (24).

To better understand the basis for the relative serum-resistance among the O:19 strains, we compared their ability to bind C3 in relation to non-O:19 strains. Because C3 binding occurs after activation by either the alternative or classical complement pathways, it is a screen for differences in these early steps. The serum-resistance of *C. fetus* is explained by the inability of C3 to bind to the cell surface (25). In contrast, C3 binding is normal in serum-resistant *Salmonella*, but the C5-9 membrane attack complex does not insert properly (26). In the present study, the lack of substantial C3 binding differences among O:19 and non-O:19 *C. jejuni* strains suggests that the early steps in complement activation are similar among strains. However, clear differences in serum-susceptibility bespeak either rapid inactivation of C3 or reduced assembly or function of the membrane attack complex in the more resistant strains. Future studies should address this point.

The increasing awareness of the importance of *C. jejuni* infection in triggering GBS is another example of how previously well-described diseases have emerged as sequelae of acute infectious illnesses. This study attempts to begin to characterize the nature of this association; however, there is much to learn about how an acute gastrointestinal infection results in ascending paralysis. One fact is quite clear: many more people are infected with *C. jejuni* than contract GBS subsequently. Perhaps some persons are predisposed to contracting GBS after infection with campylobacters that might cause only uncomplicated enteritis in another patient. Conversely, as we have suggested in this paper, some strains may be more likely than others to trigger GBS. No associations between human leukocyte antigen (HLA) types and GBS have been found (27,28). However, in Great Britain and Japan, an association between HLA type and *C. jejuni*-associated GBS has been suggested (9,29). Perhaps some combination of familial susceptibility, HLA type, strain serotype, or other host or strain characteristics together play a role in the pathogenesis of *C. jejuni*-induced GBS.

The relative serum-resistance of O:19 strains correlates with mechanism. Furthermore, the relevance of these in vitro assays to the susceptibility of organisms in vivo cannot be known with certainty. We speculate that the relative insensitivity of these strains to the lytic effects of complement allows them to trigger a heightened specific immunologic response. We further speculate that this heightened immunologic response leads to injury of peripheral nerve structures. Since only a small fraction of infections caused by *C. jejuni* O:19 lead to GBS (estimated incidence 1 in 158) (30), additional factors also must be involved in vivo.

Acknowledgments

We thank Charlotte Patton for serotyping the strains and Manfred Kist for supplying the strains from Germany.

This work was supported by grant NIH-1-K08-NS01709 of the NINDS, NIH (BMA); NIH grant KO4-AI01036 (RGW); and the Medical Research Service of the Department of Veterans Affairs (MJB).

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An Apparently New Virus (Family *Paramyxoviridae*) Infectious for Pigs, Humans, and Fruit Bats

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We isolated an apparently new virus in the family *Paramyxoviridae* from stillborn piglets with deformities at a piggery in New South Wales, Australia. In 1997, the pregnancy rate and litter size at the piggery decreased markedly, while the proportion of mummified fetuses increased. We found serologic evidence of infection in pigs at the affected piggery and two associated piggeries, in humans exposed to infected pigs, and in fruit bats. Menangle virus is proposed as a common name for this agent, should further studies confirm that it is a newly recognized virus.

Viruses in the family *Paramyxoviridae* have been associated with new diseases in a variety of species, including humans, throughout the world. Morbilliviruses related to canine distemper virus have caused disease outbreaks in seals, dolphins, porpoises, and lions (1-6). Equine morbillivirus (EMV) (also called Hendra virus or bat paramyxovirus), responsible for the deaths of horses and humans with respiratory or neurologic disease in Australia (7,8), is thought to have originated from fruit bats (*Pteropus* sp.) (9-11). La Piedad Michoacan paramyxovirus (blue eye paramyxovirus) causes encephalitis and death in piglets and reproductive disease in adult pigs in Mexico (12). We have isolated an apparently new virus in the family *Paramyxoviridae* from stillborn piglets with abnormalities of the brain, spinal cord, and skeleton at a commercial piggery with 2,600 sows in New South Wales, Australia. Serologic studies indicate that at least two humans exposed to affected pigs have been infected with the virus, possibly with resultant illness, and that fruit bats are a potential source of infection.

From mid-April to early September 1997 at the affected piggery, the farrowing rate decreased from an expected 82% to 60%; the number of live piglets declined in 27% of litters born; the proportion of mummified and stillborn piglets, some with deformities, increased; and occasional abortions occurred. The disease occurred sequentially in all four breeding units at the piggery, affecting the progeny of sows of all parities and gilts. No disease was observed in postnatal animals of any age. Affected stillborn piglets frequently had severe degeneration of the brain and spinal cord (which were almost absent in some piglets), arthrogryposis, brachygnathia, and occasionally fibrinous body cavity effusions and pulmonary hypoplasia. Histologic examination of the brain and spinal cord showed extensive degeneration and necrosis of gray and white matter associated with infiltrations of macrophages and other inflammatory cells. Neurones contained intranuclear and intracytoplasmic inclusion bodies. Some piglets had nonsuppurative myocarditis.

A virus producing cytopathic effects in cell cultures, including vacuolation of cells and formation of syncytia, was isolated at the Elizabeth Macarthur Agricultural Institute, Menangle, in baby hamster kidney cells (BHK21) from lung (n = 9 isolates), brain (n = 8), and heart (n = 5) of affected piglets. The isolates have been shown by electron microscopy to be morphologically consistent with viruses in the family

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Paramyxoviridae, which comprise spherical to pleomorphic particles 30 nm to more than 100 nm long, contain "herringbone" nucleocapsids with a diameter of 19 ± 4 nm and a pitch of 5.8 ± 0.4 nm, and are surrounded by an envelope with a single fringe of surface projections 17 ± 4 nm long (Figure). The virus grows in a wide range of cell types from many species, including porcine and human cells, and is nonhemadsorbing and nonhemagglutinating, using erythrocytes from several species; in this respect, the virus differs from La Piedad Michoacan paramyxovirus, which is hemagglutinating (13). Studies at the Australian Animal Health Laboratory, Geelong, indicate that the virus is probably a new member of the family *Paramyxoviridae*. EMV and La Piedad Michoacan paramyxovirus have been excluded on the basis of absence of specific polymerase chain reaction products and limited sequencing, and EMV has been further excluded on the basis of lack of serologic cross-reactivity and differing appearance by electron microscopy. Serologic tests at the Elizabeth Macarthur Agricultural Institute and the Australian Animal Health Laboratory on sera from sows have also excluded other porcine reproductive pathogens, such as ruminant and porcine pestiviruses (including classic swine fever virus), porcine reproductive and respiratory syndrome (Lelystad) virus, porcine parvovirus, encephalomyocarditis virus, and Aujeszky's disease virus. Pestivirus infection was also excluded by negative antigen capture enzyme-linked immunosorbent assay results on tissues from affected piglets.

A high proportion (>90%) of serum collected from pigs of all age categories at the affected piggery (n = 88) from May to September 1997 contained high titers (≥ 256) of neutralizing antibodies against the virus. In contrast, serum and plasma samples collected from pigs at the affected piggery before May 1997 (n = 120) tested negative. Porcine sera (n = 50) from two piggeries that receive only weaned pigs from the affected piggery neutralized the virus at dilutions of 16 to 4,096. Virus-neutralizing antibodies were also detected in body cavity fluids from some stillborn piglets. Testing of porcine sera (n = 1,114) from other piggeries throughout Australia, including piggeries with reproductive problems, indicates that infection is confined to the affected piggery and the two associated piggeries.

Serum from two humans—one working at the affected piggery and one working at one of the associated piggeries—had neutralizing antibody titers of 128 and 512. These workers had unexplained febrile illnesses in the weeks after exposure to potentially infective material. Further details are provided in the article by Chant et al. in this issue.

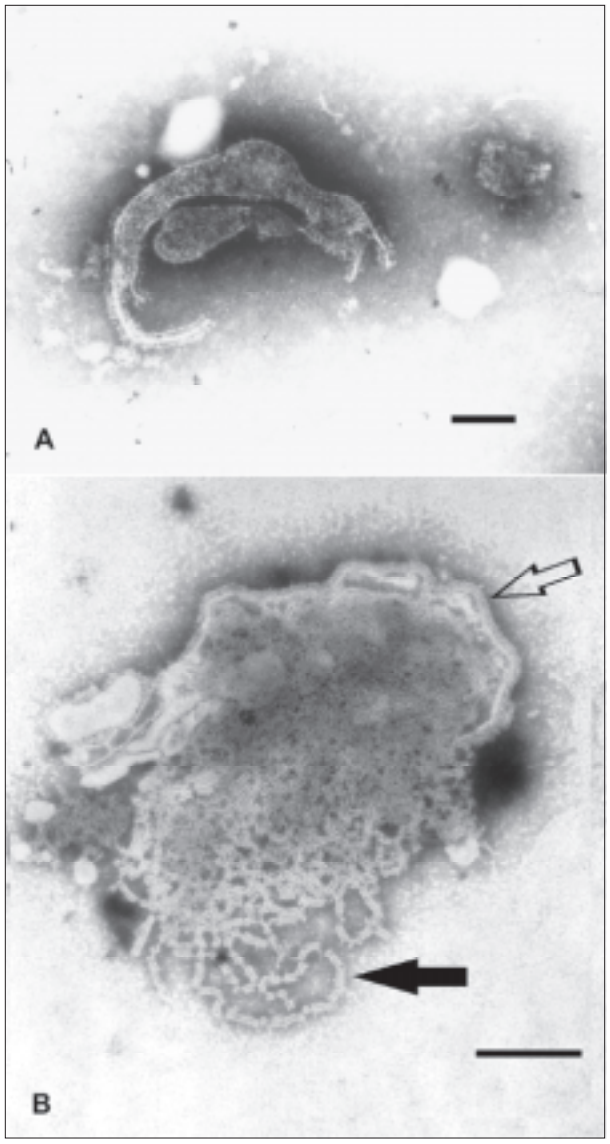

Figure. Transmission electron micrographs of Menangle virus negatively stained with 2% phosphotungstic acid. A. Depicts the pleomorphic nature of the virion; bar=100nm. B. Shows a disrupted virion, the virus envelope with surface projections (hollow arrow) and nucleocapsids (solid arrow); bar=100nm.

A large breeding colony of gray-headed fruit bats (*Pteropus poliocephalus*), as well as little red fruit bats (*P. scapulatus*), roosts within 200 m of

the affected piggery from October to April; therefore, fruit bats were investigated as a potential source of infection. In a preliminary study, 42 of 125 serum samples collected from fruit bats in New South Wales and Queensland were positive in the virus neutralization test, with titers of 16 to 256. Positive samples were from 26 of 79 gray-headed fruit bats, 11 of 20 black fruit bats (*P. alecto*), 4 of 10 spectacled fruit bats (*P. conspicillatus*), 0 of 15 little red fruit bats, and one unidentified species. This panel included positive samples collected in 1996 before the pigs were infected, as well as positive samples collected in November 1997 from a colony of gray-headed fruit bats 33 km from the piggery, supporting the hypothesis that fruit bats were the primary source of the virus. Other species in the vicinity of the affected piggery, including rodents (n = 19), birds (n = 13), cattle (n = 60), sheep (n = 70), cats (n = 25), and a dog, were seronegative.

Along with EMV (7-11) and a lyssavirus causing encephalitis (14,15), this is the third virus causing disease in humans or domesticated animals that appears to have emerged from fruit bats in Australia in recent years. Menangle virus is proposed as a common name for this agent, should further studies confirm that it is a newly recognized virus.

Acknowledgments

We thank Bryan Eaton, Peter Hooper, Jacquie Kattenbelt, Brenda King, Chris Morrissey, Paul Selleck, and Harvey Westbury from the CSIRO Australian Animal Health Laboratory, Geelong, for their invaluable contributions with serology, histopathology, and PCR and Keith Hart and Andrew Glover, Moss Vale Rural Lands Protection Board, Camden, for collection of serum samples from rodents, birds, cattle, sheep, and cats at the affected piggery. Sera from humans were provided by the Public Health Unit, South Western Sydney Area Health Service, Liverpool, under the directorship of Kerry Chant. Peter Young and Hume Field from the Animal Research Institute, Department of Primary Industries, Yeerongpilly, Queensland, kindly provided serum samples from fruit bats. We appreciate contributions by technical staff in the virology section and regional veterinary laboratory at the Elizabeth Macarthur Agricultural Institute, Menangle, and at the University of Sydney, Camden, throughout this investigation.

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Probable Human Infection with a Newly Described Virus in the Family *Paramyxoviridae*

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After an apparently new virus in the family *Paramyxoviridae* was isolated from pigs in August 1997, an investigation was carried out to assess its risk for humans. More than 250 persons with potential exposure to infected pigs were tested serologically. Two piggery workers with intense occupational exposure had high convalescent-phase antibody titers to this new virus. In early June 1997, both workers had an influenzalike illness with rash; serologic testing showed no alternative cause. Strong evidence indicates that the two men became ill from this new virus, but the mode of transmission from pigs to humans remains unknown.

Zoonotic illnesses due to viruses in the family *Paramyxoviridae* (i.e., Newcastle disease virus [1] and equine morbillivirus [2]) have been described. An apparently new virus in the family *Paramyxoviridae*, isolated from pigs in a piggery near Sydney, Australia, is described by Philbey et al. in this issue. We describe epidemiologic investigations to assess the risk the virus poses for humans and detail two probable cases of human disease.

Piggery A, from which the virus was isolated, is a large commercial piggery with animals housed in four discrete production units. At approximately 6 weeks of age, some pigs are transported to piggeries B and C for growing to slaughter weight. Animals from piggery A are also supplied to a university and a hospital for research.

After the new virus was isolated, an assay for neutralizing antibodies that allowed testing of sera from animals and humans was developed at the Elizabeth Macarthur Agricultural Institute. All 33 workers at piggery A were tested with this assay for antibodies to the new agent. Other workers who had come into contact with potentially infectious pigs from piggery A were also tested: abattoir workers (n = 142), workers at the grower piggeries B and C (n = 5), researchers and animal handlers (n = 41), veterinarians and pathology laboratory workers (n = 24), and others (n = 6). Sixty delinked sera from women receiving routine prenatal screening were tested as controls and were seronegative for the agent. Two workers were seropositive, with virus neutralizing antibody titers of 128 and 512. These results were confirmed by repeat collection and testing. Both workers received a detailed clinical review and extensive serologic testing, the results of which are described below.

In early June 1997, Patient 1 had sudden onset of malaise and chills followed by drenching sweats and fever. He was confined to bed with severe headaches and myalgia for the next 10 days. He had no cough, vomiting, or diarrhea. After the third day, he went to a locum physician,

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who prescribed amoxicillin. A day later, he noted a spotty red rash. He went to his usual physician, who noted tenderness in both hypochondria, lymphadenopathy, and a rubelliform rash and diagnosed acute Epstein-Barr virus infection. However, subsequent review of the patient's medical notes showed a positive result for Epstein-Barr virus immunoglobulin G (IgG) in 1991. He returned to work after 14 days' absence but tired easily. He reported a 10-kg weight loss during his illness.

Results of a clinical examination 2 months after this illness were normal except for mild tenderness in the right hypochondrium. Results of urinalysis, full blood count, erythrocyte sedimentation rate, C-reactive protein concentration, and blood chemistries were normal. An upper abdominal ultrasound indicated that liver size was at the upper limits of normal, and the spleen was enlarged (15 cm long).

The worker had frequent prolonged contact with birthing pigs. He reported that splashes of amniotic fluid and blood to the face were not uncommon and that he often received minor wounds to his hands and forearms. His partner tested negative for neutralizing antibodies to this agent.

Six other workers at the piggery reported an influenzalike illness during the winter months, yet records showed that only two had more than 1 day off work, the most being 4 days.

Patient 2 worked at piggery B. He also had onset of illness in early June 1997 characterized by fever, chills, rigors, drenching sweats, marked malaise, back pain, severe frontal headache, and photophobia. He had no cough, vomiting, or diarrhea. The headache resolved after 4 to 5 days. Four days after onset of the illness, he noted on the torso a spotty, red, nonpruritic rash, which lasted 7 days. He had largely recovered after 10 days, noting a 3-kg weight loss. No investigations were performed.

Results of a clinical examination 2 months after his illness were essentially normal. Results of urinalysis, full blood count, erythrocyte sedimentation rate, and C-reactive protein were within normal limits. Blood chemistry results were normal except for mildly elevated liver function. He was hepatitis C–antibody positive and IgA-deficient. An upper abdominal ultrasound showed mild hepatomegaly with normal texture. The spleen was at the upper limit of normal size.

This worker did not have contact with birthing pigs; however, he performed autopsies on pigs without wearing gloves or protective eye wear. As with Patient 1, exposure to pig secretions (e.g., feces, urine) was common. The worker had received a delivery of young pigs from piggery A the week before his illness.

Both workers had a similar illness in early June 1997. Both had high convalescent-phase neutralizing antibody titers to this new virus. Serologic testing of all 33 workers at piggery A for other human paramyxoviruses excluded cross-reactivity as a cause for this finding. Extensive serologic testing of both patients in September 1997 (Table) did not identify an alternative cause for the illnesses.

The unexplained splenomegaly in the first worker may be an incidental finding. The second worker had evidence of hepatitis C virus infection, abnormal liver function tests, hepatomegaly, and IgA deficiency. None of these findings need be implicated in the patient's presumed infection with this paramyxovirus.

While single high antibody titers must be interpreted with caution, the timing of the described illnesses in relation to the disease in pigs, the similarity of the two cases, the exclusion of cross-reactivity due to preexisting antibody to other paramyxoviruses, and the absence of serologic results suggesting another cause constitute strong evidence that the illness in these two men was caused by this new virus.

Although respiratory transmission is proposed as a mode of spread in pigs, the mode of transmission from pigs to humans is unknown. If spread to humans is by the respiratory route, infectivity appears much lower than in pig-to-pig transmission. Alternatively, a different transmission mode such as parenteral or permucosal exposure may be involved. Philbey et al. in this issue present evidence suggesting that bats may be involved in the ecology of this new virus. Neither of the two patients had contact with bats.

Human infection with this new virus seems confined to those with intense occupational exposure to recently infected pigs. Sentinel pig surveillance indicates that the virus continues to circulate at piggery A. Ongoing surveillance for influenzalike illnesses and a serologic testing program have been instituted for the workers. In January 1998, 19 of 21 previously seronegative

Table. Results of case investigations

Serologic test[a]	Titer Patient 1	Titer Patient 2
Neutralizing antibody to new agent	128	512
Antibody to equine morbillivirus	negative	negative
CMV IgG	positive	positive
CMV IgM	negative	negative
EBV-VCA IgG	positive	positive
EBV-VCA IgM	negative	negative
EBV-EBNA IgG	negative	positive
Rubella IgG	positive	positive
Rubella IgM	negative	negative
Mycoplasma (CFT)	<4[b]	4[b]
Mycoplasma IgM	negative	negative
Adenovirus (CFT)	8[b]	8[b]
Enterovirus (CFT)	8[b]	8[b]
Influenza A (CFT)	32[b]	16[b]
Influenza B (CFT)	8[b]	<4 (negative)
Leptospiral agglutinins	negative	negative
Brucella antibodies	negative	negative
Toxoplasma IgG	positive	positive
Toxoplasma IgM	negative	negative
Q Fever (phase 2 - CFT)	negative	negative
Measles IgG	positive	positive
Measles IgM	negative	negative
Mumps IgG	positive	positive
Mumps IgM	negative	negative
RSV (CFT)	<8[b]	anticomplementary
Parainfluenza 1 (CFT)	32[b]	<16[b]
Parainfluenza 2 (CFT)	16[b]	<16[b]
Parainfluenza 3 (CFT)	<8[b]	<16[b]
HBsAg	negative	negative
anti-HBc	negative	negative
anti-HCV	negative	positive[c]
anti-HIV (Western Blot)	negative	negative
anti-HIV (EIA)	negative	negative

[a]CMV, cytomegalovirus; IgG, immunoglobulin G; EBV, Epstein-Barr virus; VCA, viral capsid antigen; EBNA, Epstein-Barr nuclear antigen; CFT, complement fixation test; RSV, respiratory syncytial virus; HBsAg, hepatitis B surface antigen; HBc, hepatitis B core; HCV, hepatitis C virus; EIA, enzyme immunoassay.
[b]Not significant.
[c]By two screening enzyme immunoassays.

workers had repeat testing; all remained negative. The occupational health and safety body of the state, in conjunction with agricultural and health authorities, has issued appropriate safety guidelines for the industry.

Acknowledgments

We thank the Microbiology Department, Prince of Wales Hospital; CIDMLS, ICPMR, Westmead Hospital; Dan Russell, Mid Western NSW Public Health Unit; Paul Van Buynder, Southern NSW Public Health Unit; and staff of South Western Sydney Public Health Unit.

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Emergence of the M Phenotype of Erythromycin-Resistant Pneumococci in South Africa

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Erythromycin-resistant pneumococci have been isolated in South Africa since 1978; however, from 1987 to 1996, resistance to macrolides was only detected in 270 (2.7%) of 9,868 blood or cerebrospinal fluid (CSF) pneumococcal isolates, most of which were obtained from the public sector. In South Africa, macrolide use in the public sector is estimated at 56% of that in the private sector. Most erythromycin-resistant strains (89%) exhibited resistance to erythromycin and clindamycin (macrolide-lincosamide-streptogramin B phenotype). In the United States, most erythromycin-resistant pneumococci exhibit the newly described M phenotype (resistance to erythromycin alone), associated with the *mefE* gene. The M phenotype in South Africa increased significantly in the last 10 years, from 1 of 5,115 to 28 of 4,735 of blood and CSF isolates received from 1987 to 1991 compared with 1992 to 1996 ($p = 5x10^{-7}$). These data suggest that, although macrolide resistance in pneumococci remains low in the public sector, the *mefE* gene is rapidly emerging in South Africa.

Resistance to erythromycin in pneumococci has been observed since 1967 (1) and was first reported in South African multiresistant pneumococcal strains in 1978 (2). Until recently, the only mechanism described for resistance to erythromycin in the pneumococcus was the N^6-methylation of a specific adenine residue (A2058) in 23S rRNA, which resulted in reduced affinity between the antibiotic and the ribosome (3,4). This resistance is associated with the gene *ermAM* (5), first described in *Streptococcus sanguis* (6). Since then, other mechanisms of erythromycin resistance in the pneumococcus have been reported. In fact, most resistance in the United States appears to be due to efflux of the antibiotic from the cell, associated with the gene *mefE* (7,8). While *ermAM* confers coresistance to most macrolides, lincosamides, and streptogramin B antibiotics (resulting in the so-called MLS phenotype) (3,9), *mefE* confers resistance only to the 14- and 15-membered macrolides (resulting in the M phenotype) (7,8). We report the emergence of M-phenotype erythromycin resistance in South African blood and cerebrospinal fluid (CSF) pneumococcal isolates from 1987 to 1996.

The South African Institute for Medical Research (SAIMR), Johannesburg, South Africa, regularly receives all pneumococcal isolates from participating laboratories in eight of the nine provinces of South Africa. We examined all erythromycin-resistant blood and CSF isolates received by SAIMR from 1987 to 1996.

Erythromycin-, clindamycin-, and penicillin-resistance phenotypes were determined by using disk diffusion assays (erythromycin, 15µg/disk, clindamycin, 2 µg/disk, oxacillin, 1 µg/disk) on 5% horse blood agar plates (Mueller-Hinton base) after overnight growth at 37°C under aerobic conditions. Strains showing resistance (zone diameters ≤ 20mm for erythromycin, ≤ 18mm for clindamycin, and <20mm for oxacillin) on the disk diffusion plates were tested by the agar dilution method to obtain MICs according to the National Committee for Clinical Laboratory Standards guidelines (10). We evaluated (by the chi-square test) increases in the prevalence of erythromycin resistance and the incidence of resistance to erythromycin and susceptibility to clindamycin, which represents the M phenotype.

The M phenotype increased significantly from 1987 to 1991 compared with 1992 to 1996 in

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blood and CSF isolates—from 1 of 5,115 isolates to 28 of 4,753 isolates ($p = 5x10^{-7}$, odds ratio [OR] 30.13, 95% confidence interval [CI] 4.1-221) (Table 1; Figure).

Data on oral macrolides in the public sector (from the Division of Medical Schemes Supplies and Pharmaceutical Services of the Department of Health) show that 16.4 million defined daily doses (ddd) of macrolides were purchased for the estimated 30.3 million persons who obtain health care from the public sector (0.54 ddd per capita). Private sector use for the year ending December 1997 (Intercontinental Medical Statistics South Africa, Pty, Ltd., unpub. data) show that 7.3 million ddd of macrolide were purchased in an estimated population of 7.57 million (0.96 ddd per capita).

Table 1. Prevalence of South African erythromycin-resistant pneumococcal isolates, 1987–1996[a]

Years	Total isolates[b]	No. of E-R strains (%)[c]	No. of M strains (% of E-R strains)
1987-1991	5,115	128 (2.5)	1 (0.8)
1992-1996	4,753	142 (3.0)	28 (19.7)
Total	9,868	270 (2.7)	29 (10.7)

[a]Of 9,868 blood and cerebrospinal fluid (CSF) isolates received by the SAIMR from 1987 to 1996, 270 were fully resistant to erythromycin. While the number of erythromycin-resistant blood and CSF isolates received increased from 1987 to 1991 compared with 1992 to 1996 (2.5% to 3.0%), the increase was not significant. There was no significant relationship between erythromycin resistance and the M phenotype within any given province throughout the 10 years.
[b]Blood and cerebrospinal fluid isolates.
[c]E-R= erythromycin-resistant.

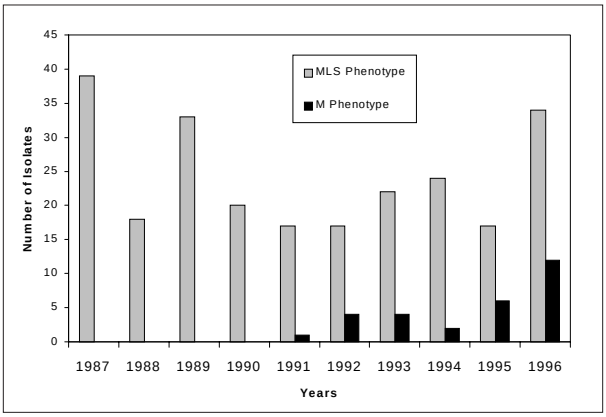

Figure. Number of erythromycin-resistant blood and cerebrospinal fluid isolates of pneumococci.

All 78 MLS isolates hybridized with the *ermAM* probe or produced a 616-bp-amplification product during polymerase chain reaction (PCR) amplification using the *ermAM*-specific primers.[1] The 12 M isolates tested contained the *mefE* gene as shown by a 348bp-amplification product when amplified using primers specific for *mefE*. There were no erythromycin-resistant isolates that contained neither the *ermAM* nor the *mefE* gene.

Erythromycin-resistant strains were serotyped by using the quellung reaction and antisera from the Staten Seruminstitut, Copenhagen, Denmark. Over the 10 years, the five most common serotypes and groups among the erythromycin-resistant isolates in decreasing order of frequency were serotype 14, serogroups 6, 19, 23, and serotype 1 (Table 2). Serotype 1 erythromycin-resistant pneumococci appeared only after 1992; serotype 14 was the most common in MLS isolates; serogroup 23 was the most common serogroup in M isolates (Table 2). Serotypes 14 and serogroups 6, 23, and 19 are the most common serotypes and groups isolated from children with serious infections (13,14). Of the 157 isolates from patients whose age was supplied, 98 (62%) were obtained from children (≤12 years). There was a trend that was not significant toward more macrolide resistance in children than adults (OR 1.17 [95% CI 0.98-1.39]). This trend may have been significant if age data had been supplied with all the isolates received. The proportion of

[1]DNA was extracted from pneumococcal isolates by using a lysis solution consisting of 0.1% sodium deoxycholate as described in (11), except that we used plate rather than broth cultures.

Seventy-eight MLS strains were probed for the *ermAM* gene by using dot blots. The probe (supplied by P. Courvalin, Pasteur Institute, Paris, France) (*Escherichia coli* JM83/pUC19 560bp *Ssp*1 intragenic fragment of *ermB*) was labeled with digoxygenin by using random primed labeling (DIG DNA Labeling and Detection Kit; Boeringer, Mannheim, Germany). Hybridization and detection were performed following manufacturer's instructions (DIG DNA Labeling and Detection Kit; Boeringer, Mannheim, Germany). PCR was also used to detect *ermAM* in 30 strains according to standard conditions, with an annealing temperature of 58°C. We used the following primers: forward primer, 5'-CGAGTGAAAAAGTACTCAACC, reverse primer, 5'-GGCGTGTTTCATTGCTTGATG).

Published primers for the *mefE* gene (5'-AGTATCATTAATCACTAGTGC, and 5'-TTCTTCTGGTACTAAAAGTGG) (12) were used to detect *mefE* through PCR amplification in 13 M strains. Amplification was performed in a Perkin Elmer Cetus DNA Thermal Cycler under standard reaction conditions, with an annealing temperature of 56°C.

Table 2. Serotype distribution among erythromycin-resistant pneumococci

Serotype/ group	No. (%) of MLS[a] isolates	No. (%) of M isolates	Total no. (%) of isolates
14	96 (39.8)	9 (31.0)	105 (38.9)
6	71 (29.5)	3 (10.3)	74 (27.4)
19	36 (14.9)	3 (10.3)	39 (14.4)
23	26 (10.8)	10 (34.5)	36 (13.3)
1	7 (2.9)	1 (3.5)	8 (3.0)
Other	5 (2.1)	3 (10.3)	8 (3.0)
Total	241	29	270

[a]MLS, macrolides-lincosamides-streptogramin B.

pediatric isolates did not change significantly over the 10-year period (63% during 1987 to 1991 and 62% the 1992 to 1996 period).

Approximately half of all the macrolide-resistant isolates were also either intermediate or fully resistant to penicillin (60 of the 128 isolates from the 1987 to 1991 period, and 72 of the 142 isolates from the 1992 to 1996 period). There was a trend (not significant) toward greater resistance to penicillin in MLS strains. Of the 78 strains with MICs available, 38 (49%) were fully resistant (MIC ≥ 2 µg/ml) to penicillin, while the rest showed intermediate resistance (1 µg ≤ MIC ≤ 0.12 µg). Previous data have indicated that penicillin-intermediate resistance is far more common in South Africa than full resistance to penicillin (15). In 1992, Friedland and Klugman (15) reported that only 3 of 35 penicillin-resistant strains showed high-level resistance.

Coresistance to penicillin limits the use of most macrolides as treatment of penicillin-resistant pneumococcal infections. New semisynthetic macrolides such as the ketolide RU 64004 (16) are being developed, however, that do not show cross-resistance to penicillin or to erythromycin.

Compared with total erythromycin resistance (middle ear fluid, blood, and CSF) in Europe (17-19), the United Kingdom (18), and the United States (19,21,22), the overall prevalence of macrolide resistance is low. In Europe erythromycin resistance varies by country. In Slovakia, almost all pneumococcal isolates are resistant (17), whereas in Portugal only 0.6% of isolates are resistant and the proportion appears to be declining (23). In the United Kingdom, erythromycin resistance increased from 3.3% to 8.6% between 1989 and 1992 in England and Wales (20). In the United States, 10% of pneumococcal isolates appear to be erythromycin resistant (21). Most isolates received by SAIMR are from the public sector, where macrolides are not normally prescribed for pneumococcal infections. Only 4 of the 128 erythromycin-resistant isolates from 1987 to 1991 and 14 of the 142 erythromycin-resistant isolates from 1992 to 1996 were from the private sector. Resistance data from the private sector may show much higher levels of macrolide resistance, a contention supported by previous South African resistance data (1986), where the carriage rates of multiresistant pneumococci were 17.7% in children from more affluent communities and 0% in children from less affluent areas (24). MIC data were available for 15 of the 20 multiresistant isolates, and all 15 were fully resistant to both erythromycin and clindamycin (24).

Before the M phenotype was observed, erythromycin resistance was assumed to indicate cross-resistance to lincosamides and streptogramin B antibiotics in the pneumococcus. The increase in the incidence of M phenotype may warrant investigation into the use of these antibiotics for the treatment of pneumococcal infections. Sutcliffe et al. (7) suggested that clindamycin be considered for the treatment of bacteremia and middle ear and sinus infections caused by *Streptococcus pneumoniae*. Treatment with clindamycin is feasible only if infection with gram-negative pathogens has been excluded and if the *S. pneumoniae* phenotype is known because the strain may show MLS resistance and studies indicate that many penicillin-resistant strains are also clindamycin-resistant (16,25). Visalli and colleagues (25) found that clindamycin concentrations of only 0.06 µg/ml were required to inhibit 90% of penicillin-susceptible strains when grown in air, while clindamycin concentrations of >64 µg/ml were required to inhibit 90% of penicillin-intermediate and -resistant strains.

Studies of streptogramin use against pneumococci show some promise. The streptogramin RP 59500, a mixture of type A streptogramin, dalfopristin, and type B streptogramin quinupristin, is active against pneumococci regardless of their susceptibilities to penicillin or erythromycin (26,27). In contrast to erythromycin, RP 59500 is rapidly bactericidal (26). Clinical and bacteriologic failure has, however, already been reported using pristinamycin (28), an oral streptogramin combination from which RP 59500 was derived.

The M phenotype is thus relatively new in South African pneumococci but is emerging as an important factor in erythromycin-resistant

pneumococci. Although the low overall rate of resistance makes the use of streptogramins and lincosamides potentially more feasible for the treatment of pneumococcal infections, coresistance to penicillin and the present high rate of MLS resistance necessitate antibiotic susceptibility testing before these antibiotics are administered.

Acknowledgments

The authors thank Patrice Courvalin for the probe used in this study, Thora Capper for assistance with MICs, Avril Wasas for performing serotyping, and Robyn Hubner for assistance with data analysis.

This work was supported by the Medical Research Council, the South African Institute for Medical Research, and the University of the Witwatersrand.

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Mycobacterium tuberculosis Infection as a Zoonotic Disease: Transmission between Humans and Elephants

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Between 1994 and 1996, three elephants from an exotic animal farm in Illinois died of pulmonary disease due to *Mycobacterium tuberculosis.* In October 1996, a fourth living elephant was culture-positive for *M. tuberculosis.* Twenty-two handlers at the farm were screened for tuberculosis (TB); eleven had positive reactions to intradermal injection with purified protein derivative. One had smear-negative, culture-positive active TB. DNA fingerprint comparison by IS*6110* and TBN12 typing showed that the isolates from the four elephants and the handler with active TB were the same strain. This investigation indicates transmission of *M. tuberculosis* between humans and elephants.

Mycobacterium tuberculosis and *M. bovis*, related organisms of the *M. tuberculosis* complex, infect a wide variety of mammalian species. *M. bovis* is pathogenic for many animal species, especially bovidae, cervidae, and occasionally carnivores. Human disease with *M. bovis* is well described and historically was a common cause of tuberculosis (TB) transmitted by infected dairy products. As a result of milk pasteurization and TB eradication programs in most industrialized countries, zoonotic transmission of *M. bovis* through domestic livestock is now rare. In contrast, a similar eradication program has not been conducted for wild or exotic animals, which therefore remain an uncommon source for *M. bovis* exposure. Zoonotic transmission of *M. bovis* has been reported from seals, rhinoceros, and elk (1-4).

M. tuberculosis, the most common species to cause TB, classically causes disease in humans. Animal infection with *M. tuberculosis*, while uncommon, has been described among species (e.g., birds, elephants, and other mammals) with prolonged and close contact with humans (5-10). Transmission of *M. tuberculosis* between animals and humans has not been reported. This paper describes *M. tuberculosis* transmission from elephants to humans.

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The Outbreak

In March 1996, five elephants from an exotic animal farm in Illinois were in California as part of a circus act. One elephant (with chronic, unexplained weight loss since October 1995) died under anesthesia on August 3, 1996, during a diagnostic dental work-up. Necropsy showed widespread consolidation of lung tissue with caseous necrosis of the lungs and mediastinal lymph nodes. Short, fat, relatively scant numbers of acid-fast bacilli were observed in necropsy tissues. A presumptive diagnosis of *M. tuberculosis* was made. The remaining four elephants were recalled to the farm in Illinois. A second elephant died en route on August 6, 1996. Necropsy revealed copious respiratory and trunk exudates and caseous necrosis of the lung.

To determine the risk for and possibility of infection among the animal trainers and caretakers, an epidemiologic investigation was initiated. The remaining elephants in the herd and the elephant handlers and trainers who were

still traveling were recalled to the farm and examined for evidence of *M. tuberculosis* infection. All elephants were empirically begun on antituberculous therapy in early December 1996.

Epidemiologic Investigation

The exotic animal farm was visited on numerous occasions to evaluate the type and degree of contact between elephants and employees. The farm, located in a rural area and surrounded by barbed wire and trees, originally housed 18 Asian and 2 African elephants. Thirteen elephants were tethered on a chain in one large barn, four were housed in a separate large room (two in a common stall), and a baby elephant was in a third room with 5-6 tigers. A separate barn housed approximately 80 tigers.

TB Screening of Employees

The animal handlers (trainers and caretakers) who had direct contact with the elephants were administered purified protein derivative (PPD) skin tests. Initial screening was performed in August 1996, with subsequent screenings in December 1996 and March, June, and September of 1997. Testing was performed by the McHenry County Department of Health, except in two handlers who had subsequent skin tests performed elsewhere. As part of the screening process, handlers were questioned about their risk factors for TB, including previous bacillus Calmette-Guérin (BCG) vaccination.

Handlers were tested by the two-step method using 5 tuberculin units of PPD (0.1 ml) by intradermal injection in the flexor surface of the forearm. A positive result was defined as an induration of >5 mm. Handlers with positive skin tests were evaluated by a TB health-care worker and had chest radiographs taken. Sputum samples from any handler with a chest radiograph consistent with TB were submitted to the Illinois Department of Public Health Laboratory. Samples were examined by direct microscopy for acid-fast organisms, stained with fluorochrome, and processed for culture by standard methods.

Examination of Isolates

The human isolate and the four elephant isolates were sent to the National Tuberculosis Genotyping and Surveillance Network at the Michigan Community Public Health Agency for restriction fragment length polymorphism (RFLP) analysis. Southern blots of Pvu II restricted whole chromosomal DNA, resolved in 1% agarose gels, were probed with a DNA fragment corresponding to the right side of IS*6110* and detected by chemiluminescence (11). The number and size of the hybridizing fragments for each isolate were compared in the same gel. Isolates with identical RFLP patterns or with ≤ 2 band differences were considered to represent the same strain. Additionally, Pvu II digested DNA was similarly typed after probing with the repetitive element TBN12.

Epidemiologic Findings

Elephant handlers worked in very close proximity with the elephants around the clock, whereas tiger handlers had little direct contact with the elephants. Most of the elephant handlers lived on the farm in a separate section of the barn; four lived in trailers on the grounds. The handlers' living quarters had a separate ventilation system from the elephants'; however, the doors between the two quarters were open for unknown periods. Handlers indicated that they held social events in a building connected to the elephant barn.

Necropsies of elephants were performed on the farm and were attended by a number of elephant and tiger handlers (including the handler with the active case). The necropsy of the elephant that died in 1994, also performed on the farm, showed caseous necrosis of the lungs and pleural exudates whose culture yielded *M. tuberculosis*.

In addition to the three elephants that died of *M. tuberculosis* infection, a fourth living elephant was also infected with the mycobacterium; this infection was diagnosed in late December 1996 from a trunk culture obtained in October 1996. Subsequent cultures from this and the other animals have been negative for mycobacteria. Another elephant from this farm died of *M. tuberculosis* infection in 1981 (5), but contact between this elephant and the present herd or any of the handlers could not be established.

Twenty-two handlers at the exotic animal farm had moderate to frequent contact with the infected animals; 12 were elephant handlers and 10 were tiger handlers. Initial PPD testing was performed for 14 handlers in August 1996, 2 in October 1996, and 5 in December 1996. One who was PPD-positive in November 1995 reported receiving BCG more than 10 years before.

Eleven (50%) of 22 handlers were found PPD-

positive as part of this investigation. Eight of the 11 had positive PPD skin test results upon initial testing, with a median induration of 12 mm (range, 10 to 19 mm). Four of the eight were elephant handlers and four were tiger handlers. The skin test reaction of three handlers converted from negative to positive with a median induration of 12 mm (range, 8 to 15 mm). The three PPD converters were initially tested in August 1996; one was positive upon retesting in January 1997, and two tested positive in April 1997 (Table).

Eight of the 11 handlers reported that they had negative skin tests in the past and had not received BCG. The other three reported some type of reaction from a previous skin test in the past but did not know the results. All three also reported receiving BCG more than 10 years before. Eight of the 10 handlers with negative PPD skin tests had at least one negative follow-up test at 3 months; two left the farm and did not receive follow-up testing.

The attack rates were approximately equal for the elephant and tiger handlers. Of the 12 elephant handlers tested, 6 (50%) were PPD-positive with two conversions documented in April 1997; of the 9 tiger handlers, 5 (56%) were PPD-positive, with one conversion documented in January 1997. Overall, a very high rate (52%) of handlers tested positive.

All 12 handlers with positive PPDs (including the one with the known positive PPD) received an evaluation and chest radiograph; one had irregular nodules and interstitial changes in the right apex without retraction of the lungs, consistent with active TB, but no cough, chest pain, fever, night sweats, weight loss, or fatigue.

Three sputum samples were smear-negative for acid-fast bacilli, although one yielded *M. tuberculosis* upon culture. Isoniazid (INH), rifampin, pyrazinamide, and ethambutol treatment was initiated in September 1996, and after 2 months, was reduced to INH and rifampin when the isolate showed no resistance to antituberculous medications. Subsequent chest radiographs revealed improvement or clearing of the initial lesions. Nine of the remaining 11 PPD-positive handlers were prescribed INH prophylaxis; two declined because of the risk for adverse reactions.

Table. TB PPD[a] skin test results of animal handlers, Aug 1996–Sep 1997

	Positive	Negative
Previously positive	1	
Elephant handlers	4	6
Tiger handlers	4	4
Elephant handlers (converted)	2	
Tiger handlers (converted)	1	
Total	12	10

[a]Tuberculin purified protein derivative.

Molecular Analysis of Elephant and Human Isolates

The sputum isolate from the handler with active TB was compared with the isolates from the three animals that died and the living elephant whose infection was diagnosed during the investigation. The isolates had identical IS*6110* RFLP pattern, differing by ≤ 2 bands (Figure 1). Additionally, all isolates had the identical TBN12 RFLP pattern, except the isolate from the elephant that died in August 1996, which demonstrated a shift of one band (Figure 2).

Conclusions

Infection with *M. tuberculosis* or *M. bovis* has not been reported in nondomesticated Asian or African elephants. *M. tuberculosis* infection in domesticated elephants was first reported in 1875 by Garrod and has been recognized in the ancient Ayurvedic literature (10); humans have been considered the source of infection. A trainer with cavitary TB was suspected as the source of infection (8) for one Asian elephant that died of *M. tuberculosis*, although subsequent analysis showed the animal and human isolates to be of two different phage types.

This report describes the first case of zoonotic *M. tuberculosis* transmission. The epidemiologic investigation strongly suggests *M. tuberculosis* transmission between humans and elephants, as evidenced by DNA fingerprinting. RFLP analysis comparing Southern blots of chromosomal DNA probed with IS*6110* and TBN12 indicated that four elephant isolates had identical patterns with the human isolate, differing by ≤ 2 bands. The addition or loss of a single band has been demonstrated in other outbreak settings, and the repetitive element that generates patterns has characteristics of a mobile genetic element (11).

Eleven (50%) of 22 employees screened were skin-test positive, with no difference between tiger and elephant handlers. This is a higher rate of positives than documented in animal handlers exposed to *M. bovis*-infected animals (3,4). Since the handlers had no accurate

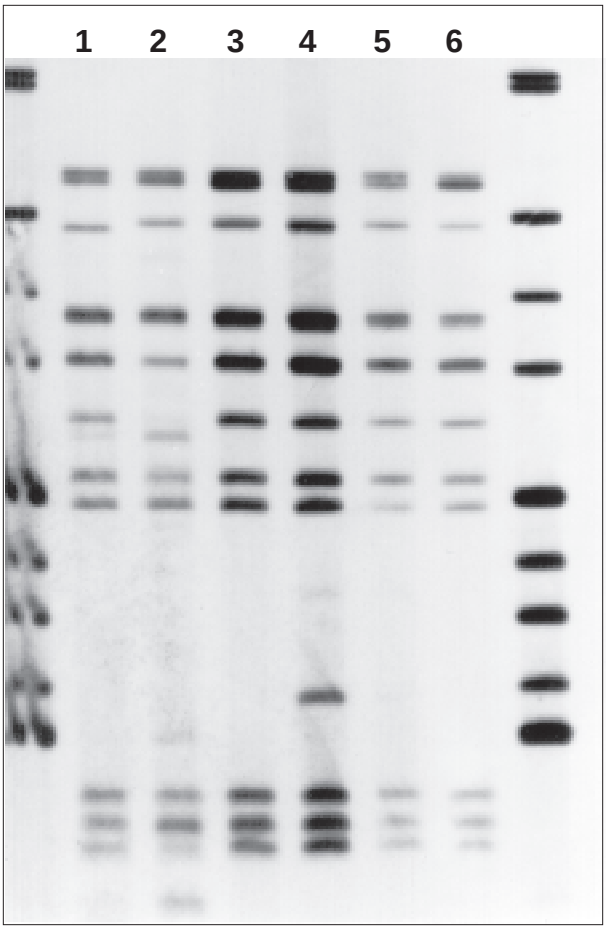

Figure 1. IS*6110* restriction fragment length polymorphism results. Lane1, elephant isolate (died August 6, 1996); Lane 2, elephant isolate (died 1994); Lane 3, living elephant trunk culture (October 1996); Lane 4, elephant lung tissue isolate (died August 3, 1996); Lane 5, elephant lymph node tissue isolate (died August 3, 1996); Lane 6, human sputum isolate (September 1996). Provided by State of Michigan Community Public Health Agency.

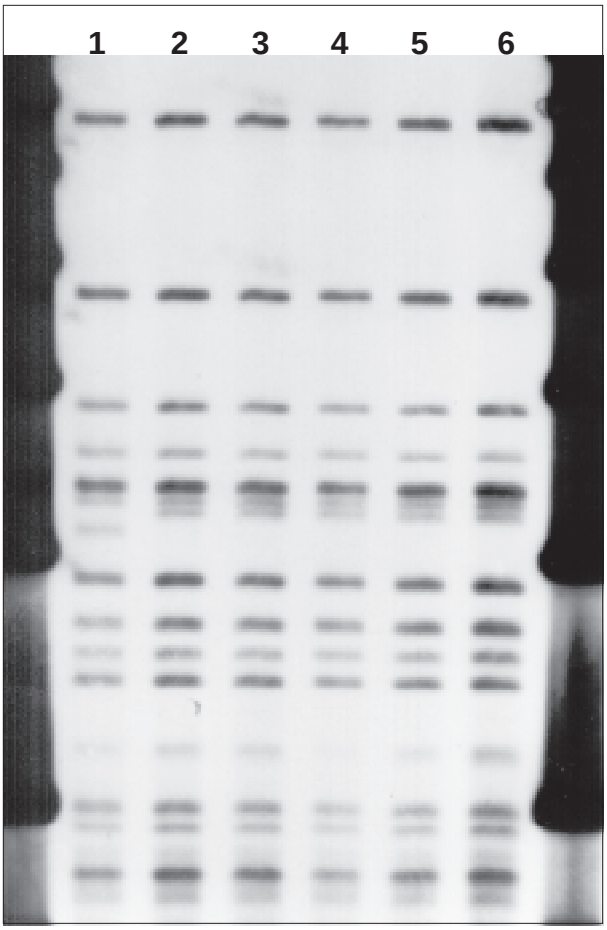

Figure 2. TBN12 restriction fragment length polymorphism results. Lane1, elephant isolate (died August 6, 1996); Lane 2, elephant isolate (died 1994); Lane 3, living elephant trunk culture (October 1996); Lane 4, elephant lung tissue isolate (died August 3, 1996); Lane 5, elephant lymph node tissue isolate (died August 3, 1996); Lane 6, human sputum isolate (September 1996). Provided by State of Michigan Community Public Health Agency.

history of tuberculin skin testing, it was not possible to determine when conversions took place. The original source of infection for both elephants and humans is unknown.

The possible mechanisms of transmission include close contact while handling and training elephants, cleaning the barn, participating in elephant necropsies, and living in close proximity to the elephant barn.

Human-to-human transmission of TB is unlikely because the only handler with active disease did not have cough. Of the three sputum samples initially collected, two were smear- and culture-negative; the third had low numbers of acid-fast bacilli manifested by a negative sputum smear, thus posing a low infectivity risk to others. In contrast, the three elephants that died had evidence of widespread pulmonary disease and, therefore, represented a greater risk for dissemination.

Three handlers converted from negative to positive during the course of the investigation; their relevant exposure is unknown. The source may have been one elephant found antemortem to be culture-positive for M. *tuberculosis*, although this animal did not return to the farm

until November. Contact with this animal is unlikely for handlers whose PPD tests converted in December and unknown for the two handlers whose test results were positive in April (the latter two had not been retested since August).

TB is transmitted through close prolonged contact with a person (or animal) with active TB. The risk for TB transmission from an animal with a case of active TB is higher for daily handlers than for persons with only brief contact, e.g., members of the public viewing a performance or receiving elephant rides. In this outbreak, screening of all persons who had (or thought they had) contact with an elephant that died of *M. tuberculosis* identified three PPD-positive cases but no cases of active TB (8). Because the real risk for transmission to the general public was poorly understood, this case received considerable media attention as well as mention in the medical literature (7,12).

Veterinary practices should be initiated to reduce the risks for exposure to animals infected with *M. tuberculosis.* No data are available on TB incidence among domesticated elephants in the United States. An estimate can be derived from a retrospective study of 379 zoo elephants of which eight (2.3%) had *M. tuberculosis* infection (10).

Reliable diagnosis and prevention of TB in all domesticated and exhibited animals is ideal. Short of this, possible ways to prevent and decrease zoonotic spread of any mycobacterial infection (*M. tuberculosis* or *M. bovis*) include 1) regular skin testing of handlers or keepers; 2) a high index of suspicion of TB in elephants with unexplained weight loss, cough, or rhinorrhea; 3) public health measures of contact tracing and notification; and 4) active and effective treatment of infected personnel and animals (13).

Acknowledgments

We thank Stephen Dietrich and Laura Mosher, North Central Area TB-RFLP Laboratory, Michigan Department of Public Health; George J. Dizikes, Chief of Molecular Diagnostics, Illinois Department of Public Health, Division of Laboratories; and Janet Payeur, National Veterinary Services Laboratory, U.S. Department of Agriculture, MB Section. This research was supported in part by the Centers for Disease Control and Prevention, National Tuberculosis Genotyping and Surveillance Network cooperative agreement.

Kathleen Michalak is the Director of Nursing for McHenry County Department of Health. She has worked to improve the use of community resources, and improved service to client, providers, and the community as a whole.

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New Vectors of Rift Valley Fever in West Africa

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After an outbreak of Rift Valley fever in Southern Mauritania in 1987, entomologic studies were conducted in a bordering region in Sénégal from 1991 to 1996 to identify the sylvatic vectors of Rift Valley fever virus. The virus was isolated from the floodwater mosquitoes *Aedes vexans* and *Ae. ochraceus*. In 1974 and 1983, the virus had been isolated from *Ae. dalzieli*. Although these vectors differ from the main vectors in East and South Africa, they use the same type of breeding sites and also feed on cattle and sheep. Although enzootic vectors have now been identified in West Africa, the factors causing outbreaks remain unclear.

Rift Valley fever (RVF) virus belongs to the genus *Phlebovirus*, family Bunyaviridae. RVF virus epizootics, which cause abortions and deaths in young ungulates and epidemics with hemorrhagic fever and other symptoms, have occurred throughout sub-Saharan Africa and Egypt (1). The virus was first isolated in 1930 in Kenya. The most recent outbreaks occurred in Egypt in 1977-1978 and 1993, South Mauritania in 1987, Madagascar in 1990-1991 (2), and Northern Kenya and Somalia in 1997 (3). The virus is transmitted by mosquitoes and by aerosols of viremic blood during hemorrhage.

After the virus was isolated from livestock, humans, and mosquitoes, transmission cycles were proposed for East and South Africa (1). In East and South Africa, the virus is transmitted from floodwater *Aedes* (subgenera *Aedimorphus* and *Neomelaniconion)* to vertebrates or from mosquito to mosquito by vertical transmission. Vectors (*Ae. cumminsii, Ae. circumluteolus,* and *Ae. mcintoshi)* breed in temporary flood ponds, which flood extensively during heavy rainfall. After such rainfall, the number of mosquitoes increases dramatically, and epizootic and epidemic cycles can occur. During epidemics, other mosquito species can serve as vectors; transmission also occurs by aerosol from the blood of viremic vertebrates. Data obtained in the last few years, however, have shown that the pattern of RVF transmission is different in West Africa (4).

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Table 1. Rift Valley fever virus isolates in West and Central Africa

Host	No. of isolates	Location	Year(s)
Aedes dalzieli	3	Kédougou, Sénégal	1974
Ae. dalzieli	1	Kédougou, Sénégal	1983
Ae. ochraceus	3	Barkedji, Sénégal	1993
Ae. vexans	10	Barkedji, Sénégal	1993
Ae. cumminsii	1	Burkina-Faso	1983
Ae. furcifer	1	Burkina-Faso	1983
Culex antennatus	1	Nigeria	1967-70
Culicoides sp.	2	Nigeria	1967
Ae. palpalis	1	Central African Republic	1969
Mansonia africana	1	Central African Republic	1969
Amblyomma variegatum (on cattle in a slaughterhouse)	1	Central African Republic	1983
Humans	2	Sénégal	1975
	1	Sénégal	1980
	201	Mauritania	1987
	12	Central African Republic	1971-90
Bats	2	Guinea	1981-83
Sheep	1	Barkedji, Sénégal	1993
Bovine	1	Kolda, Sénégal	1993

Before 1991, RVF virus had been isolated from mosquitoes, humans, and bats in different West African countries (Table 1). Serologic data have demonstrated active transmission of the virus throughout West Africa (5).

In Sénégal, RVF virus was isolated from *Aedes dalzieli* in 1974 and 1983. RVF virus had never been isolated from this species in East and South Africa. The only known RVF outbreak, resulting in more than 200 human deaths, occurred in Southern Mauritania near the village of Rosso, on the Sénégal River (6). Serologic surveys of cattle after the outbreak showed that the epizootic was widespread. Animals with positive immuglobulin (IgM) were recorded in The Gambia 340 km south of the epidemic (7). However, surveys conducted 1 to 2 years after the outbreak showed a decrease in RVF seroprevalence, which suggested that the transmission had ceased (8). No human cases were observed in Sénégal after the outbreak. In 1995 and 1996, IgM-positive sheep and cattle were observed again along the Sénégal River, demonstrating that the virus remains present in the region (Thonnon, unpub. data). Because the virus was not isolated from approximately the 500,000 mosquitoes captured in the Rosso area the year after the outbreak, we decided to identify the mosquitoes or other arthropod species involved in the RVF-endemic cycles. We wanted to determine where the virus was when there were no visible manifestations in humans or cattle and whether the mosquito vectors were the same there as in East Africa.

On the basis of the epidemiology of the RVF virus in East and South Africa and the few isolations from West Africa, we selected for a study of the sylvatic vectors of RVF two sites in different bioclimatic areas in Sénégal: Kédougou, where the virus had been isolated before, and Barkedji, where temporary ground pools occur. We focused on mosquitoes and sand flies because RVF is a phlebovirus and research had shown that *Phlebotomus duboscqi* can transmit the virus (9). This research also first identified in the sahelian region the sylvatic vectors of RVF (10), which are different from those in East and South Africa.

Entomologic surveys were conducted from 1991 to 1996. The Kédougou area (12°ll'N, 12°33'W), in southeastern Sénégal in the Sudano-Guinean bioclimatic zone, has a rainy season (May through October) and an average rainfall of 1,100 mm. The Barkedji area (15°17'N, 14°17'W), in northern Sénégal in the sahelian Ferlo region, has a short rainy season (July to September) with an average rainfall of 250-350 mm. Temporary ground pools fill soon after the first rains and remain the only source of water during the dry season until January.

Hematophagous arthropods were collected each year in July, October, and November in Kédougou, and monthly in Barkedji. Insects were captured by four methods: when they landed to bite human volunteers from 17:30 to 22:30; with Centers for Disease Control and Prevention (CDC)–dry ice light traps; with CDC light traps located in sheepfolds, and with animal-baited (one sheep or three chickens) intermittent light traps. In Kédougou, arthropods were collected in villages and in a forest, and in Barkedji, at the edge of three temporary ground pools.

Arthropods were sorted and pooled by species, sex, location, and date in the field. Pools of arthropods (fewer than 100) were put in liquid nitrogen and stored at -70°C. Viruses were isolated on AP61 *(Ae. pseudoscutellaris)* and Vero cell cultures. Some mosquito pools were injected into suckling mice. Viruses were detected by immunofluorescence assay that used specific mouse immune ascitic fluids (11). Viruses were identified by complement fixation and neutralization tests. Blood-fed mosquitoes collected in the traps were preserved so that the blood meal source could be identified by enzyme-linked immunosorbent assay (ELISA) (12).

More than 228,000 mosquitoes from 52 species in Barkedji and 250,000 mosquitoes from 102 species in Kédougou were collected and tested for virus isolation. Additionally, 233,000 sand flies from 11 species and 35,000 sand flies from 25 species were caught in Barkedji and Kédougou, respectively (Table 2). In Barkedji, *Aedes* species represented 28.8% of the mosquitoes collected. *Ae.(Aedimorphus) vexans* was the most abundant *Aedes* species collected, followed by *Ae.(Adm) ochraceus; Ae. (Neomelaniconion) mcintoshi* and *Ae. (Adm) dalzieli* were rare. Sand flies were abundant during the dry season (December through May). In Kédougou, *Aedes* species represented 50.6% of the mosquitoes collected. *Ae. dalzieli* was the predominant *Aedes* species; *Ae. vexans, Ae. mcintoshi,* and *Ae. ochraceus* were much less abundant.

In Barkedji, 10 RVF virus isolates came from *Ae. vexans* and 3 from *Ae. ochraceus* collected around three temporary ground pools and near

Table 2. Arthropod collections in Kédougou and Barkedji, 1991 to 1996

		Mosquitoes		*Aedes* spp.		Sand flies	
Location	Year	No.	(Pools)	No.	(Pools)	No.	(Pools)
Barkedji	1991	34,327	(1,042)	11,233	(338)	3,370	(56)
	1992	42,804	(1,534)	5,782	(352)	30,547	(269)
	1993	64,810	(2,023)	6,056	(300)	72,104	(651)
	1994	22,470	(927)	8,701	(365)	42,730	(416)
	1995	40,952	(1,413)	16,124	(561)	66,300	(667)
	1996	23,041	(764)	16,243	(453)	18,934	(192)
Total		228,404	(7,703)	64,139	(2,369)	233,895	(2,251)
Kédougou	1991	48,377	(1,264)	28,579	(730)	200	(2)
	1992	37,685	(1,364)	26,889	(804)	1,032	(9)
	1993	71,992	(2,493)	31,224	(1,136)		
	1994	48,751	(1,877)	23,839	(956)		
	1995	17,756	(1,014)	12,590	(616)	23,122	(238)
	1996	25,768	(1,284)	14,869	(679)	11,215	(115)
Total		250,329	(9,296)	126,659	(4,914)	35,569	(364)
Total	(1991-96)	478,733	(16,999)	190,798	(7,283)	269,464	(2,615)

cattle droves in CO2-CDC light traps in October and November 1993. In November, an RFV virus isolate was obtained from one of the sheep. West Nile, Ngari, and Wesselsbron viruses were also isolated from vectors or potential RVF vectors. Five viruses were isolated from sand flies (Table 3).

In Kédougou, no RVF virus was isolated from any vector during the study period, although the virus had been found four times in earlier isolates obtained in 1974 and 1982 from *Ae.dalzieli*. This study found other viruses isolated from *Ae. dalzieli* including 42 viral strains belonging to seven different viruses, mostly alphavirus and flavivirus, one strain of Wesselsbron virus was isolated from *Ae. ochraceus*, and five different viruses were isolated from sand flies (Table 3).

Even if the vectorial competence of *Ae.vexans*, *Ae. ochraceus*, and *Ae. dalzieli* had not been experimentally confirmed, they would be likely enzootic vectors of RVF virus in Sénégal. These three species are different from the main known East African vectors—*Ae.cumminsii*, *Ae. circumluteolus*, and *Ae. mcintoshi*—which are also in West Africa, but whose role in RVF virus transmission has not been demonstrated. A much lower number of *Ae. mcintoshi* than *Ae.vexans* or *Ae. ochraceus* were captured each year: when RVF virus was isolated in 1993 in Barkedji, 6,958 *Ae. vexans*, 1,069 *Ae. ochraceus*, but only 58 *Ae. mcintoshi* were captured and tested.

Other Sénégalese floodwater zoophilic mosquitoes such as *Ae.(Adm) minutus*, *Ae. (Adm) fowleri*, and *Ae. (Adm) argenteopuntatus* should be suspected as potential sylvatic RVF vectors because they belong to the same *Aedes* subgenera of known vectors and have almost the same breeding sites and trophic behavior (10). Moreover, females from a colony of Sénégalese *Ae. fowleri* were able to transmit RVF virus experimentally (13). Mosquito species belonging to other genera *(e.g., Culex, Mansonia)* should be implicated during an outbreak only after the virus is amplified, as in East Africa (3).

The taxonomic status of *Ae. vexans*, the chief enzootic vector of RVF virus, has to be clarified. *Ae. vexans* has a worldwide distribution. In 1975, G.B. White wrote that the African specimens of *Ae. vexans* belong to the same subspecies *Ae. vexans arabiensis*, which was recorded in Mauritania, Sénégal, The Gambia, Ghana, Nigeria, Sudan, Ethiopia, Somalia, and South Africa (14). In Barkedji, in the dry sahelian region of Sénégal, female *Ae. vexans* lay eggs on the soil of temporary ground pools. The adult *Ae. vexans* appear only 4 days after the first rain, a very short larval development period. *Ae. vexans* can be abundant (more than 4,000 per light trap). However, their density decreases quickly about 2 months after the ground pools flood, while the density of other species, such as *Culex poicilipes*, *Mimomyia splendens*, or *Mansonia africana*, increases at the end of the rainy season (Figure).

Evaluating the trophic preferences of mosquitoes is difficult and highly dependent on the sampling methods. Some species are highly host-specific, but most species feed on a range of different host animals. Despite numerous human bites by *Ae. vexans* and *Ae. ochraceus* around the

pools, these species seemed not very host-specific. Females that fed on cattle, sheep, goats, horses, and even chickens were identified. Other sylvatic vectors or potential vectors of RVF virus also showed equally low host specificity (Table 4).

Because of low host specificity, many different viruses, including RVF, can be transmitted to many vertebrate species. More than 30 viruses belonging to different groups have been isolated from *Ae. vexans* throughout the world (15)—the three from West Africa include West Nile virus (a flavivirus from birds) and Ngari virus (isolated from ill humans). Fifteen viruses, also from different arbovirus groups, have been isolated from *Ae. dalzieli* in West Africa (16). Few viruses were isolated from *Ae. ochraceus* and *Ae. mcintoshi*, probably because of the lower number of pools tested.

The risk for a new outbreak of RVF in Sénégal is highly speculative. An increase in IgG prevalence in livestock in different West African countries the year before the 1987 Mauritanian outbreak demonstrated that the virus was present in endemic cycles in this area (17). This epizootic was caused by three factors: a dam was built near Rosso on the Sénégal River, mosquito density increased probably because of the flooding of the river bank in 1987, and the livestock density increased. In 1993, when RVF

Table 3. Potential Rift Valley fever sylvatic vectors and viral isolates, Sénégal, 1991 to 1996

Vector species	No.	No. pools	Virus isolates[a] (No. strains)
Barkedji			
Aedes vexans	42,055	1,428	WN-NRI (1), RVF (10), WN (2)
Ae. mcintoshi	758	88	NRI (1)
Ae. ochraceus	3,672	228	WSL (1), RVF (3)
Ae. dalzieli	105	34	(0 virus)
Phlebotominae spp.	233,895	2,251	SAB (63), CHP (7), GF (5), Ar D 88909 (1), Ar D 95737 (12)
Kédougou			
Ae. vexans	1,194	81	(0 virus)
Ae. mcintoshi	536	107	(0) virus)
Ae. ochraceus	915	110	WSL (1)
Ae. dalzieli[b]	31,809	821	CHIK (8), BBK (1), WSL (2), KED (6), BOU (1), PGA (2), ZIKA (22)
Phlebotominae spp.	35,569	364	SAB (11), CHP (4), TETE (1), Ar D 111740 (1), Ar D 95737 (2)

[a]WN: West-Nile virus, NRI: Ngari virus, RVF: Rift Valley fever virus, WSL: Wesselsbron virus, SAB: Saboya virus, CHP: Chandipura virus, GF: Gabek Forest virus, CHIK: Chikungunya virus, BBK: Babanki virus, KED: Kédougou virus, BOU: Bouboui virus, PGA: Pongola virus, ZIKA: Zika virus, TETE: Tete virus. Ar D 88909, Ar D 95737, and Ar D 111740: not yet identified viruses.
[b]4 RVF strains were isolated in 1974 and 1982.

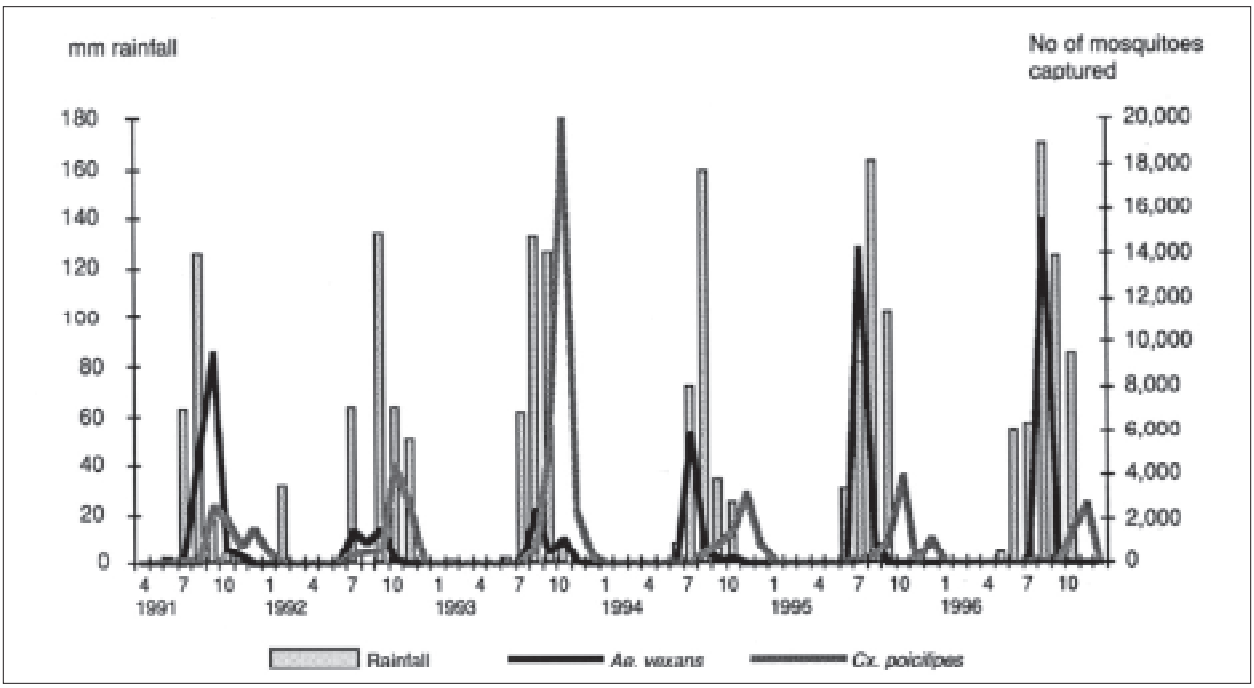

Figure. Distribution of *Aedes vexans* and *Culex poicilipes* captured by monthly rainfall, Barkedji, Sénégal, 1991-1996.

Table 4. Host choices of Rift Valley fever virus vectors captured with light traps or animal-bait traps in Sénégal, 1991-1996

	No. of host-specific blood feedings				
Vector	Cow	Sheep	Horse	Chicken	Human[a]
Aedes vexans	35	33	38	7	+ + +
Ae. ochraceus	8	18	5	1	+ +
Ae. mcintoshi	7	20	2	2	+ +
Ae. dalzieli	52	114	1	21	+ + +

[a]++: numerous bites; +++: very numerous bites.

virus was isolated in Barkedji from floodwater *Aedes* and from one sheep, the situation was different. No environmental or climatic changes were identified; in particular, the rainfall was not higher than in previous years. However, a 15% IgM prevalence was observed in 1994 and 1995 among herds studied each year since 1990 along the Sénégal River, demonstrating RVF virus circulation. Enzootic vectors of RVF virus are now identified in West Africa, but factors causing outbreaks need to be further studied.

Acknowledgments

We thank Leonard Munstermann, who organized the Emerging Vectors of Emergent Diseases symposium {sponsored by the American Commitee of Medical Entomology, held in Orlando during the XX meeting of the American Society of Tropical Medicine and Hygiene (7-11 December 1997), where these data were presented and Scott McKeown for help with the English translation.

This work was supported by the Institut Francais de Recherche Scientifique pour le Développement en coopération (ORSTOM) and by the Dakar Pasteur Institute.

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

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Plasmodium vivax malaria reemerged in the Republic of Korea in 1993. The number of cases has tripled each year since, with more than 1,600 cases reported in 1997. All 27 cases in U.S. troops resolved uneventfully with chloroquine/primaquine therapy. Disease is localized along the western Demilitarized Zone and presents minimal risk to tourists.

Plasmodium vivax malaria has been endemic on the Korean peninsula for centuries (1). The primary vector is believed to be *Anopheles sinensis*, a zoophilic and facultatively anthropophilic mosquito, which breeds in fresh, sun-exposed water such as that found in rice fields (2). During the Korean War (1950–1953), U.S. troops were exposed to malaria (3) and received weekly chloroquine chemoprophylaxis. This regimen generally succeeded in preventing malarial attacks while U.S. troops were in the Republic of Korea. As the troops returned home, however, U.S. military hospitals were inundated with cases of malaria—at one point, as many as 629 cases per week (3). More than 3,000 cases of malaria were documented in U.S. troops that served during the war. Subsequent U.S. military research led to the current use of primaquine therapy (4). The outbreak of malaria continued on the peninsula after the armistice. In the 1960s and 1970s, the Republic of Korea government combined active and passive case detection with extensive use of pesticides in an ambitious eradication project (1). Indigenous transmission of malaria appeared to cease in the mid-1970s, and in 1979 the Republic of Korea was declared malaria-free by the World Health Organization (5).

In 1993, *P. vivax* malaria reemerged in the Republic of Korea along the western edge of the Demilitarized Zone (DMZ) (6). Since the reemergence, the annual number of cases has increased geometrically, tripling (1995, 1996) or quadrupling (1997) in each of the past 4 years (Table 1). In 1997, the Republic of Korea Army administered antimalarial chemoprophylaxis to approximately 8,000 of its troops at greatest risk (U.S. Army/Republic of Korea Army/Republic of Korea Malaria Symposium, pers. comm.). U.S. troops in Korea were not placed on antimalarial chemoprophylaxis in 1997 after local and U.S. Army experts concluded that personal protective measures and community mosquito control would adequately limit transmission (7).

Cases in 1997 were distributed as expected from June to October, with a peak of 518 cases in August (Table 2). As in past years, more than 80% of the cases were associated with military service along the western DMZ. During 1997, 34 cases of *P. vivax* malaria in U.S. troops were attributed to exposure in the Republic of Korea. This number includes 27 cases in U.S. Forces-Korea troops, one case in a soldier stationed in Okinawa, Japan, who was temporarily assigned to the Republic of Korea in August 1997, and six cases in U.S. troops exposed in the summer of 1996. The U.S. Forces-Korea incidence of *P. vivax* in all malarious areas

Table 1. Annual number of *Plasmodium vivax* malaria cases in U.S. Forces-Korea (USFK), Republic of Korea (ROK) Army, and ROK Civilians, 1993–1997

Year	USFK	ROK Army	ROK Civilians	Total
1993	0	1	1	2
1994	1	18	20	39
1995	1	98	19	118
1996	11	285	71	367
1997	27	1,154	461	1,642

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Table 2. Monthly distribution of *Plasmodium vivax* malaria cases in the Republic of Korea (ROK), by onset of disease, 1997

Month	USFK[a]	ROK Army	ROK Civilian	Total
Jan	0	0	0	0
Feb	0	0	0	0
Mar	0	5	2	7
Apr	1	10	3	14
May	0	31	4	35
Jun	0	145	38	183
Jul	2	260	102	364
Aug	11	317	190	518
Sep	10	320	75	405
Oct	3	55	38	96
Nov	0	9	9	18
Dec	0	2	NA[b]	2

[a]USFK, U.S. Forces-Korea.
[b]NA, Not available.

between July 1, 1997, and September 30, 1997, was estimated to be 0.5 cases per 1,000 per week. The incidence northwest of the Imjin River was approximately one case per 1,000 per week. Site-specific rates, as well as rates for the Republic of Korea Army, cannot be released because of security concerns; however, the Republic of Korea Army has 50 times more exposed soldiers than does U.S. Forces-Korea.

The epidemic remains focused along the western DMZ. Before 1997, almost all civilian cases were attributed to exposure in areas within 10 km of the DMZ. In 1997, however, dozens of cases occurred among civilians farther than 10 km from the DMZ. The area where U.S. troops are exposed is strictly controlled; tourists are not allowed in this area after dark. Other areas under the Republic of Korea Army control, e.g., Yonchon and Chorwon, are accessible to tourists but are seldom visited. The risk to foreign tourists, therefore, appears to be negligible at this time.

Of the 27 U.S. Forces-Korea cases, 18 were in U.S. soldiers and 9 were in Korean soldier augmentees to the U.S. Army. All cases were in men 19 to 40 years of age (median 21.5 years). With one exception, the cases occurred in junior enlisted soldiers. As in the past 3 years, no cases occurred in African American soldiers, who account for almost 30% of the exposed U.S. troops. This is consistent with the known natural immunity against *P. vivax* in African Americans, most of whom do not have the Duffy antigen on their red blood cells (8). This relative immunity of African Americans was also seen during the Korean War (3). The time from onset of symptoms to diagnosis was 1 to 25 days (median 5 days). Three patients with mild illness were treated as outpatients. All patients quickly recovered after standard chloroquine therapy. All patients were also treated with primaquine.

The Korean strain of *P. vivax* malaria has been well characterized by both Korean and U.S. medical personnel (1,3). This type of malaria is termed "temperate climate" *P. vivax* malaria (3). Transmission is restricted to only a few summer months, when the Korean peninsula is warm enough to support the parasite's reproductive cycle in the mosquito (1,3). This Korean *P. vivax* malaria is remarkable in that 40% to 50% of cases have no symptoms until 6 to 9 months after infection (3,9). The long incubation period interferes with determining the start of the transmission season. Malaria cases begin to occur in the late spring or early summer, before the weather is warm enough to support malaria transmission. These early season cases, arising from infections acquired the previous fall, blend into and fuel infections transmitted each summer. Another result of this long incubation period, given the extraordinary (>90%) annual turnover in U.S. troops, is the return of infected soldiers to the United States before any symptoms appear.

Malaria in the Republic of Korea is also characterized as epidemic or unstable because conditions usually do not support widespread disease (10). Factors contributing to this instability include the extremely short transmission season and the zoophily of the major vector. Areas with unstable malaria usually have low to very low rates of disease but can occasionally have high rates in response to climatic, livestock, or sociopolitical aberrations (10). The current epidemic may fit this pattern. Heavy rains from 1994 to 1996 led to widespread crop failures in the Democratic Peoples Republic of Korea. As a result, domestic farm animal populations, the preferred source of blood for *A. sinensis*, fell sharply. These factors may have contributed to the geometric increase of disease, but the lack of data makes this explanation speculative.

Most Korean officials perceive the current epidemic to be a Democratic Peoples Republic of Korea public health problem spilling over the DMZ and discount the importance of indigenous transmission in the Republic of Korea. Broad-scale epidemiologic investigation is necessary to confirm indigenous transmission. Although it is

not known if the epidemic will continue its geometric increase and geographic expansion in 1998, a decrease in disease without extensive intervention seems unlikely.

Acknowledgments

The authors thank Colonel Daniel F. Perugini, U.S. Forces-Korea Surgeon, and Colonel Kenneth G. Torrington, Deputy Commander, 18th Medical Command, for their support and critical review of this manuscript.

All equipment, supplies, and author salaries were received from the U.S. Army.

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Molecular and Epidemiologic Analysis of Dengue Virus Isolates from Somalia

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Nucleotide sequence analysis was performed on 14 dengue virus isolates (13 dengue-2 viruses and 1 dengue-3 virus) recovered from febrile soldiers in Somalia in 1993. The dengue-2 viruses were most closely related to dengue-2 virus recovered in Somalia in 1984. However, differences in nucleotide sequence (0.35% to 1.35%) were evident among the 1993 isolates. These differences were closely associated with the geographic location of the infection as well as with different times of infection at the same location. Genetic difference between strains was not associated with differences in clinical features. Molecular analysis of dengue viruses is a useful adjunct to epidemiologic investigation of their distribution over distance and time.

Dengue fever was a major cause of illness among U.S. troops in Somalia during 1993 (1). We identified 14 virus isolates from soldiers with dengue fever: 12 dengue-2, 1 dengue-3, and 1 mixed dengue-2 and -3 (2). Nucleotide sequence followed by phylogenetic analysis was performed to further characterize these isolates. The genotype of these virus isolates was subsequently combined with information collected during epidemiologic investigation of the febrile patients. The molecular epidemiologic analysis showed that endemic strains of dengue-2 virus were segregated in specific geographic locations (Figure 1).

Analytic Approach

Ninety patients with temperatures of at least 38.1°C who were admitted to the 86th Evacuation Army Hospital in Somalia from 27 February to 3 April 1993 underwent a standardized clinical evaluation (history and physical examination) and were asked to fill out an epidemiologic questionnaire at admission. Clinical evaluations were repeated daily until discharge. Serum samples were obtained from patients at admission and discharge and frozen at -70ºC for later testing. For this report, the geographic site of infection for each patient was defined as the site of principal activity 2 weeks before onset of illness and was assigned without knowledge of the dendrogram.

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Figure 1. Map of Somalia showing towns from which dengue viruses were recovered.

Virus isolations were performed on 81 available admission sera by injecting them into *Toxorhynchites splendens* mosquitoes and C6/36 cells (*Aedes albopictus* mosquito cell line) (3). Dengue virus isolates were identified by indirect immunofluorescence assay (IFA), by nested reverse transcriptase-polymerase chain reaction (RT-PCR) for dengue virus, and by virus neutralization (3). Virus seeds were obtained from culture fluids collected 14 days after incubation on C6/36 cells (single amplification in C6/36 cells) and stored at -70°C for genotypic analysis.

Nucleotide sequencing of the envelope (E) gene from position 639 to 1,233 was obtained for all 14 Somalia isolates. This genomic region was amplified directly by RT-PCR from extracted viral RNA and sequenced by the dye-terminator method of ABI (Applied Biosystems, Foster City, CA) (5). The nucleotide sequence was resolved by a 377 automated DNA sequencer (Applied Biosystems). The sequences, combined with the E-gene database of other viruses (5,6), were phylogenetically analyzed by genetic distance or discrete character-state methods (7). Viruses were classified into a specific serotype and genotype according to phylogenetic clustering with other dengue viruses.

Findings

The patients were men ages 19 to 39 years (median age 22); 12 were Caucasian and 2 (patients 11 and 12) were African American; all had signs and symptoms consistent with dengue fever (Table 1). The sequence analysis identified 13 dengue-2 viruses and one dengue-3 virus among the 14 isolates. These results supported previous identification by IFA and RT-PCR, except that no mixed infection with dengue-2 and -3 was found in the isolate from patient 12 after amplification in C6/36 cell culture.

The single dengue-3 isolate was recovered from a soldier stationed at the port in Mogadishu. The sequence was dengue-3 genotype III, which closely resembles Mozambique 85 strain (6) and strains from Sri Lanka, India, and Samoa.

The 13 dengue-2 isolates were all genotype IV and most closely related genetically to the 1984 isolate from Somalia (Figure 2). Phylogenetic clustering of genotype IV viruses was supported by 98% bootstrap confidence level, which also included viruses from Burkina Faso, Indonesia, Sri Lanka, and the Seychelles (5). An apparent genetic drift of 1.18% to 1.85% nucleotide sequence difference within dengue-2 viruses was observed between 1984 and 1993 (data not shown). A similar level of genetic difference (0.34% to 1.35%) was also observed among the 13 dengue-2 virus isolates collected in 1993.

Nucleotide sequence alignment from E gene position 638 to 1,233 showed 24 nucleotide changes with four amino acid alterations at the E-291, 300, 352, and 409 between 1984 and 1993 isolates (Table 2). Transition mutations were the predominant changes with a transition to transversion ratio of six to one. All 1993 isolates shared six unique nucleotide changes at position 792, 871, 885, 1,074, 1,137, and 1,161. However, only position 871, C to A change, altered amino

Table 1. Patients from whom dengue-2 virus isolates were recovered

Patient No[a]	Location	Unit	Dates of illness (1993)	Max. temp (°F)	Head-ache	Body-ache	Eye pain	Rash
23	Mogadishu/Airport	711 Postal	5-9 Mar	102.0	No	Yes	Yes	Yes
20	Kismayu/ICRC Hospital	2/87th	4-10 Mar	102.0	Yes	No	No	No
21	Kismayu/ICRC Hospital	2/87th	4-8 Mar	102.6	Yes	No	Yes	Yes
02	Kismayu/Airport	710 MSB	27 Feb-5 Mar	103.0	Yes	Yes	Yes	Yes
11	Kismayu/Airport	3/14th	27 Feb-2 Mar	102.0	Yes	Yes	NA[b]	Yes
12	Kismayu/Airport	129th Postal	28 Feb-4 Mar	104.2	Yes	Yes	Yes	No
13	Kismayu/Airport	3/14th	27 Feb-3 Mar	101.8	Yes	Yes	NA	Yes
56	Mogadishu/Airport	LSB	1-4 Apr	103.0	Yes	Yes	No	No
57	Mogadishu/Airport	70th Transport	2-5 Apr	102.4	Yes	Yes	Yes	No
58	Mogadishu/Stadium	1/7th	3-6 Apr	102.8	Yes	Yes	Yes	No
30	Afgoi	984 MP	11-15 Mar	103.4	No	Yes	Yes	No
39	Mog/Checkpoint 57	1/7th	21-25 Mar	103.8	Yes	Yes	Yes	No
46	Mog/Checkpoint 57	1/7th	26-28 Mar	103.2	Yes	Yes	Yes	Yes

[a]Patients are grouped according to the degree of homology of isolate sequences. Each group represents a single branch in the phylogenetic dendrogram.
[b]NA = not available

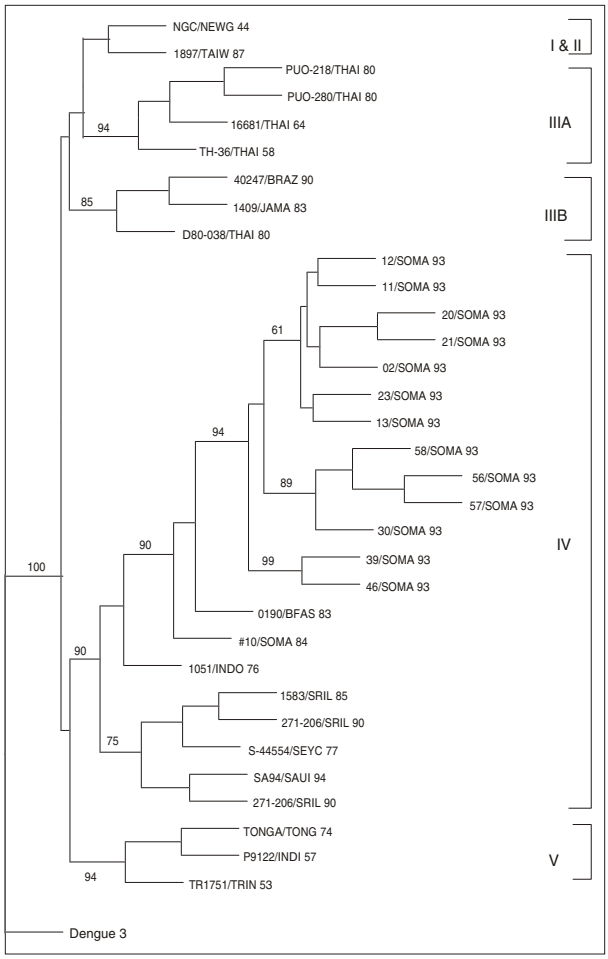

Figure 2. Phylogenetic tree of the dengue-2 viruses, including Somalia 1993 virus isolates, using nucleotide sequence from position 639 to 1,233 of the envelope protein gene. This strict consensus tree was constructed by the following programs of the PHYLIP 3.5 package: SEQBOOT, DNAPARS, CONSENSUS, and DRAWGRAM. Strain ID is listed first, followed by abbreviation for country of isolation, and the last two digits of the year of isolation.

acid sequence of Gln in the Somalia 1984 isolate to Lys in the 1993 dengue-2 virus isolates.

Viruses with certain nucleotide sequence differences appeared to be segregated by location. Virus isolates from Kismayu had a unique U change at position 1,167 that the Somalia 1984 dengue-2 virus and the Mogadishu and Afgoi isolates did not have (Table 2). Identical strains were isolated from soldiers infected in the same location at the same time. Distinguishable strains were collected from patients apparently infected at different locations within the same city, e.g., during a 1-week period at the ICRC hospital and the airport in Kismayu (Tables 1, 2). Yet, greater differences were found between dengue-2 viruses recovered from cases at different times from the same location within the city of Mogadishu. Sequences from viruses recovered in March (patient 23) or in April (patients 56, 57) were as dissimilar as sequences from viruses collected at the same time from different locations.

Conclusions

The data show distinct clustering of dengue-2 viruses within specific locales in Somalia. Identical dengue-2 viruses were acquired at the same time in the same locale, as shown by the four isolates from persons presumably infected at the airport in Kismayu. Moreover, virus isolates recovered from persons infected in three widely dispersed towns (Mogadishu, Kismayu, and Afgoi) were clearly distinguishable by nucleotide sequence analysis. The number of virus isolates studied in this analysis is small, and there is some imprecision in assigning the geographic location of infection. However, these data suggest a selective preference for a single circulating dengue-2 virus in a particular geographic location.

The distances between towns exceeded the flight range of mosquito vectors, unless they were transported by vehicles or by air. Movement of vectors is refuted by the consistency of isolates recovered at different locations. Additionally, no large-scale movements of troops occurred during this study. The local population was stable, and no large refugee movements occurred. Hence, the isolates reflect viruses circulating among the indigenous populations, a consequence of moving populations unexposed to dengue into endemic-disease locations.

The analysis also shows that dengue-2 viruses with unique genetic sequences may circulate at different times within the same location. The six isolates recovered from soldiers infected in Mogadishu show consistent differences over time during the short course of this analysis (2 months). These findings suggest spatial and temporal clustering of dengue viruses causing infections within Somalia. These differences are not readily explained by immigration of infective vector mosquitoes. The distances between locations within towns (e.g., 8 km) exceeded the flight range of *Aedes aegypti*. However, surveillance for vector mosquitoes was not routinely performed during the time of this

Table 2. Nucleotide sequences at the E-639 to 1233 region of Somalia dengue-2 isolates

E gene position nt/aa[a]	SOM 1984[b] nt/aa	Patient[c] 11	13	02	12	21	20	23	30	39	46	56	57	58
654/218	uuA/Leu									G	G			
661/221	Cug/Leu											U	U	
720/240	uuC/Phe							U						
723/241	aaA.Lys									G	G			
729/243	ccU/Pro								C					
783/261	caU/His					C	C							
792/264	cuC/Leu	U	U	U	U	U	U	U	U	U	U	U	U	U
801/267	gcC/Ala									U	U			
870/290	gaC/Asp								U					
871/291	Caa/Gln→Lys	A	A	A	A	A	A	A	A	A	A	A	A	A
885/295	aaA/Lys	G	G	G	G	G	G	G	G	G	G	G	G	G
900/300	UCu/Ser								A			A	A	A
972/324	guA/Val					G	G							
1,050/350	cgC/Arg								U					
1,056/352	auU/Ile					C	C		A			A	A	A
1,074/358	guU/Val	C	C	C	C	C	C	C	C	C	C	C	C	C
1,079/360	gAa/Glu→Gly									G	G			
1,082/361	aAa/Lys→Arg													G
1,104/368	gaA/Glu					G	G							
1,137/379	auC/Ili	U	U	U	U	U	U	U	U	U	U	U	U	U
1,161/387	cuG/Leu	A	A	A	A	A	A	A	A	A	A	A	A	A
1,167/389	cuC/Leu	U	U	U	U	U	U	U						
1,209/403	gaG/Glu							A						
1,225/409	Gcg/Ala→Pro							C						

[a]nt/aa = nucleotide/amino acid.
[b]Somalia 1984 dengue-2 virus.
[c]Capital letter (A, U, G, C) = different nucleotide sequence; empty cell indicates identical nucleotide sequence.

analysis, and mosquitoes may have been carried by transport vehicles within the town.

No apparent differences in clinical features of the illness caused by these different dengue-2 strains were observed. This may reflect the small sample size, intrinsic variability in host response, and restricted time of analysis. Reports from Australia suggest that clinical features may be modified over the course of a dengue epidemic, perhaps reflecting a change in virulence of circulating virus (8). Only sporadic cases of dengue fever were detected, reflecting endemic transmission during this investigation; hence we cannot directly comment on transmission during epidemics.

Isolation of dengue viruses in C6/36 insect cells from viremic serum was required to increase virus titer for genetic analysis. Amplification of the virus isolates on insect cells may have induced or selected specific genetic changes. A comparison of the RNA T1 fingerprints of the New Guinea C isolate and C6/36 cell passage showed that under the conditions of laboratory passage in experimental hosts, during a single infection in humans, dengue-2 was subject to very little selective pressure (10). In this study, the identical isolation procedure was followed, and four identical gene sequences representing four sets of independent isolates were obtained for all isolates (Table 2); these results indicate that, if present, the same selection process was exerted on all isolation attempts.

Hence, nucleotide sequences obtained by direct sequencing of RT-PCR amplified product from serum samples of viremic patients were certainly representative of original virus genotype. Dengue-2 was the dominant virus in the mixed dengue-2, and -3 infected serum from patient 12. Failure to recover dengue-3 virus from this patient may be due to a direct growth competition of viruses in C6/36 cells. Titer of dengue-3 virus after C6/36 cell passage was insufficient to be amplified by RT-PCR directly.

The stability or relatedness of strains over time can only be surmised from these limited data. The dengue-2 viruses recovered in 1993

from this investigation are genetically distinct from the Somalia 1984 dengue-2 isolate. The extent of homology preserved for 10 years suggests that only 6 (1%) of 596 positions (Table 2) of nucleotides changed because of genetic drift. Remarkable genetic stability has been proposed for dengue viruses (10). The ability of genotyping methods to reliably resolve small (<1%) differences in sequence and the expanding access to these methods are powerful epidemiologic and clinical tools. These methods may further refine the ability to track the circulation or spread of dengue viruses within a city or country (9), as has been done with other viruses (11). These techniques may also provide insight into the evolution of dengue viruses at specific locations and perhaps detect the frequency of dual infections or the presence of recombination within dengue viruses (12).

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Molecular Characterization of a Novel *Rickettsia* Species from *Ixodes scapularis* in Texas

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A novel *Rickettsia* species of undetermined pathogenicity was detected in *Ixodes scapularis.* DNA sequencing showed the highest nucleotide sequence similarities with *R. australis* for the 17 kDa gene, *R. helvetica* for *gltA*, and *R. montana* for *rompA*. The new organism, provisionally designated as genotype Cooleyi, is highly divergent in three conserved genes from recognized *Rickettsia* species.

Rickettsia spp., obligately intracellular, gram-negative, vector-transmitted bacteria, are divided antigenically into the typhus group (TG) and the spotted fever group (SFG). In nature, TG rickettsiae inhabit either flea (*Rickettsia typhi* and *R. felis*), louse (*R. prowazekii*), or tick (*R. canada*) arthropod hosts. Except for *R. akari*, whose invertebrate host is a mite, all SFG rickettsiae reside in tick hosts. SFG rickettsiae, excluding *R. akari*, are maintained in nature by a cycle involving both transovarial and transstadial passage of the microorganism in the tick and, for some species, amplification in small mammal hosts. Ticks infected with pathogenic rickettsiae (i.e., *R. rickettsii, R. conorii, R. sibirica, R. australis, R. japonica, R. africae,* and *R. honei*) that feed on humans, who are dead-end hosts, may transmit an SFG rickettsiosis (i.e., Rocky Mountain spotted fever [RMSF], boutonneuse fever, North Asian tick typhus, Queensland tick typhus, Japanese spotted fever, African tick bite fever, or Flinders Island spotted fever, respectively).

In the United States, *Dermacentor andersoni* and *D. variabilis* are the classic vectors of *R. rickettsii* to humans; other recognized vectors include *Rhipicephalus sanguineus* for *R. rickettsii* in Mexico and for *R. conorii* in Eurasia and Africa, *Amblyomma cajennense* for *R. rickettsii* in Brazil, and *Ixodes holocyclus* for *R. australis* in Australia. Other rickettsiae, such as *R. parkeri* and *R. amblyommii,* have been isolated from human-biting ticks; however, these rickettsiae have not been proven pathogenic for humans. The presence of apparently nonpathogenic rickettsiae whose vector host and geographic distribution overlap with pathogenic rickettsiae may interfere with the transmission of virulent rickettsiae to humans, as may be the case with *R. peacockii* (nonpathogenic) (1,2) and *R. rickettsii* in *D. andersoni* in the Bitterroot Valley of Montana.

The black-legged tick, *I. scapularis*, is of major public health importance because it feeds indiscriminately on numerous vertebrate hosts, including humans, and transmits *Borrelia burgdorferi*, *Babesia microti*, and human granulocytotropic ehrlichia to humans. Rickettsiae have been detected in *I. scapularis* (3-8); however, the ability of black-legged ticks to serve as vectors of rickettsiae to humans or to other small mammals important in maintaining and spreading rickettsiae in nature has not been examined. Because rickettsiae are regularly detected by immunofluorescence staining in *I. scapularis* specimens sent to the Texas Department of Health (5,7) and because the range of *I. scapularis* and RMSF cases overlaps in Texas (9), we characterized the immunofluorescence-detected microbial agent in this tick collected from Anderson County in eastern Texas.

We examined the field-collected *I. scapularis*, preserved in alcohol, to determine species, sex,

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and stage of development. Midgut tissues were removed and smeared on two glass slides. One tissue smear was screened for rickettsiae by the Gimenez staining technique. The smear was further analyzed by using a fluorescein isothiocyanate–labeled rabbit antibody against *R. rickettsii* that cross-reacts with many other SFG rickettsiae (10).

DNA from the tick was extracted with phenol:chloroform (1:1) by the standard method (11) after proteinase K and sodium dodecyl sulfate digestion of homogenized tick tissue. The DNA was resuspended in 50 µl of sterile water.

A volume of 10 µl of the polymerase chain reaction (PCR) product was separated by electrophoresis on 6% polyacrylamide gels. After electrophoresis, gels were stained with ethidium bromide, and amplicons were visualized with UV transillumination. Each PCR product was sequenced after DNA purification by a QIAquick Spin PCR Purification Kit (Qiagen, Santa Clarita, CA). Perkin Elmer Ready Reactions (Foster City, CA) and AmpliTaq FS with a Perkin Elmer ABI Prism 377 Automated DNA Sequencer (Foster City, CA) were used for sequencing.

Nucleotide and deduced amino acid sequences were aligned by using the PILEUP program (12). Phylogenetic analyses were conducted by using the PAUP parsimony algorithm (13), and the NEIGHBOR and FITCH distance matrix programs were implemented in the PHYLIP package (14). Parsimony analysis was implemented by using the heuristic algorithm and both ordered (transition:transversion = 3:1) and unordered characters. Because nucleotide sequences generally differed by less than 20%, distance methods used the Jukes-Cantor formula (15). Sequences were added at random with 10 replications. Bootstrap resampling with 200 replicates was used to place confidence values on groupings within trees.

Rickettsiae were visualized in a Gimenez-stained tick smear. A duplicate slide contained rickettsiae stained by an SFG-specific fluorescein isothiocyanate–labeled antibody (Figure 1).

PCR amplification was performed on the fluorescent antibody-positive tick, and the DNA sequences of the *Rickettsia*-specific 17 kDa gene, *gltA*, and *rompA* were determined. Comparison of the highly conserved 17 kDa gene sequences showed the highest level of nucleotide sequence similarity (94.0% identical) between the *I. scapularis* rickettsia and

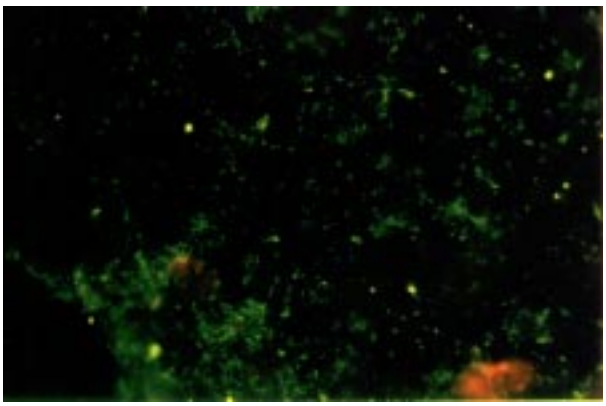

Figure 1. Photomicrograph of crushed midgut tissues of the fluorescent antibody-positive *Ixodes scapularis* specimen. Slides were incubated with fluorescein isothiocyanate–conjugated rabbit antibodies to *Rickettsia rickettsii* and examined by fluorescent microscopy. Magnification 400X.

R. australis (Figure 2A). Comparison of the *I. scapularis* rickettsia *gltA* partial nucleotide sequence showed the highest nucleotide sequence similarity (95.6% identical) with *R. helvetica* (Figure 2B). Comparison of the amplified 532 bp-product of *rompA* over a length of 441 nucleotides with all GenBank's *rompA* sequences showed the highest nucleotide sequence similarity (87.8% identical) with the established species *R. montana* (Figure 2C). A portion of *rompB* was not amplified for the rickettsia in this tick. The expected PCR product of 650 bp was obtained from the positive control, *R. rickettsii* DNA.

All three genes (17 kDa, *gltA*, and *rompA*) analyzed from this rickettsia had distinct sequences. Indeed, comparison of 17 kDa, *gltA*, and *rompA* partial nucleotide sequences showed at least 7.5%, 4.4%, and 12% differences, respectively, with the closest rickettsial species for each of the three genes analyzed. Both parsimony and distance phylogenetic methods indicated that the most closely related rickettsia was different at each of the three genes. However, bootstrap values for grouping the *I. scapularis* rickettsia with other rickettsiae were low, with the exception of its pairing with *R. montana* for the *rompA* gene (bootstrap value 99%, Figure 2C), perhaps because only relatively short partial sequences of the three genes were used for analysis. Otherwise, the three phylogenetic methods generally yielded trees with similar branching patterns; minor differences were

observed within the SFG and in the position of the *R. akari*, *R. australis*, and *R. felis* groups for the *gltA* gene (Figure 2B). Figures 2A and B generally support the monophyletic nature of SFG. The few exceptions were the grouping of *R. australis* and *R. akari* with *R. felis* at the *gltA* gene (Figure 2B). This grouping of *R. australis* and *R. akari* with TG rickettsiae was supported by a bootstrap value of only 47%. In contrast, TG sequences never made up a monophyletic group

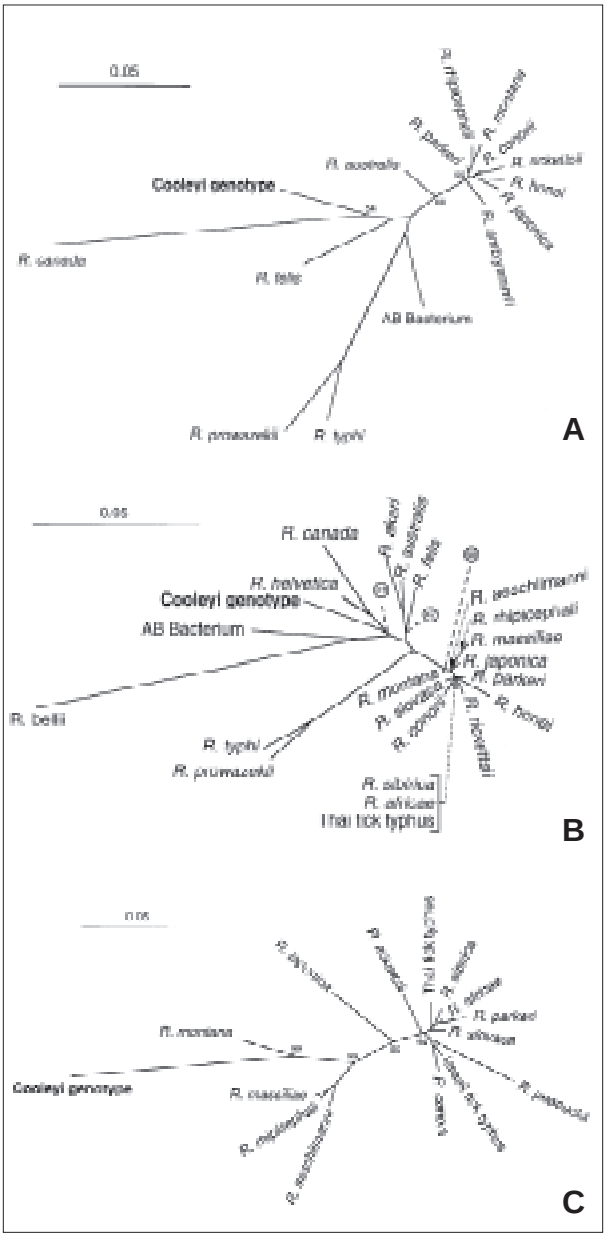

Figure 2. Unrooted phylogenetic trees showing relationship of Cooleyi genotype to other rickettsiae. Scale bar represents genetic distance of 5% by using the Jukes-Cantor formula. Bootstrap values were derived by using the Fitch algorithm. Circled bootstrap values are for nodes indicated by the dashed line. Dotted lines show the actual position of some closely related spotted fever group species. A. 17 kDa. B. *gltA*. C. *rompA*.

A 10-µl aliquot of phenol:chloroform-extracted DNA was used as a template to amplify portions of four rickettsial genes: 17 kDa protein gene, *gltA* (citrate synthase), rickettsial outer membrane protein A (*rompA*), and rickettsial outer membrane protein B (*rompB*). In all amplification reactions, *R. rickettsii* DNA was used as a positive control. A 434 bp-portion of the *Rickettsia* genus-specific 17 kDa protein was amplified (16). A 381 bp-portion of the rickettsial *gltA* gene was amplified from extracted DNA by using primer pairs Rp CS.877p and Rp CS.1258n (17). A 532 bp-portion of the rickettsial *rompA* gene was amplified from extracted tick DNA by using primer pairs Rr190.70n and Rr190.602p (17). Primer pair BG1-21 and BG2-20 (BG-12) was used in a polymerase chain reaction (PCR) for the amplification of a 650 bp-portion of the *rompB* gene (18).

GenBank accession numbers for the portions of the *I. scapularis* rickettsia 17 kDa, *rompA*, and *gltA* gene sequences are AFO31534, AFO31535, and AFO31536, respectively. GenBank accession numbers for the available 17 kDa gene sequences included in phylogenetic analyses are *R. canada*, M82879; *R. felis*, M82878; *R. prowazekii*, M28482; *R. typhi*, M28481; AB Bacterium, U04162; *R. amblyommii*, U11013; *R. japonica*, D16515; *R. honei*, M99391; *R. rickettsii*, M16486; *R. conorii*, M28480; *R. montana*, U11017; *R. rhipicephali*, U11020; *R. parkeri*, U17008; and *R. australis*, M74042. GenBank accession numbers for the available *gltA* sequences included in phylogenetic analyses are AB Bacterium, U20242; *R. bellii*, U59716; *R. typhi*, U20245; *R. prowazekii*, U20244; *R. montana*, U74756; *R. slovaca*, U59725; *R. conorii*, U20243; *R. sibirica*, U59734; *R. africae*, U59733; Thai tick typhus rickettsia, U59726; *R. rickettsii*, U59729; *R. honei*, AFO22817; *R. parkeri*, U59732; *R. japonica*, U59724; *R. massiliae*, U59719; *R. rhipicephali*, U59721; *R. aeschlimanni*, U74757; *R. felis*, U33922; *R. australis*, U59718; *R. akari*, U59717; *R. canada*, U20241; and *R. helvetica*, U59723. GenBank accession numbers for the *rompA* sequences included in phylogenetic analyses are *R. massiliae*, U43793; *R. rhipicephali*, U43803; *R. aeschlimanni*, U43800; *R. conorii*, U43794; Israeli tick typhus rickettsia, U43797; *R. peacockii*, U55821; *R. slovaca*, U43808; *R. parkeri*, U43802; *R. africae*, U43790; *R. sibirica*, U43807; Thai tick typhus rickettsia, U43809; *R. rickettsii*, U55822; *R. japonica*, U43795; and *R. montana*, U43801.

when either *gltA* or 17 kDa sequences were analyzed by any of the phylogenetic methods. However, groupings placing TG sequences with sequences for other organisms such as *R. bellii,* AB Bacterium, or *I. scapularis* rickettsia were only weakly supported by bootstrap analyses.

When *R. helvetica* sequence information becomes available and is analyzed, it will be interesting to view the placement of the *I. scapularis* rickettsia and *R. helvetica* within the 17 kDa and *rompA* phylogeny. The closest rickettsial species at the 17 kDa, *R. australis*, and at the *gltA* gene, *R. helvetica*, are both *Ixodes*-associated rickettsiae. That a segment of the *rompB* was not amplified when the BG-12 primer pair was used is not surprising; the *rompB* segments of *R. akari, R. bellii, R. helvetica, R. massiliae,* and the *I. scapularis* rickettsia identified recently in Minnesota were also not amplified when this primer pair was used (8,18). Indeed, the highest sequence similarity of the *gltA* gene was with *R. helvetica*, which occurs in Swiss *I. ricinus* ticks (19).

Because *I. scapularis* readily feeds upon humans in the northeastern United States and occasionally in Texas, this rickettsia, genetically distinct from *R. rickettsii*, may cause an unrecognized rickettsiosis manifesting as a clinical disease different from the severe illness caused by *R. rickettsii*. In fact, *I. scapularis* has been associated epidemiologically with a fatal illness in Texas considered to be RMSF (5). Elliott and colleagues reported a fatal case of SFG rickettsiosis in a patient who jogged with a friend at a state park. Ticks collected at that site were only *I. scapularis*, some infected with an SFG rickettsia. An *I. scapularis* tick removed from the scalp of the patient's friend during an interview was later found to contain a rickettsia. In addition, rickettsiae of lower virulence may exist in Texas. A serologic study by Taylor and others suggested that 9% to 21% of sixth-grade students in Burleson and Mansfield Counties of Texas had prior immune stimulation by a rickettsial agent (7). None of the 32 children with serologic evidence of prior rickettsial infection had ever been treated for or diagnosed with RMSF. This study implies that these children were infected with a rickettsia of low virulence, or contrarily, that these children experienced asymptomatic immune stimulation with rickettsial antigens.

On the other hand, if not pathogenic for humans, the *I. scapularis* rickettsia could interfere with the transmission of *R. rickettsii* by other ticks to animals by inducing cross-protective immunity or by an interference mechanism such as that of *R. peacockii* (1,2). Noda et al. have proposed that a rickettsia found in laboratory-reared *I. scapularis* from Minnesota is an endosymbiont of the tick because the microorganism appears to be localized only to the ovaries (8). The potential pathogenicity of a rickettsia confined to the ovaries raises the question of whether or not it could escape from this intracellular niche to become infectious. Also, naturally occurring dual infections of rickettsiae in a tick have never been established. If this rickettsia is restricted to the ovaries, this tick may be precluded from serving as a vector of rickettsiosis under natural conditions. However, because the black-legged tick is a three-host tick, it may potentially serve as a vector of pathogenic rickettsiae to a wide range of susceptible hosts, including mammals, birds, and reptiles, from which other tick vectors could become infected after a rickettsemic blood meal. SFG rickettsiae have been detected in 10.2% to 16.7% of *I. scapularis* ticks in Texas (5,7), 5.6% in Connecticut (4), and 11.1% to 66.7% in South Carolina (3). While the numbers of ticks in these collections have been low, the prevalence of SFG rickettsiae in these ticks is among the highest recorded. Because rickettsiae have been regularly detected in unengorged black-legged ticks from different geographic locations (Texas, South Carolina, and Connecticut), we believe that *I. scapularis* is infected with SFG rickettsiae and that infection in these ticks likely represents more than ingestion in blood meals from rickettsemic animals.

Because for each of the three genes analyzed phylogenetically a robust relationship with other known *Rickettsia* species does not appear to exist, we believe that this rickettsia is genetically unique, and therefore, we provisionally designate this agent genotype Cooleyi after Dr. Robert A. Cooley, who served the Montana State Board of Entomology from 1899 to 1944. Isolation of this new rickettsial agent, characterization of its pathogenicity as the potential etiologic agent of an emerging infectious disease, and investigation of its role in its ecologic niche are subjects of interest for future study.

Acknowledgments

The authors thank Josie Ramirez and Kay Kantowski for their expert secretarial assistance. This research was supported by grants from the National Institutes of Health (AI 21242 and AI 31431-05A1).

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

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Well-documented cases of simultaneous human infection with more than one tick-borne pathogen are rare. To our knowledge only two dual infections have been reported: simultaneous human infection with the agent of human granulocytic ehrlichiosis and *Borrelia burgdorferi* and simultaneous human infection with *B. burgdorferi* and *Babesia microti* (1-2). Rocky Mountain spotted fever has long been known to be endemic in North Carolina; cases of human ehrlichial infection were recognized there soon after *Ehrlichia chaffeensis* was recognized as an important cause of tick-borne disease in the southeastern United States. Because both Rocky Mountain spotted fever and ehrlichiosis are prevalent in North Carolina, occasional cases of simultaneous human infection by rickettsial and ehrlichial agents would not be surprising; however, no such cases seem to have been reported.

Case Report

A 44-year-old man with a history of hepatitis C infection and regular use of cocaine was asymptomatic until June 6, 1995, when he noted the acute onset of myalgia, headache, arthralgia, fever (40°C), nonproductive cough, nausea, and vomiting. The following day he sought medical care and received oral trimethoprim/sulfamethoxazole for a presumed respiratory tract infection. Over the next 3 days, his symptoms worsened; on the fourth day he noticed a maculopapular rash and came to our medical center. Initial examination showed tachycardia (pulse 125 beats per minute), fever (38.6°C), and a macular red rash localized to his right arm. The patient reported a tick bite 1 day before his symptoms began. In addition, he reported removing an engorged tick from his pet dog and crushing it between his fingers 4 days before he became ill. Laboratory studies showed leukopenia (white blood cell count 2,700/mm^3), thrombocytopenia (platelet count 69,000/mm^3), hyponatremia (serum sodium 130 meq/dL), and mildly elevated serum concentrations of hepatic enzymes (serum aspartate aminotransferase level of 160 IU/L [normal range 10 to 60 IU]). A screening test for HIV infection was negative. Therapy with intravenous doxycycline was begun (100 mg twice daily). A skin biopsy showed a perivascular lymphocytic infiltrate in the superficial and deep dermis. Direct immunofluorescent antibody staining of the fresh frozen skin biopsy with antiserum against *Rickettsia rickettsii* demonstrated organisms typical of spotted fever group rickettsiae within endothelial cells. Sections were also stained with antiserum against human albumen as a negative control. Parallel staining of a section of positive control tissue was also performed (3). Three days after admission, the patient was afebrile and asymptomatic. He was discharged and instructed to continue therapy with oral doxycycline for an

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additional 7 days. At follow-up 12 weeks later, he felt well. His white blood count was 7,600/mm^3, and his platelet count was 166,000/mm^3.

Serologic Tests

Serum samples obtained 5, 24, and 55 days after onset of illness were tested for antibodies to *Ehrlichia chaffeensis* and *R. rickettsii* antigens by immunofluorescence assay (IFA) except that *E. chaffeensis* (Arkansas strain) was used instead of *E. canis* (4,5).

A fourfold IFA antibody rise (1:32 to 1:128) occurred against *E. chaffeensis* antigen, but antibodies against *R. rickettsii* antigen were not detected in either the acute- or first convalescent-phase serum sample. However, a serum sample collected 55 days after onset contained an antibody titer of 1:40 to *R. rickettsii* antigen. Significant in vitro T-cell proliferation was seen in response to the antigens of both *E. chaffeensis* and *R. rickettsii* (Table 1).

Cell Culture Isolation and In Vitro Lymphocyte Stimulation

A sample of blood taken before the patient was treated with doxycycline was centrifuged in two steps to remove red blood cells and to pellet buffy coat cells. A portion of cellular material was then resuspended in plasma and injected into Vero cells and 030 cells (North Carolina State University canine monocytoid cell line). Vero cell cultures were then maintained with medium 199 and 030 cells with RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, and 0.075% sodium bicarbonate. Cultures were examined weekly for rickettsia-like organisms (5) or morulaelike structures (6) by light microscopy. Cell suspensions were stained by Gimenez, Wright, and direct immunofluorescent antibody (DFA) for *R. rickettsii* (rabbit anti-*R. rickettsii*, Centers for Disease Control and Prevention [CDC], Atlanta, GA) and *E. chaffeensis* (rabbit anti-ehrlichia fluorescein isothiocyanate-labeled conjugates, J.E. Dawson, CDC, Atlanta, GA) (6,7).

Table 1. Patient's T-lymphocyte proliferation when stimulated with antigens of *Rickettsia rickettsii* or *Ehrlichia chaffeensis*

Mitogenic stimulus	Quantity of antigen (µg)	Stimulation index
R. rickettsii	2.0	17.9
	1.0	26.7
	0.2	14.8
E. chaffeensis	4.0	65.3
	2.0	19.1
	0.4	48.7
Phytohemagglutinin	5.0	271.2
	1.0	8.2

Approximately 12 weeks after onset of illness, venous blood was obtained and placed in tubes with EDTA anticoagulant. The specimen was shipped overnight on wet ice and then processed as follows. An equal volume of medium was added to a 10-ml aliquot of the blood sample, loaded onto 6 ml of Ficoll-paque (Pharmacia, Uppsala, Sweden), and centrifuged at 770 x g for 20 min at room temperature. The upper layer of plasma was removed. Then the interface (containing most of the lymphocytes) and the Ficoll-paque (containing most of the monocytes) were harvested and washed once with medium. The pellet was suspended in medium (Dulbecco modified Eagle medium [DMEM]) containing 10% fetal bovine serum, 2 mM glutamine, 5 x 10^{-5} M 2-mercaptoethanol, 10 µM HEPES buffer, and 50 µg/ml of gentamicin. The concentration of cells was adjusted to 1 x 10^6/ml in DMEM. A 0.1-ml portion of these cells was then aliquoted into each well of a 96-well plate. Serial dilutions of inactivated intact rickettsial or ehrlichial antigen (0.2µg to 4 µg/well) were added to individual wells in the same plate in triplicate. The plate was then incubated at 37°C in a 5% CO_2 atmosphere for 6 days. Afterwards, 3H-thymidine was added to each well (1 microcurie/well), and the plates were again incubated at 37°C overnight. On the next day the cells were harvested and dried; ß-fluor cocktail scintillation fluid was then added, and the mixture was counted in a ß-counter. Count per minute rates greater than threefold of the unstimulated control were considered positive.

After 8 days of incubation in Vero cells, a spotted fever group rickettsia was detected by direct immunofluorescent antibody from a sample of blood obtained immediately before doxycycline therapy. However, the rickettsial organism did not thrive with subsequent in vitro passage, and further attempts to recover the organism in vitro or by injecting into guinea pigs were unsuccessful. Subsequent attempts to amplify rickettsial 17kDa protein gene and the 16SrRNA gene by PCR with stored cell culture materials from passages 1 and 2 were negative. In addition, morulae consistent with ehrlichial organisms were detected by light and direct fluorescence in blood taken from the patient before antimicrobial therapy.

Subsequent attempts to isolate ehrlichiae with further passage in 030 cells were unsuccessful.

Polymerase Chain Reaction (PCR) Amplification/Sequencing

DNA was extracted from acute-phase blood with a commercially available kit (IsoQuick; Orca Research Inc., Bothell, WA) for preparation of DNA from whole blood, according to manufacturer's instructions. The dried pellet was then resuspended in DNAase-free water in preparation for PCR analysis.

A pair of primers that recognize sequences of the *nad*A gene of *E. chaffeensis* was used to analyze the patient's sample (Table 2). The PCR products were then separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized under UV light (Figure A).

The amplification conditions for the 120 kDA protein gene were essentially the same as the ones used for the *nad*A gene, except that nested PCR was performed (9-10). The outside primers were pXCF3 and pXAR4, and the nested primers were pXCF3b and pXAR5 (Table 2). The nested primers obtained a product similar to the 1.1 kb product amplified from the Arkansas strain and one repeat unit larger than the Sapulpa strain (Figure B).

Nested PCR amplification of the 120 kDa protein gene of *E. chaffeensis* yielded a PCR product from the patient's blood similar to the repeat region of the *E. chaffeensis* Arkansas strain. The *nad*A gene of *E. chaffeensis* was amplified by a single pair of primers; the sequence of the *nad*A segment was determined to be similar in size to that of *E. chaffeensis* (Figure).

Case Findings

This patient had clinical features of infection with both *R. rickettsii* and *E. chaffeensis*. His rash, although limited and purely macular, was compatible with *R. rickettsii* infection (11). Compelling data support the conclusion that the patient was infected with a spotted fever group rickettsia: 1) IFA staining of a skin specimen showed organisms typical of spotted fever group rickettsia, 2) a spotted fever group rickettsia was initially recovered in tissue culture from a blood sample taken before therapy, and 3) T-cell lymphocytes harvested from a convalescent-phase blood sample contained specific memory cells reactive to *R. rickettsii* from which a T-lymphocyte cell line was subsequently established.

We do not know why a typical vigorous convalescent-antibody response to *R. rickettsii* did not develop in this patient, even though antibody response to *E. chaffeensis* was typical. It is possible, but highly unlikely, that the spotted fever group rickettsia isolated from the patient was a species other than *R. rickettsii*. We cannot state with certainty that the isolate detected in vitro in Vero cells and then lost in subsequent passage was indeed *R. rickettsii* and not a closely related species such as *R. amblyommii* (12). It is also possible that coinfection with *E. chaffeensis* suppressed a normal antibody response to *R. rickettsii* antigens. Alternately, if infection with *E. chaffeensis* was transmitted by a tick bite several days before the second bite by a tick infected with a spotted fever group rickettsia, antimicrobial therapy may have blunted a typical antibody response to rickettsial—but not to previously present ehrlichial—antigens (early antimicrobial therapy can impair or blunt the antibody response in patients with *R. rickettsii* infection [13]). In addition, a previous report described a patient with documented rickettsemia who was treated within 12 hours of onset of symptoms and failed to develop a convalescent-phase antibody response (14).

Had we not performed a skin biopsy or attempted to isolate rickettsiae from the blood, we might have logically (but erroneously) concluded that the illness was due solely to *E. chaffeensis* infection.

Concurrent infection with *E. chaffeensis* is supported by three different lines of evidence: 1) a

Table 2. Sequences of primers used in amplification of the *Nad*A gene and the 120 kDa protein gene of *Ehrlichia chaffeensis*

Gene	Primers	Sequence
*Nad*A gene	ECHNADA1	5' TCATTTCGTGCTTTCTTATTG 3'
	pXCR6	5' TGCCCACATATGCGTTTG 3'
120 kDa protein gene	pXCF3	5' CAGAATTGATTGTGGAGTTGG 3'
	pXAR4	5' ACATAACATTCCACTTTCAAA 3'
120 kDa protein gene nested	pXCF3b	5' CAGCAACAGCAAGAAGATGAC 3'
	pXAR5	5' ATCTTTCTCTACAACAACCGG 3'

greater than fourfold rise in IFA titer against *E. chaffeensis* antigens, 2) in vitro stimulation of T cells harvested from a convalescent-phase blood sample with the antigen of *E. chaffeensis* causing a strong lymphoproliferative response, and 3) PCR products similar in size to the repeat

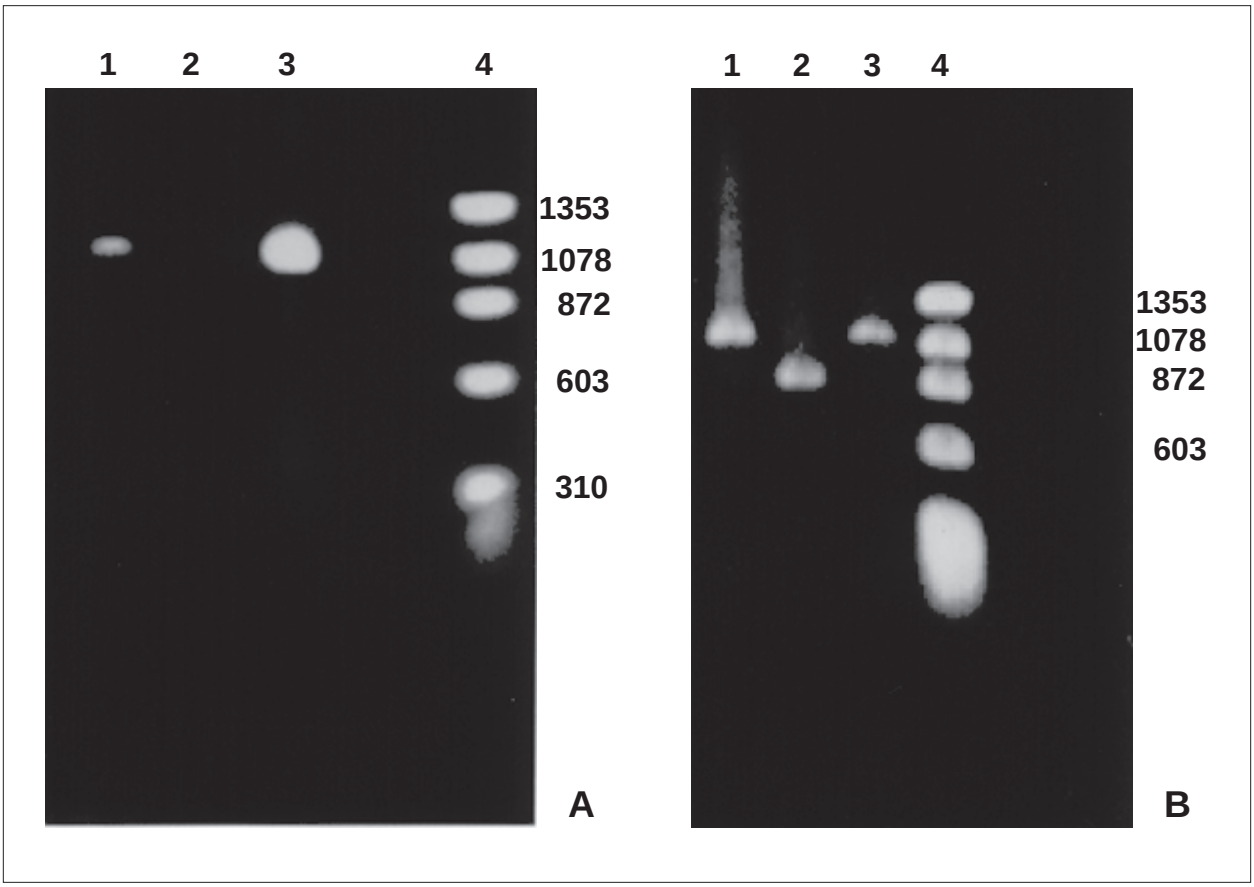

Figure. Polymerase chain reaction (PCR) products. A. With primers for the *Nad*A gene. Lane 1: Patient's sample with an approximately 1.02-kb product; Lane 2: Negative control; Lane 3: Positive control. *Ehrlichia chaffeensis* (Arkansas strain)–infected DH82 cells; Lane 4: Molecular size markers: ϕX174 phage DNA cleaved with HaeIII. B. With nested primers for the 120 kDa protein gene. Lane 1: Patient's sample with an approximately 1.1-kb product; Lane 2: Positive control. *E. chaffeensis* (Sapulpa strain)–DH82 infected cells; Lane 3: Positive control. *E. chaffeensis* (Arkansas strain)–infected DH82 cells; Lane 4: Molecular size markers: ϕX174 phage DNA cleaved with HaeIII.

Amplification conditions. Ten microliters of the patient's DNA was added to a 90 -µl PCR reaction tube containing 10 µl of 10X PCR buffer (Boehringer Mannheim, Indianapolis, IN), 1 µl of PCR primers ECHNADA1 and pXCR6, final concentration 1 µM each; 2 µl deoxynucleotide triphosphates (final concentration, 200 µM); and 1 µl of *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN). Water was added to bring the volume to 100 µl. The mixture was placed in a Progene FPROGO2Y thermocycler (Techne, Princeton, NJ). A DNA lysate prepared from *E. chaffeensis*-infected DH82 cells was used as a positive control. The cycling program consisted of 3 min at 94°C followed by 30 cycles, each of 30 sec at 94°C, 1 min at 55°C, and 2 min at 72°C, and an additional cycle with an extension step of 3 min at 72°C.

To confirm the identity of the PCR product, we sequenced the 590 bp of the *nad*A gene product. After amplification, the PCR products were purified by QIAquick, a PCR purification kit (QIAgen, Santa Clarita, CA). The nucleotide sequence was then determined by the dideoxynucleotide method of cycle sequencing with *Taq* polymerase (ABI Prism 377 DNA sequencer, Perkin-Elmer Corp., Foster City, CA). The sequencing reaction was carried out for each strand of DNA. The sequencing data were analyzed by Genetics Computer Group, Wisconsin Package software; 99.8% identity of the 590-bp overlap with *nad*A nucleotide sequence of Arkansas strain (Gen Bank accession number U90900) was obtained (8).

region of the 120 kDA surface protein gene of *E. chaffeensis* Arkansas strain and specific for the *E. chaffeensis nad*A gene recovered from a sample of blood obtained before therapy. Although we did not sequence amplicon generated from the 120 kDa protein gene, the oligonucleotide primers that were used in PCR amplification are derived from regions of the 120 kDa protein gene that are unique to *E. chaffeensis*. These regions are not present in other bacteria, including closely related *Ehrlichia* species.

We are unable to determine if the patient was infected by two different ticks or one tick simultaneously infected with *E. chaffeensis* and *R. rickettsii* or another spotted fever group rickettsia. Coinfection of *Ixodes scapularis* ticks with the agent of human granulocytic ehrlichiosis or a closely related organism and *B. burgdorferi* has been described (15-17); however, to our knowledge, simultaneous infection of a tick with *R. rickettsii* and *E. chaffeensis* has not. Furthermore, the major vector of *E. chaffeensis* in North Carolina is thought to be *Amblyomma americanum* (18), and the primary vector for *R. rickettsii* in this area is *Dermacentor variabilis*. Although *E. chaffeensis* has been detected on one occasion in *D. variabilis* (19), the evidence that *A. americanum* is a vector for Rocky Mountain spotted fever elsewhere is circumstantial; to our knowledge, *R. rickettsii* has never been isolated from *A. americanum* (W. Burgdorfer, pers. comm.).

Most likely *E. chaffeensis* was transmitted to the patient from *A. americanum*, and the spotted fever rickettsia was transmitted from *D. variabilis*. Indeed, a walk in the fields of the Piedmont region of North Carolina in the spring frequently results in attachment of numerous ticks of both species. Since the patient had direct contact or bites with two different ticks before his illness, we suspect that he was infected by separate ticks rather than by one tick infected with both pathogens.

Finally, this case is remarkable in an additional way. Despite coinfection with two pathogens, the patient had a relatively mild illness. He did not have shock, major organ dysfunction, or central nervous system involvement; and with doxycycline therapy, recovery was prompt and uneventful.

On the basis of this case report, we cannot predict that future cases will have a benign course or an impaired or absent convalescent-phase antibody response to one or more of the infecting agents. However, the patient's illness illustrates that coinfection with a spotted fever group rickettsia and *E. chaffeensis* may occur in areas where both diseases are endemic. The frequency of human coinfection with ehrlichial and rickettsial agents is unknown. Serologic studies have demonstrated that approximately one third of patients with antibodies to *E. chaffeensis* also have antibodies to one or more rickettsiae (20). Whether this phenomenon is due to the presence of cross-reactive antigens or actual immune stimulation by simultaneous or sequential multiple tick-borne pathogens is unknown.

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Molecular Fingerprinting of Multidrug-Resistant *Salmonella enterica* Serotype Typhi

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For epidemiologic investigations, the primary subdivision of *Salmonella* Typhi is vi-phage typing; 106 Vi-phage types are defined. For multidrug-resistant strains the most common types have been M1 (Pakistan) and E1 (India, Pakistan, Bangladesh, and the Arabian Gulf); a strain untypable with the Vi phages has been responsible for a major epidemic in Tajikistan. Most often, isolates from the Indian subcontinent have been resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracyclines, and trimethoprim; but in the 1997 Tajikistan outbreak, the epidemic strain was also resistant to ciprofloxacin. For multidrug-resistant strains, subdivision within phage type can be achieved by plasmid profile typing and pulsed-field gel electrophoresis.

In the United Kingdom, the incidence of multidrug-resistant (MDR) *Salmonella* Typhi isolates has increased from 1.5% in 1989 to 34% in 1995 (1). The most common MDR Vi-phage types have been M1 and E1, isolated from patients who recently returned from Southeast Asia or the Indian subcontinent (1). Although the increasing incidence of MDR isolates of Vi-phage types El and M1 has been particularly noteworthy, outbreaks caused by drug-sensitive strains of these Vi-phage types have continued to be identified. For example, all four patients in a July 1997 outbreak had recently traveled to the Dominican Republic; the causative strain was drug sensitive and belonged to Vi-phage type E1 (2).

Discrimination within phage type is desirable for studying the epidemiology of MDR strains of *S*. Typhi and for determining their phylogenetic relationships with established drug-sensitive types. Insertion sequence *200* profiling can differentiate between drug-sensitive and drug-resistant strains of Vi-phage types M1 and E1 but is unsuitable for subdivision within these phage types (3).

We used plasmid profile typing and pulsed-field gel electrophoresis (PFGE) for the molecular characterization and subdivision of isolates of *S*. Typhi of Vi-phage types E1, M1, and untypable Vi strain (UVS) from outbreaks and sporadic cases. We selected a panel of 18 strains comprising the type strains of *S*. Typhi Vi-phage types E1 and M1, as well as drug-sensitive and drug-resistant isolates of these phage types from patients infected in several countries of the Indian subcontinent. We also included UVS and Vi-phage type E1 isolates from the 1997 epidemic in Tajikistan and an isolate of Vi-phage type E1 from an infection acquired in the Dominican Republic in 1997 (Table). The type strains of Vi-phage types E1 and M1 were isolated in Russia and Canada in 1918 (E1) and 1939 (M1), which demonstrates the subsequent global dissemination of these phage types over a prolonged period.

All drug-sensitive isolates of Vi-phage types E1 and M1 were plasmid-free, whereas MDR isolates were characterized by a plasmid of approximately 120 megadaltons (MDa). The two UVS isolates also had a 120-MDa plasmid and an additional plasmid of 80 MDa. Conjugation and incompatibility testing showed that the 120-MDa plasmids encoded resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracyclines, and trimethoprim and belonged to incompatibility group H_1. In contrast, the 80-MDa plasmids in the two UVS isolates from Tajikistan did not code for drug resistance and were not further characterized by incompatibility

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Table. Drug-resistant and drug-sensitive isolates of *Salmonella* Typhi phage types E1, M1, and UVS

Laboratory accession number	Phage type	Country of origin	Year of isolation	Antibiogram[a]	Mol wt of plasmid DNA	Pulsed-field profile
E1	E1	Russia	1918	$	-[b]	X1
P3047990	E1	India	1993	$	-	X2
P2967750	E1	India	1992	ACSSuTTm	120	X2a
P3639160	E1	Pakistan	1994	ACSSuTTm	120	X2a
P3640980	E1	Pakistan	1994	ACSSuTTm	120	X2a
P4632360	E1	Bangladesh	1997	ACSSuTTm	120	X2a
P4663970	E1	Bangladesh	1997	ACSSuTTm	120	X2a
P4405140	UVS	Tajikistan	1997	ACSSuTTmCp	120 80	X2a
P4405200	UVS	Tajikistan	1997	ACSSuTTmCp	120 80	X2a
P4466680	E1	India	1997	ACSSuTTm	120	X3
P2951290	E1	Bangladesh	1992	ACSSuTTm	120	X4
P3166750	E1	Bangladesh	1993	ACSSuTTm	120	X4
P4219910	E1	Pakistan	1997	ACSSuTTmCp	120	X4
P4405160	E1	Tajikistan	1997	ACSSuTTm	120	X5
P4543210	E1	Dominican Republic	1997	$	-	X6
M1	M1	Canada	1939	$	-	X7
P3112100	M1	Pakistan	1992	$	-	X8
P3044890	M1	Pakistan	1992	ACSSuTTm	120	X8a

[a]A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulphonamides; T, tetracyclines; Tm, trimethoprim; Cp, ciprofloxacin; $, drug-sensitive.
[b]Plasmid DNA not detected.

grouping. In isolates of Vi-phage type E1 and UVS with additional resistance to ciprofloxacin, resistance to this antimicrobial was chromosomally encoded.

PFGE following digestion of genomic DNA with *Xba* I showed 19 to 23 fragments per strain, with 13 fragments common to each and 1 to 12 band differences between patterns. Several differences were identified, both between and within phage types (Figure 1). For Vi-phage type E1, six pulsed-field profiles (PFPs) (*S.* Typhi PFP *Stp* X1 through PFP *Stp* X6) and one subtype (PFP *Stp* X2a) were identified and defined. For PFPs *Stp* X1, X2, X5, and X6, subdivision was based on the numbers of fragments (from 150 kb to >500 kb) and for PFPs X3 and X4, subdivision was based on the number and size of fragments in the 20-kb to 50-kb range, with PFP X3 also lacking a 300-kp band present in PFP X4. The PFPs of the two UVS isolates from Tajikistan were indistinguishable from PFP X2a. For Vi-phage type M1, two PFP types (PFPs X7 to X8) and one subtype (PFP X8a) were identified. PFPs X7 and X8 were designated as separate types on the basis of the numbers of fragments in the 250-kb to 500-kb range. PFP X2a could be distinguished from PFP X2, and PFP X8a could be distinguished from PFP X8 by the presence of three additional fragments of approximately 50 kb, 30 kb, and 25 kb. As these three fragments were observed in isolates of PFPs X2a, X3, X4, X5, and X8a, all of which had incompatibility group H_1 plasmids but were not identified in isolates of PFPs X1, X2, X6, X7, and X8 (none of which possessed H_1 plasmids), it is likely that these three fragments are plasmid-derived. These plasmid-containing derivatives of PFPs X2 and X8 have been designated PFPs X2a and X8a, respectively.

A dendogram (Figure 2**)** analyzed by pairwise fragment comparison using the Dice coefficient and by data clustering using the unweighted pair group arithmetic averaging method (UPGMA) clearly illustrated the relationships described above. The Dice coefficient values, excluding plasmid DNA, were from 66.7% to 97.6%. The PFPs of Vi-phage type E1 and UVS and Vi-phage type M1 clustered as two distinct groups with the branch-point for the E1 and UVS PFPs X1-X6 cluster at approximately 80.5% and the M1 PFPs X7-X8 cluster at approximately 86%. Within Vi-phage type E1, PFPs X1 and X6 (isolates from Russia and the Dominican Republic) are distinct from the closely related PFP X2-X5 cluster (the Indian subcontinent and Tajikastan), which

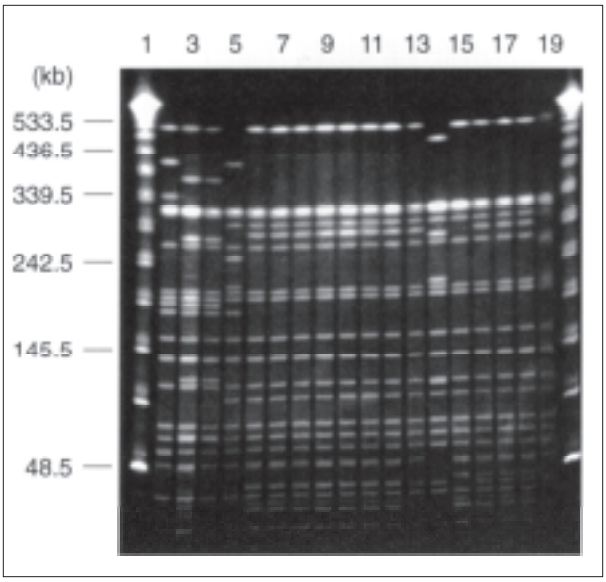

Figure 1. Pulsed-field gel electrophoresis profiles of *Xba* I-digested genomic DNA from drug-resistant and drug-sensitive isolates of *Salmonella* Typhi Vi-phage types E1, M1, and UVS. Tracks 1-20 contained 1 and 20, lambda 48.5-kb ladder (Sigma); 2, M1 type strain (*S*. Typhi PFP *Stp* X7); 3, P3044890 (PFP X8a); 4, P3112100 (PFP X8); 5, E1 type strain (PFP X1); 6, P3640980 (PFP X2a); 7, P3639160 (PFP X2a); 8, P2967750 (PFP X2a); 9, P4405140 (PFP X2a); 10, P4405200 (PFP X2a); 11, P4632360 (PFP X2a); 12, P4663970 (PFP X2a); 13, P3047990 (PFP X2); 14, P4543210 (PFP X6); 15, P4466680 (PFPX3); 16, 3166750 (PFP X4); 17, P2951290 (PFP X4); 18, P4219910 (PFP X4); and 19, P4405160 (PFP X5). PFGE conditions: 180V (5.4 V/cm); 45h; Ramp, 0.5 - 45s.

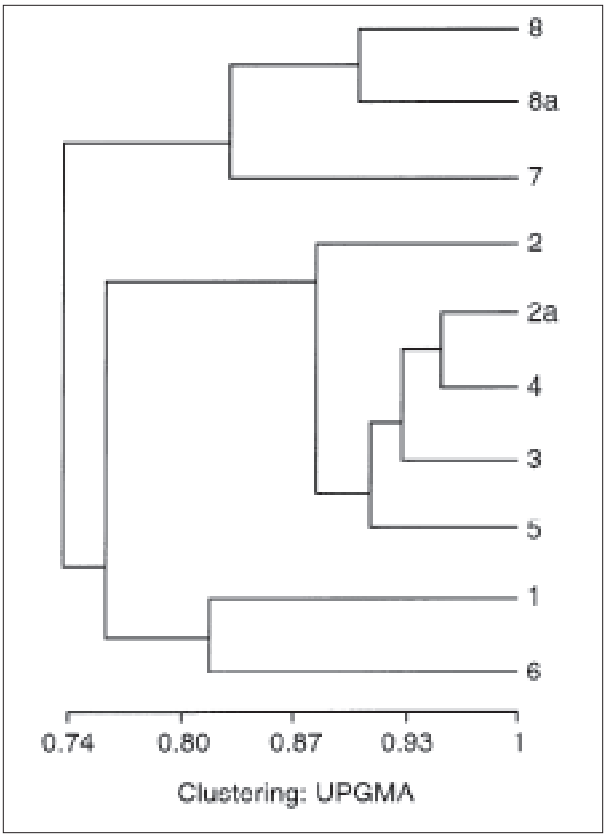

Figure 2. Dendogram of *Salmonella* Typhi pulsed-field profiles *Stp* X1 -X8a. Genetic similarity was calculated by the Dice coefficient (*S*) and clustered by unweighted pair group arithmetic averaging method.

suggests a recent evolutionary divergence in Asia within this universally common phage type.

These results demonstrate that PFGE, coupled with plasmid profile analysis, is a useful method for discriminating MDR isolates of *S*. Typhi Vi-phage type E1. The results also suggest that isolates of Vi-phage type E1 with the X2a pulsed-field profile have been derived within the Indian subcontinent from a drug-sensitive strain of Vi-phage type E1 already endemic in this area. This drug-sensitive progenitor strain is genotypically distinct from the type strain of Vi-phage type E1, isolated in Russia in 1918 and more recently in the Dominican Republic. In turn, isolates of Vi-phage type E1 of PFPs X3-X5 are closely related to PFP X2 and could have been derived from PFP X2 in the course of its recent epidemic history. The two UVS isolates from Tajikistan had a PFP indistinguishable from PFP X2a but differed by plasmid profile and antibiogram and by at least three bands from that of the MDR Vi-phage type E1 isolate from Tajikistan (PFP X5). This suggests that despite the recent observation that MDR strains of Vi-phage type E1 and UVS are in circulation in Tajikistan (4), the epidemic ciprofloxacin-resistant UVS strain is more likely to have originated in the Indian subcontinent than in Tajikistan. Likewise, the drug-sensitive and drug-resistant isolates of Vi-phage type M1 from Pakistan are genotypically distinct from the type strain of Vi-phage type M1 isolated in Canada in 1939. Again, this suggests that MDR strains of Vi-phage type M1 have been derived in Pakistan from a drug-sensitive progenitor strain of Vi-phage type M1 already endemic in that country.

A previous PFGE-based study of *S*. Typhi from Southeast Asia demonstrated that multiple genetic variants associated with sporadic cases and occasional outbreaks are simultaneously

present in that area (5). The study also demonstrated considerable genetic diversity between strains, suggesting considerable movement of these strains within endemic-disease regions. However, the study did not use Vi-phage typing for the preliminary identification and subdivision of isolates. In our study, we combined Vi-phage typing with plasmid analysis and PFGE to provide a method of subdividing MDR isolates of Vi-phage types El and M1 endemic in India, Pakistan, and Bangladesh; we also compared the resulting patterns with those from historic isolates and isolates from recent outbreaks of MDR *S*. Typhi in Tajikistan. Although further work is needed to establish the applicability of combining phage typing with plasmid typing and PFGE for studying the epidemiology of MDR *S*. Typhi, our studies demonstrate that subdivision within the most common phage types can be achieved and may be useful both for investigating outbreaks and for providing information about the evolutionary history of MDR epidemic strains.

Acknowledgments

We thank Drs. D. Murdoch and N. Banatvala of MERLIN for providing the isolates of *S*. Typhi from the 1997 epidemic in Tajikistan.

M.D. Hampton is a biomedical scientist.

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Clostridium septicum Infection and Hemolytic Uremic Syndrome

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Five cases of *Clostridium septicum* infection secondary to *Escherichia coli* O157–induced hemolytic uremic syndrome have been reported. We report on three cases (one of which is included in the above five) of dual *Cl. septicum* and *E. coli* infection; all three patients were exposed to farm animals. A common zoonotic source for *Cl. septicum* and *E. coli* O157 infections should be considered. Patients with hemolytic uremic syndrome should be treated aggressively and monitored closely for *Cl. septicum* superinfection.

Severe infection with *Clostridium septicum* in humans is relatively rare. In recent years, the annual incidence of reported bacteremic infection in England and Wales has been 0.4 to 1.0 cases per million population, although compared with other clostridial bacteremias, *Cl. septicum* infection has been second only to *Cl. perfringens* (Public Health Laboratory Service, Communicable Disease Surveillance Centre, Colindale: pers. comm.).

In 1877, *Cl. septicum*, then known as the "vibrion septique," was the first pathogenic anaerobe cultured by Pasteur and Joubert (1). The organism reportedly caused much of the gas gangrene in wounded soldiers during the First and Second World Wars (2), although the bacteriologic techniques of the time were primitive, and findings varied. In addition to gas gangrene, *Cl. septicum* can cause other severe focal or disseminated infections by spontaneous invasion from the gut of compromised patients (3,4). Strong associations have been established between these spontaneous forms of infection and colonic malignancy (especially in the cecum) (5), acute leukemia or cyclical neutropenia (with the development of neutropenic enterocolitis) (6,7), and diabetes. The organism may be more likely than other clostridia to establish infection in viable tissues by virtue of its aerotolerance and ability to establish infection (at least in animal models) from a small inoculum (4).

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We have diagnosed serious *Cl. septicum* infection (three bacteremic) in four English patients in a period of 13 years. One of these infections developed as a complication of *Escherichia coli* O157-induced hemolytic uremic syndrome (HUS); with four similar case reports (8-11), this condition appears to be emerging as one with a high risk of *Cl. septicum* superinfection. Details of five published reports of HUS-associated invasive *Cl. septicum* infection are shown in the Table. Mucosal damage and thrombocytopenia from, respectively, Shiga toxin-producing *E. coli* enteritis and HUS appear to provide highly suitable conditions for invasion by *Cl. septicum* in infected patients (5). Caya et al. (7) speculate that thrombocytopenia may impair endothelial resistance to clostridial invasion, and there is gathering evidence for the importance of platelets in host defense against infection (12). When antibiotics are not given, as in the management of *E. coli*-associated HUS, clostridia have more opportunity to thrive unhindered. The five patients in this case sequence did not show granulocytopenia, a feature thought to potentiate invasive clostridial infection in those with neutropenic enterocolitis (6) and in leukemic patients (7); in the latter group, the thrombocytopenia that commonly coexists with neutropenia might be relevant in the evolution of infection. Four of the five patients died, and the three examined at autopsy had colonic ulceration and hemorrhagic necrosis. Unusual clinical features included intracranial infection with *Cl. septicum* in four and clostridial cellulitis in the abdominal wall in two.

Table. Hemolytic uremic syndrome–associated *Clostridium septicum* infection

Country, year (ref)	Sex,[a] age (yrs)	*E. coli*[b] in feces	*Cl. septicum* isolated from	Cytopenias[c] granulo-	Cytopenias[c] thrombo-	Clinical features—outcome
USA, 1988 (8)	M, 1	NS[d]	Rash aspirate, PM[e] blood	No	Yes	Abdominal rash, sepsis, convulsions, cerebritis, necrosis colon—died
England, 1993 (9)	M, 4	O157	Blood, PM brain	No	Yes	Convulsions, cerebritis, necrosis colon—died
USA, 1993 (10)	F, 2	*E. coli*	Blood, cerebrospinal fluid	No	Yes	Sepsis, cerebritis, meningitis, necrosis colon—died
USA, 1995 (11)	M, 2	O157	Abscess pus	No	Yes	Brain abscess—recovered
England, 1997 (this study)	M, 2	O157	Blood	No	Yes	Septic shock—died

[a] M, male; F, female.
[b] *Escherichia coli*.
[c] At HUS = at the time that hemolytic uremic syndrome was evident.
[d] NS, not stated.
[e] PM, postmortem.

Both *Cl. septicum* and *E. coli* O157 have herbivore animal reservoirs. We propose as a possible sequence of events for our three cases a) acquisition of the organisms from animals, b) colonization of the colon of the patient, leading to c) invasion by *Cl. septicum* as described above. Two of three cases of serious *Cl. septicum* infection also were linked to sheep; the remaining case, in an elderly man from a suburban setting, who had carcinoma of the cecum and *Cl. septicum* septicemia, resembles the typical case descriptions recorded by others (3,5).

In May 1997, a 2-year-old boy was hospitalized with a 7-day history of vomiting and blood-flecked watery diarrhea; *E. coli* O157 phage-type 1, VT-type 1+2 had been isolated from fecal specimens. On admission, the patient was pale and uncomfortable with a petechial rash on the trunk and an oral temperature of 38.2°C; the abdomen was soft with no tenderness, and blood pressure was normal. Investigations showed hemoglobin 9.5 g/dL with some burr cells and microspherocytes present, elevated total white cell count, and thrombocytopenia. Renal function was impaired, with blood urea elevated; serum creatinine was within the reference range. Blood cultures were taken on admission. After diagnosis of HUS, the patient was given intravenous fluid replacement and was transferred to the local teaching hospital where blood cultures were repeated and antibiotics were initially withheld.

Peritoneal dialysis initially produced clinical improvement, but 32 hours after admission, the patient suddenly became irritable and confused and had a large hematemesis; his blood pressure fell to 100/40 mm Hg, and he exhibited clinical shock. After transfusion with fresh frozen plasma and albumen, cefuroxime, and metronidazole, he was transferred to the intensive care unit, intubated, and ventilated. The pulse rate rose to 163/min and the circulating white blood cell count to 34.0 x 10^9/L. The abdomen became distended, and at the peritoneal dialysis catheter entry site, an area of superficial skin ischemia resembling fasciitis developed, which later progressed over the right side of the abdomen. A plain radiograph showed dilated loops of bowel with thickened walls but no free gas. A diagnosis of ischemic bowel was made, but 8 hours after his sudden deterioration and before an exploratory laparotomy could be performed, the patient went into cardiorespiratory arrest and died. Both sets of blood cultures yielded *Cl. septicum*, the first becoming positive after 48 hours of incubation. An autopsy was not performed.

The boy had played on a dairy and sheep farm, where *E. coli* O157 with identical banding on digested DNA pulsed-field gel electrophoresis analysis to that from the boy was isolated from five cows, but no search was made for *Cl. septicum*.

A 64-year-old hiker in August 1984 slipped against a rock on a sheep trail and cut her leg a

few centimeters above the ankle. Emergency treatment consisted of wound suturing and a tetanus toxoid booster. Three days later, the patient returned to the Emergency Department flushed and sweating with a grossly swollen, discolored, blistered, and foul-smelling leg. The limb was clearly gangrenous to the lower third of the thigh with patchy erythema and edema in the buttock and lower abdominal wall; radiographs showed large quantities of gas in the soft tissues of the leg. Findings included an oral temperature of 38.8°C, blood pressure 100/60 mm Hg, hemoglobin 14.1 g/dL, white blood cell count 5.3 x 10^9/L (including lymphopenia of 0.58 x 10^9/L), and thrombocytopenia (platelets 101 x 10^9/L). *Cl. septicum* was isolated in pure culture from blood as well as from swabs of blisters on the leg, where it was found together with *E. coli* and *Bacillus cereus*.

The patient's leg was amputated mid thigh, and she received gas gangrene antiserum, blood transfusion, and intravenous benzyl penicillin and metronidazole. Postoperative complications included hypotension, atrial fibrillation, and acute renal failure, but she responded to treatment and recovered.

A 71-year-old male, sheep-farm laborer was cleaning out sheep dipping pits in September 1985 when he caught his arm in the slurry machinery, causing a traumatic amputation above his elbow joint with dislocation of the head of the humerus. Emergency hospital treatment included general resuscitation, blood transfusion, and surgical excision of damaged bone and soft tissues together with reduction of the dislocated shoulder.

Despite postoperative treatment with cefuroxime and sodium fusidate, *Cl. septicum*, *E. coli*, and enterococci were repeatedly isolated from the wound; by the sixth postoperative day, a copious and very offensive watery discharge exuded from the wound, with crepitus under the edges. At this time the patient's general condition remained good except for an oral temperature of 38.5°C and circulating white cell count of 16.5 x 10^9/L; platelet counts remained within the normal range. Surgical exploration revealed local necrosis of soft tissues in the wound down to the bone; devitalized tissues were excised, and drainage was promoted. Intravenous benzyl penicillin, ampicillin, and metronidazole led to rapid resolution of infection and a good recovery.

Opinions differ as to whether *Cl. septicum* is part of the normal human fecal flora (5,13). Studies have reported the presence of the organism in fecal specimens of 2% to 3% of healthy persons consuming relatively large quantities of beef (14), but it is uncertain whether the organism was resident or transient. Earlier studies (14) involving very small numbers of healthy persons are unconvincing. Human infection with *Cl. septicum* is assumed to be essentially autogenous, and the possibilities of environmental or foodborne exposure originating from animals have not been adequately discussed. *Cl. septicum* is found in the feces of herbivores, and it is readily cultivated from soil in areas where they graze; the dominant causative clostridium in malignant edema (posttraumatic gangrene) in domestic animals, it causes a form of necrotizing enteritis (braxy) in young sheep grazing on frozen vegetation during the autumn and winter months (15).

The three cases described above suggest an infection of animal origin. In the first case, *E. coli* O157 was almost certainly acquired by the young boy from a farm contaminated by sheep and cattle, suggesting coexposure to clostridia. The hiker's wound was probably contaminated with spores from an area of intensive sheep grazing; Northern England is considered an area of high risk for *Cl. septicum* infection in sheep (16). In the third case, organisms from the slurry or sheep dip were probably injected directly into the wound; sheep dips can become highly contaminated with *Cl. septicum*, causing high incidence of infection (15).

We need to learn more about the role of animals in the epidemiology of colonization and disease with *Cl. septicum* in humans. *E. coli* O157 and associated HUS appear to be increasing in many parts of the industrialized world; severe *Cl. septicum* infection can occur as a secondary disease. The occurrence of at least five such dual infections suggests important elements linking the evolution and pathogenesis of the two infections; furthermore, acquisition of both organisms from a common zoonotic source remains a possibility. A high level of suspicion for *Cl. septicum* superinfection in patients with HUS, rapid diagnosis, prompt antimicrobial therapy, and urgent surgical attention, as required, can improve the survival rate of patients with this life-threatening infection.

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Persistent Infection of Pets within a Household with Three *Bartonella* Species

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We monitored by blood culture and immunofluorescence assay (IFA) bartonella infection in one dog and eight cats in a household to determine the prevalence and persistence of the infection as well as its transmissibility to humans. Ectoparasite control was rigorously exercised. During a 3-year period, *Bartonella clarridgeiae* was recovered from one cat on two occasions, and *B. henselae* was isolated from another cat on four occasions. During a 16-month period, *B. vinsonii* subsp. *berkhoffii* was isolated from the dog on 8 of 10 culture attempts. Despite extensive household contact, the pet owner was seronegative to all three species by IFA for *Bartonella*-specific immunoglobulin G.

Bartonella species are hematotropic bacteria that have been isolated from humans, cats, dogs, wildlife species, rodents, and arthropods. The mammalian reservoir for *B. clarridgeiae* and *B. henselae* is the domestic cat, in which asymptomatic infection can be maintained for long periods (1–3). Feline infection with *B. henselae* is widespread throughout the world, and *B. clarridgeiae* has been isolated from cats in Europe and various regions of the United States (4–6). Chomel et al. have demonstrated flea transmission of *B. henselae* between cats (7). Prolonged bacteremia has been demonstrated in cats, but to our knowledge persistent asymptomatic *B. vinsonii* subsp. *berkhoffii* infection in dogs, as described in this report, has not been previously documented. Human exposure to *B. henselae* can result in cat-scratch disease (CSD) or other medical conditions (8), and *B. clarridgeiae* was recently implicated as the cause of CSD in a veterinarian (6).

B. vinsonii was originally isolated by Baker in 1946; splenic homogenates were cultured from voles that inhabited a former immigrant quarantine station on Grosse Isle, Canada (9). No other isolates of *B. vinsonii* were described until 1995 when we recovered a similar organism from a dog with valvular endocarditis (10). Subsequent genotypic evaluation of the vole and canine isolates resulted in taxonomic division of the species into subspecies. The vole and canine isolates were designated *B. vinsonii* subsp. *vinsonii* and *B. vinsonii* subsp. *berkhoffii*, respectively (11). Although the type strain of *B. vinsonii* subsp. *berkhoffii* was isolated from a dog with cardiac disease, the organism can also cause subclinical infection. The reservoir for *B. vinsonii* subsp. *berkhoffii* has not been established, but seroepidemiologic evidence supports the potential for tick transmission (12).

We describe infection of three pets within the same household with three different *Bartonella* species and the subclinical persistence of *B. vinsonii* subsp. *berkhoffii* infection in a dog for 14 months. This report is a component of other ongoing studies of *Bartonella* infection in animals. In conjunction with an earlier study, a veterinary student offered her cats as controls for an investigation into the prevalence of *B. henselae* associated with cases of CSD (1). Three cats and the dog were subsequently monitored for bacteremia and seroreactivity during a bartonella antimicrobial efficacy study, in which the cats received 22.7 mg enrofloxacin q12h PO for 28 days and the dog received 25 mg doxycycline q12h PO for 28 days (13).

Blood samples were collected from the dog and additional cats acquired later by the student. At each collection, all animals were assessed for general health. For the dog, packed cell volume and total plasma protein were determined from EDTA blood samples.

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Sera were analyzed by immunofluorescence antibody assay (IFA) for reactivity against *B. clarridgeiae*, *B. henselae*, and *B. vinsonii* subsp. *berkhoffii* (1). All positive serum samples (dog, cat, and human) were reassayed at least three separate times.

Blood samples from the dog and cats were placed into 1.5-ml pediatric isolator tubes (Wampole Laboratories, Cranbury, NJ) for *Bartonella* culture on rabbit blood agar (1). Isolates were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the 16S rRNA gene and 16S-23S intergenic spacer (ITS) region using *Dde* I, *Mnl* I, *Hae* III, and *Alu* I restriction endonuclease (6). DNA from the type strains of *B. clarridgeiae* (ATCC 51734), *B. henselae* (ATCC 49882), and *B. vinsonii* subsp. *berkhoffii* (ATCC 51672), in addition to *B. vinsonii* subsp. *berkhoffii* strains cultured from two healthy dogs, was analyzed for comparison.

The dog, cats, and pet owner remained healthy throughout the study. The packed cell volume and total plasma protein values of the dog remained within reference ranges. These values were not routinely assessed in the cats. The source, age, and order of introduction of animals into the household are depicted in Table 1. *Bartonella*-specific immunoglobulin G (IgG) was detected at various titers in the animals (Table 2).

During the 3-year period (December 1993 to January 1997), *B. clarridgeiae* was recovered from one cat on two occasions, and *B. henselae* was isolated from another cat on four occasions. *B. vinsonii* subsp. *berkhoffii* was isolated from

Table 1. Animals included in the study

Animal	Breed[a]	Sex[b]	Birth-date	Date acquired	Source/state
Cats					
A	DSH	M/C	10/91	12/91	Pet store/CA
B	DSH	F/S	10/90	12/90	Stray/DE
C	DLH	F/S	6/92	6/92	Stray/NC
D	DSH	M/C	4/93	5/93	Stray/NC
E	DSH	F/S	5/93	7/93	Stray/NC
F	DLH	M/C	4/94	6/94	Stray/NC
G	DLH	F/S	6/95	10/95	Stray/NC
H	MC	M/C	6/96	9/96	Family/NC
Dog					
I	PM	F/S	4/95	6/95	Family/NC

[a]DSH, Domestic shorthair; DLH, Domestic longhair; MC, Maine coon; PM, Pekingese mix.
[b]M/C, Male/castrated; F/S, Female/spayed.

Table 2. Blood culture and immunofluorescence antibody assay results

Date	ID	Culture results[a]	Colony counts/ml	Serologic results Bh	Bc	Bvb
12/4/93	A	+ (Bh)	97	256	32	512
	D	–		ND[b]	ND	ND
1/15/94	C	+ (Bc)	67	<16	<16	<16
5/25/94	A	+ (Bh)	13	128	16	256
	C	+ (Bc)	113	128	<16	<16
	D	–		256	1024	512
8/19/94	A	cont		128	<16	128
	C	+ (Bc)	240	64	<16	<16
9/18/94	F	nd		<16	<16	<16
12/9/94[c]	A	–		32	<16	128
12/13/94	C	–		32	<16	64
	D	–		128	1024	512
5/6/95[d]	A	–		64	<16	64
	C	+ (Bc)	9	32	<16	32
	F	–		<16	<16	<16
5/27/95	A	–		64	16	64
	C	–		<16	<16	<16
	F	–		<16	<16	<16
6/10/95	A	–		32	<16	64
	C	–		32	<16	<16
	F	–		<16	<16	<16
6/22/95	A	–		32	<16	128
	C	–		<16	<16	32
	F	–		<16	<16	<16
7/30/95	A	–		32	<16	256
	C	–		32	<16	<16
	F	–		<16	<16	<16
11/19/95	A	–		16	<16	256
	B	–		<16	<16	32
	C	–		32	<16	32
	D	–		64	<16	64
	E	–		<16	<16	256
	F	–		<16	<16	32
	G	–		32	<16	128
	I	+ (Bvb)	>1000	128	<16	512
2/25/96	I	+ (Bvb)	369	64	<16	256
3/24/96	I	+ (Bvb)	164	32	<16	256
4/21/96	I	+ (Bvb)	154	16	<16	128
6/22/96	I	+ (Bvb)	357	16	<16	128
8/24/96	I	+ (Bvb)	12	<16	<16	64
10/21/96	I	–		<16	<16	64
12/18/96	I	+ (Bvb)	6	16	<16	64
1/22/97	student		nd	32	16	32
1/14/97	A	–		16	<16	128
	B	–		<16	<16	<16
	C	–		<16	<16	16
	D	–		64	<16	64
	G	–		<16	<16	32
	H	–		64	<16	256
1/30/97[e]	I	+ (Bvb)	1	16	<16	64
3/21/97	I	–		16	<16	32

[a]Bh, *B. henselae*; Bc, *B. clarridgeiae*; Bvb, *B. vinsonii* subsp. *berkhoffii*; cont, contaminated; nd, not done.
[b]ND, no data.
[c]Four weeks before sample was drawn, the cat received amoxicillin clavulanate (62.5 mg q12h, for 10 days) for the treatment of an oral abscess.
[d]After collection of blood samples, these animals received enrofloxacin at 22.7 mg q12h for 28 days.
[e]After collection of blood samples, the dog received 28 days of doxycycline hyclate at 25 mg q12h for 28 days.

the dog on 8 of 10 culture attempts during 16 months of observation (Table 2). Only one species of *Bartonella* was identified from any individual animal. In all three animals, blood cultures performed after completion of antimicrobial therapy were negative.

When compared with type strains, PCR-RFLP analysis of the 16S rRNA gene and the 16S-23S ITS region identified the feline isolates as *B. clarridgeiae* or *B. henselae*. Endonuclease digestion of the 16S gene of the isolates from the dog as well as two isolates from other subclinically bacteremic dogs produced restriction patterns identical to the type strain of *B. vinsonii* subsp. *berkhoffii*; however, digestion of the ITS region resulted in a different restriction profile for the subclinically bacteremic dogs (Figure).

We did not detect bacteremia temporally associated with exposure to known bacteremic animals in any pet newly introduced to the household. Chomel et al. (7) reported that as few as five infected fleas could transmit *B. henselae* infection between cats, but normal daily contact between infected and uninfected cats in ectoparasite-free surroundings was insufficient to produce bacteremia in the uninfected cats. Despite their immunologic status (seronegative) and the occasional presence of fleas, some cats in this household did not seroconvert or become bacteremic after continued exposure to infected resident animals. Although we have observed that infection with *B. henselae* is not cross-protective against *B. clarridgeiae* (Kordick and Breitschwerdt, unpub. data) and that dual infection with *B. clarridgeiae* and *B. henselae* can occur in cats (2,6), similar data are not available with regard to *B. vinsonii* subsp. *berkhoffii*. *B. vinsonii* subsp. *berkhoffii* bacteremia has not been reported in cats. Intradermal injection of *B. henselae* into dogs has induced seroconversion but not bacteremia (14). It is therefore not surprising that *B. vinsonii* subsp. *berkhoffii* was not recovered from any of the cats in the study or, conversely, that *B. henselae* or *B. clarridgeiae* were not recovered from the dog.

Interpretation of *Bartonella* IFA serologic results for species identification has been compromised because of presumed cross-reactivity. The dog in this study never seroconverted to *B. clarridgeiae* or *B. henselae;* however, the cat infected with *B. henselae* had a consistently elevated level of antibodies reactive against *B. vinsonii* subsp. *berkhoffii* antigen. The issue

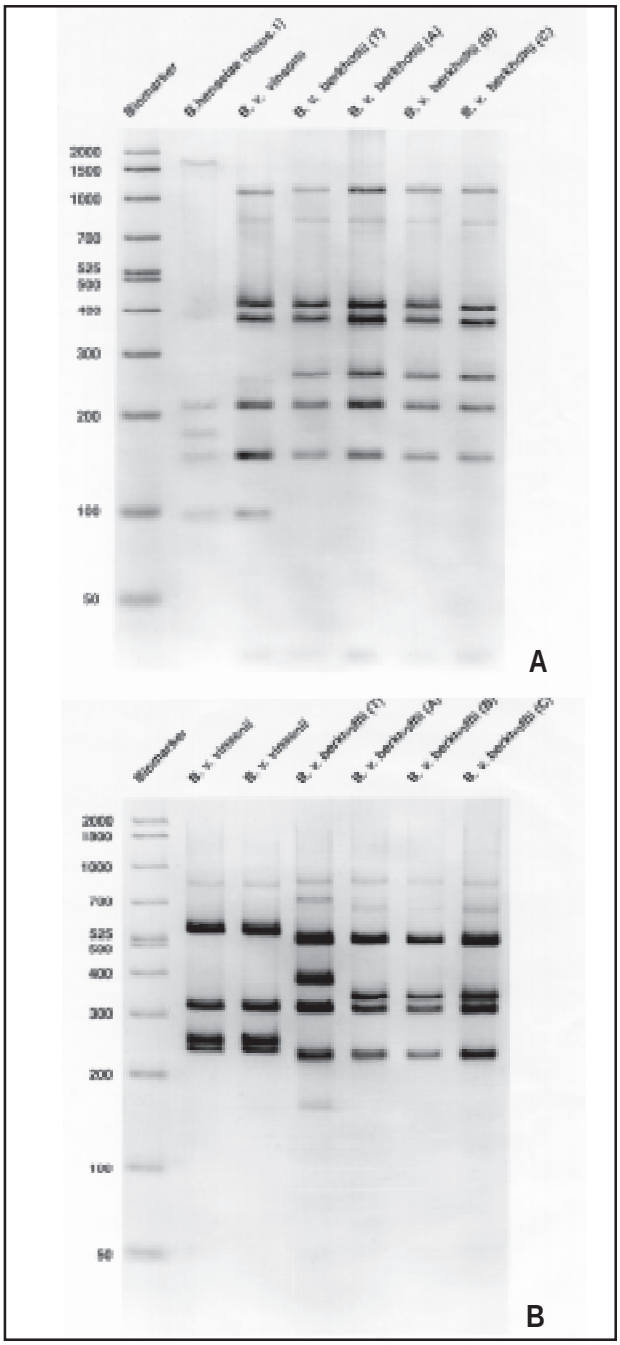

Figure. A. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the 16S rRNA gene of *B. vinsonii* subsp. *berkhoffii* isolates using *Dde* I demonstrating differentiation between subspecies of *B. vinsonii*. B. PCR-RFLP analysis of the 16S-23S intergenic spacer region of *B. vinsonii* subsp. *berkhoffii* isolates using *Hae* III. Strain differences were detected between *B. vinsonii* subsp. *berkhoffii* (T), which was the type strain cultured from a dog with endocarditis, and isolates A (described in this report), B, and C from subclinically infected dogs.

of serologic cross-reactivity among *Bartonella* species deserves additional study.

Subtyping of bacteria after endonuclease digestion of the 16S-23S rRNA ITS region has been performed by other investigators because the spacer region, not appearing to encode functional proteins, demonstrates sequence variability in an operon that has been useful for taxonomic classification (15,16). In this study, different restriction profiles were obtained from isolates derived from asymptomatic dogs compared with the type strain obtained from a dog with endocarditis. Since our sample size was small, further examination of these isolates by pulsed-field gel electrophoresis or bacteriophage analysis may determine whether PCR-RFLP analysis is epidemiologically useful in predicting the pathogenicity of different strains.

The pet owner (student) remained healthy throughout the study; however, she reported that 2½ years before the study she had debilitating fatigue of 1 month duration without fever or lymphadenopathy. At the time, she lived in California and had recently acquired Cat A. A mononucleosis test was negative; her physician suspected a viral infection. Whether the fatigue was related to *Bartonella* exposure (CSD) or the cat was bacteremic is not possible to determine retrospectively; however, the cat was bacteremic with *B. henselae* when cultured 2½ years later as part of another study (1). *Bartonella* transmission from dogs and cats to humans appears to be relatively inefficient. It is not surprising, therefore, that despite continuous exposure to three *Bartonella* species, the student's serum sample was minimally reactive to *Bartonella* antigens.

Acknowledgments

The authors thank Jennifer Goetz for her cooperation in obtaining samples for this study and Craig E. Greene and David A. Bemis for providing their canine isolates of *B. vinsonii* subsp. *berkhoffii* for analysis. Financial support was provided by Pfizer Animal Health (Exton, PA), Bayer AG (Leverkusen, Germany), and the State of North Carolina.

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Using Nurse Hot Line Calls for Disease Surveillance

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Nurse hot line calls are a potential source of public health surveillance data and may help identify epidemics of emerging infectious diseases. In this study, nurse hot line data from Milwaukee, Wisconsin, showed more than a 17-fold increase in calls for diarrhea during the 1993 Milwaukee cryptosporidiosis outbreak. Moreover, consistent patterns of seasonal variation in diarrhea- and vomiting-related calls were detected from the Baltimore, Maryland, and Albuquerque, New Mexico, hot lines. Analysis of nurse hot line calls may provide an inexpensive and timely method for improving disease surveillance.

Most disease outbreaks are identified by an alert health care provider or citizen (1-3); some are identified by analyses of laboratory-confirmed reportable diseases (4,5)—in these outbreaks, because of delays in specimen collection and analysis, laboratory confirmation is often received by public health agencies days to weeks after the onset of illness, and consequently, outbreak investigations are delayed. Some outbreaks may not be detected at all because of limitations of the disease surveillance system (6). Several new surveillance methods for more timely detection of outbreaks have been proposed (6,7). Among these is the analysis of calls to nurse hot lines, also known as nurse call lines.

During the past 5 years, computerized hot line systems for sorting patient calls have been developed and marketed. Patient calls concerning illness or injury are received by trained nurses, who categorize symptoms and provide advice on the basis of approved protocols. The computerized hot line systems integrate structured interview, record keeping, and treatment protocol. These systems have been expanding—one nurse hot line vendor alone has installed more than 240 systems throughout the United States and Canada. These systems have been highly successful not only because they provide patient services, but also because they save physicians' time (8-10).

During the structured interview, nurses follow symptom-based guidelines developed by the software vendors and modified and approved by local physicians. As part of the interview process, information about the illness and the patient is computerized and can usually be retrieved with minimal cost. For example, two hot line systems in Albuquerque, New Mexico, receive more than 50,000 calls each per year; the systems' software can provide summary reports of calls. A major advantage of nurse hot line systems for disease surveillance is, therefore, their ability to provide timely information from a large number of callers. Disadvantages are that hot lines provide symptom-based rather than pathogen-based reports and that persons who call several times can be counted as separate callers. For hot lines connected with health maintenance organizations, the data may be expressed in terms of the population, whereas call systems that serve an unknown number of clients cannot.

Our study, which provides preliminary data on the potential value of nurse hot line data for disease surveillance, had two specific goals: to evaluate whether nurse hot line calls for diarrhea-related illness during the 1993 Milwaukee cryptosporidiosis outbreak increased and could have identified the outbreak earlier than other methods and to evaluate whether nurse hot line calls for diarrheal diseases reflected an expected seasonal variation.

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We telephoned directors of three nurse hot lines: Covenant Healthcare System (CHS), Milwaukee, Wisconsin; Lovelace Health Hot Line (LHH), Albuquerque, New Mexico; and Helix Telehealth Center (THC), Baltimore, Maryland; they agreed to provide hot line call data at no cost. CHS and THC used the Ask-A-Nurse system, which defined diarrhea as a sudden increase in number and volume of bowel movements causing rapid evacuation of water and electrolytes. When the caller described multiple symptoms, detailed guidelines were used to choose the major symptom, and the call was documented in only one category. LHH used the Sharp Focus System and defined diarrhea as five or more watery stools every 6 hours. Records of daily CHS adult and pediatric (age < 13 years) diarrhea-related calls for two Milwaukee hospitals were examined for the period March 4 to May 10, 1993, to determine whether the number of calls increased during the cryptosporidiosis outbreak (11). Hot line calls to LHH and THC from July 1994 to July 1996 for pediatric (ages < 13 years) diarrhea and adult (ages > 18 years) vomiting were plotted for each month. Temporal patterns of calls for respiratory conditions and skin rashes were also examined.

From March 1 to April 1, 1993, and from April 25 to May 10, 1993, the CHS hot line in Milwaukee received an average of 0.66 adult diarrhea calls per day. An increase in diarrhea calls more than four standard deviations higher than the background rate ($p < 0.001$) was noted on April 2 (Figure 1), 4 days before a television news story about dramatic increases in cases of diarrhea (7). The number of calls remained high through April 16, with a peak in adult calls on April 10, 3 days after the Milwaukee Health Department reported the outbreak. Adult diarrhea calls peaked at more than 17 times the average number of calls received per day before and after the outbreak and returned to this number on April 25. Pediatric diarrhea-related calls increased from 18 in March to 38 in April and declined to 17 in May and 11 to 14 per month from June through August (not shown).

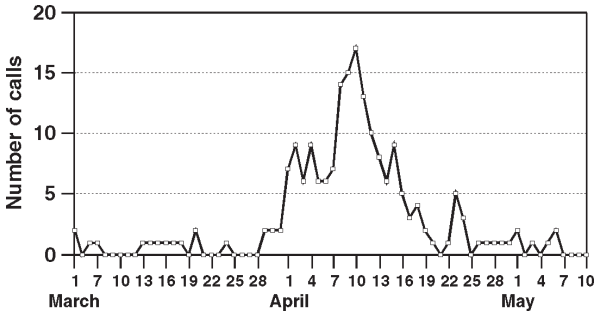

Figure 1. Calls to Covenant Healthcare System hot line, Milwaukee, Wisconsin, March 1–May 10, 1993.

Analysis of the calls by zip code indicated that most callers lived near the two hospitals (located in the north and northwest parts of the city), which were least affected by the outbreak. These data may not represent illness rates in different parts of the city.

To determine whether hot line calls reflect seasonal disease patterns, adult vomiting- and pediatric diarrhea-related calls to the THC and LHH hot lines were plotted for 24 consecutive months (Figure 2). Each condition showed a strong seasonal pattern in both health-care systems. Adult vomiting-related calls to LHH (Figure 2) and to THC (Figure 2) showed increases during November and December. Pediatric diarrhea calls to LHH showed a major increase from October to December each year (Figure 2), whereas calls to THC peaked from February to May (Figure 2). Respiratory conditions peaked in the winter, and skin rashes peaked in the summer (not shown).

The diarrheal disease outbreak in Milwaukee (first noticed by a pharmacist on April 1, 1993 [7], and reported by the Milwaukee Health Department on April 7 [11]), was the largest reported waterborne outbreak in U.S. history, affecting almost half the city's population. Nurse hot line data showed an increase in diarrhea-related calls on April 2, a day after the pharmacist noted an increase in antidiarrhea drug sales and 5 days before the health department reported the outbreak. Media exposure on April 4 did not affect the initial increase detected by hot line data but may have affected the peak on April 10. The hot lines detected an increase in diarrhea even though almost all the callers lived in neighborhoods least affected by the outbreak. Since hot lines serve the areas in which they are located, no citywide analysis of disease patterns was possible.

The collection of hot line data was passive, but because reports came regularly, no data were missed. Unlike systems monitoring school absenteeism or nursing home illnesses, nurse hot lines provide symptom-based illness reports. Although they do not identify specific pathogens, hot line calls can provide a sentinel of increased illnesses for various conditions, including diarrhea,

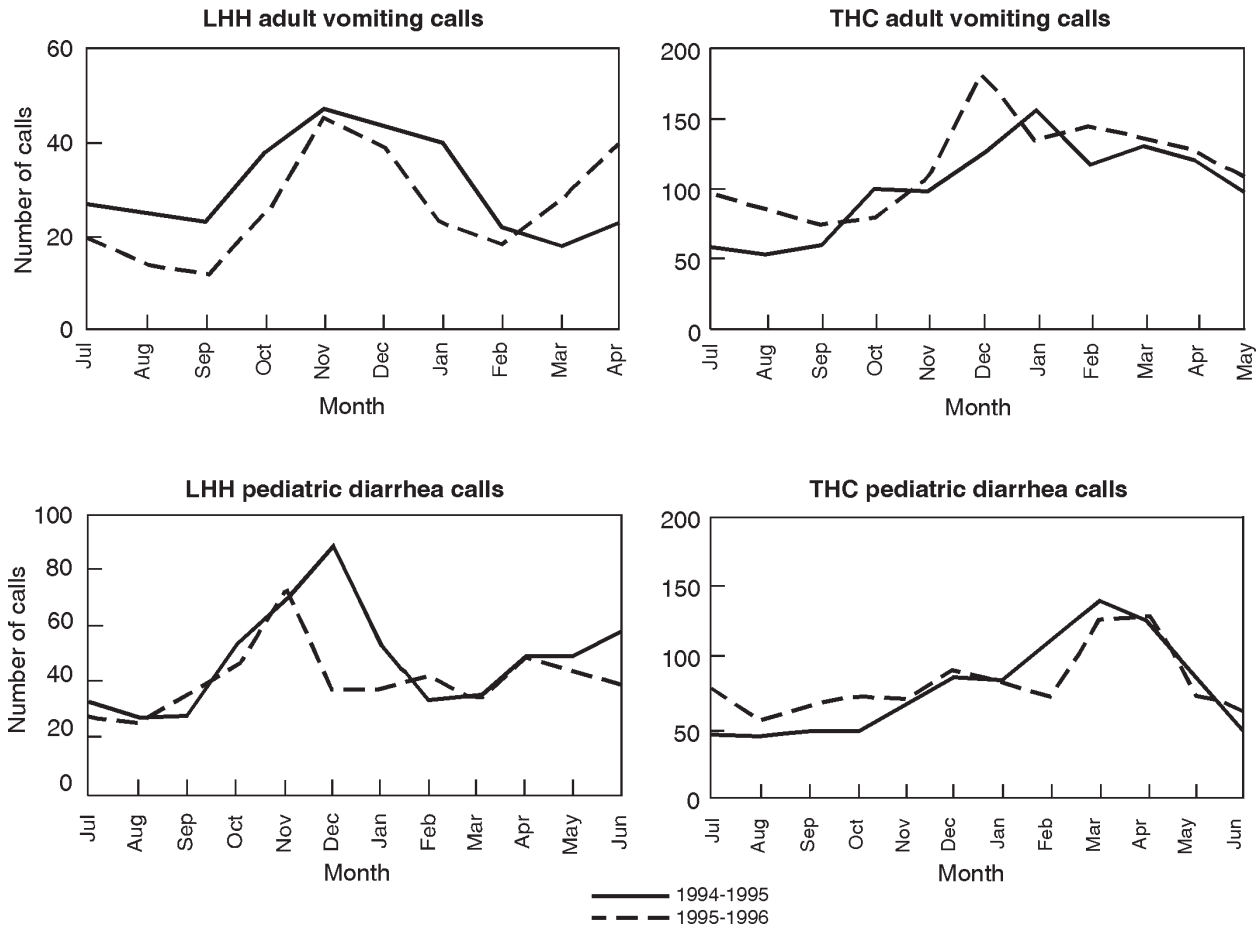

Figure 2. Seasonal variation of calls to Lovelace Health Hot Line (LHH) and Helix Telehealth (THC), 1994–1996.

vomiting, and respiratory illness. Information on symptoms can be obtained rapidly.

The dramatic increase in the number of hot line calls during the Milwaukee cryptosporidiosis outbreak indicates that call volume may prospectively identify an outbreak. If hot lines serve a large area, they could also provide geographic information on the populations affected. As use of hot line data becomes more established, better estimates of expected daily and monthly variation in call volume should emerge, making detection of excess illness easier. Analysis of another outbreak may confirm the results of our study. However, a survey of state public health epidemiology programs found that no state uses or plans to use nurse hot line information for disease surveillance (12). With the decline in resources for state disease surveillance programs (13), nurse hot lines can inexpensively enhance existing surveillance programs.

Acknowledgments

The research described in this article has been supported by the U.S. Environmental Protection Agency through assistance agreement CR 824227 to The Lovelace Institutes.

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Vector-borne Disease Surveillance and Natural Disasters

Natural disasters, such as hurricanes and floods, are frequently followed by a proliferation of mosquitoes and requests from residents and government agencies for widespread application of insecticides. However, nuisance mosquito species, which often require emergency control measures, rarely present a threat to public health (1). Natural disasters in the continental United States have rarely been accompanied by epidemics of mosquito-transmitted disease (2). Nevertheless, public health disaster response policies should include a provision for monitoring increases in the prevalence of potentially infectious mosquitoes and the risk for arboviral disease in the affected areas.

Four major arboviruses (viruses transmitted by mosquitoes or other arthropods) are of human and veterinary public health importance in the United States: eastern equine encephalomyelitis (EEE), western equine encephalomyelitis (WEE), St. Louis encephalitis (SLE), and LaCrosse (LAC) encephalitis. Because of its unique ecology, LAC virus does not occur in an epidemic form and would not increase as a result of floods or hurricanes. The three viruses of primary concern (EEE, SLE, WEE) overlap in their distribution, but each has a distinct ecology, involving different mosquito species and avian amplifier hosts (3)[1]. Despite these differences, populations of primary or secondary vector species of each virus may increase significantly in response to heavy rainfall or flooding. Therefore, under certain circumstances, disasters might produce increases in disease risk.

Several major floods and hurricanes have been evaluated since 1975 (Table). The major geographic and ecologic regions of the United States, some of which contain each of the common epidemic arboviruses (EEE, SLE, WEE), are included. With the exception of the Red River flood of 1975, disasters have not increased transmission of arboviruses to humans or domestic animals. The single case of EEE associated with Hurricane Fran in 1996 may have been acquired before the hurricane. Similarly, cases of EEE and WEE in domestic animals, especially horses and emus, do not appear to increase after floods or hurricanes (SLE does not cause disease in most domestic animals).

Despite the fact that epidemics of arboviral encephalitis have rarely followed hurricane- or flood-related disasters in the United States, these events can increase the risk for human arboviral disease in certain circumstances. In nine of the ten events in which surveillance has been conducted (Table), arbovirus activity was detected in surveillance programs initiated after the event, which indicates the existence of an enzootic transmission cycle in the area. Under these circumstances, increases in vector mosquito population density can enhance transmission and increase the prevalence of potentially infectious mosquitoes, which in turn increases the risk for human disease, particularly when coupled with expanded human exposure to mosquitoes after a disaster (e.g., residents and recovery workers removing debris, restoring housing, and living in substandard conditions).

Public health policies dealing with flood-related disasters must include provisions addressing mosquito-transmitted disease. Preferably, data from an existing arbovirus surveillance program can be evaluated to determine the status of transmission cycles before the event and determine the risk for disease. For example, California's long-term surveillance has established a baseline to which post-disaster virus activity can be compared (4-7). After the 1994-95 winter flood in California, Moore (unpub. data) used those data to determine that WEE seroconversion rates in sentinel chickens did not differ from rates in previous years. However, in most hurricane- and flood-prone areas, routine surveillance is not conducted.

In the absence of an existing program, arbovirus surveillance should be initiated as part of the disaster response plan. At the very least, these programs should determine population indexes of key vector species, virus infection rates in those species, and seroprevalence in sentinel or wild animals. Additional useful information

[1]Additional information may be obtained at http://www.cdc.gov/ncidod/dvbid/arbor/arboinfo.htm

Table. Natural disasters in the continental United States since 1975[a]

Year	State/region	Event	Surveillance done?	Activity detected?	Human cases	Veterinary cases
1975	N.D., Minn.	Red River flood	Yes	WEE[b] in mosquitoes	55 WEE, 12 SLE[c]	281 WEE (estimated)
1989	S.E. United States	Hurricane Hugo	Yes	EEE[d] in mosquitoes	None	(No data)
1992	Fla., La.	Hurricane Andrew	Yes	None	None	None
1993	Ariz.	Gila River flood	Yes	SLE, WEE in mosquitoes	None	None
1993	Midwestern United States	Mississippi, Missouri river flooding	Yes (7 states)	WEE - S.D., SLE - Il.	None	None
1994	Ala., Fla., Ga.	Tropical storm Alberto	Yes	EEE-Al, Fl	None	EEE in horses, emus - Al, Fl
1995	Calif.	Winter & spring floods	Yes	WEE, SLE in sentinel flocks	None	WEE
1996	Calif.	Winter flood	Yes	WEE, SLE in chickens, WEE in mosquitoes	None	None
1996	Oregon, Wash.	Winter flood	No	No	None	None
1996	N.C.	Hurricane Fran	Yes	EEE in mosquitoes	1 EEE	EEE in horses
1997	Colo.	Summer floods	Yes	WEE in chickens	None	None
1997	N.D., Minn.	Red River flood	Sporadic	None reported	None	None

[a]Surveillance data collected by the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention (CDC). State and local health departments assisted during emergency response. Federal Emergency Management Agency and Emergency Response Coordination Group, National Centers for Environmental Health, CDC, provided field support.
[b] Western equine encephalitis
[c] St. Louis encephalitis
[d] Eastern equine encephalitis

includes prevailing weather conditions, time of year, human exposure, human population at risk, historical patterns of arboviral disease, and the potential for imported diseases (3).

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Similarity of Chemokines Charge and the V3 Domain of HIV-1 env Protein

To the Editor: Most clinical HIV-1 isolates can infect CD4+ peripheral blood T lymphocytes, monocytes, and cultured macrophages (macrophage or M-tropic) but not transformed T-cell lines. In contrast, HIV-1 strains adapted for growth in transformed T-cell lines (T-cell line or T-tropic) do not infect primary monocytes or macrophages. This difference in tropism appears to be a consequence of specific amino acid changes in the env protein. Changes in env responsible for an M- to T-tropism shift often involve the acquisition of multiple positively charged residues in the hypervariable V3 loop domain (1). However, some non-V3 determinants are also important for viral tropism. Although both types of viruses use CD4 as receptor, the CXCR4 chemokine receptor (previously designated LESTR/fusin) is the unique cofactor for entry of T-tropic HIV-1 strains (2). The CCR5 chemokine receptor was subsequently demonstrated to be the cofactor for M-tropic HIV-1 isolates (3). Although some direct evidence for cell surface association of the CD4-env complex and the CXCR4 coreceptor was obtained (4), little detail is available on the molecular forces responsible for these protein-protein interactions. In particular, there is no direct evidence to indicate that the V3 loop binds to the chemokine receptor.

Jiang (5) reported that the extracellular domain of the CXCR4 coreceptor for T-tropic HIV-1 isolates is more negatively charged than the CCR5 coreceptor. Because T-tropic isolates have evolved a positively charged V3 domain, it was suggested that coreceptor-env binding involves the interaction between oppositely charged residues. We now expand this analysis by showing that the chemokines corresponding to the different receptors have a similarly unbalanced composition of charged amino acids. So far, the CXCR4 receptor has been demonstrated to bind only the SDF-1 chemokine (6). The CCR5 receptor binds more than one chemokine, including RANTES, MIP-1α, and MIP-1ß (7). The amino acid sequence of these four chemokines is presented in the Figure. We listed the number of positive residues (arginine [R] and lysine [K]) and negative residues (aspartic acid [D] and glutamic acid [E]), and calculated the net charge. SDF-1 appears to have the highest number of positive residues and the lowest number of negative residues, resulting in a net charge of +11. All other chemokines have much less positively charged amino acids, resulting in a net charge for MIP-1α and MIP-1ß of -2 and -1, respectively. The RANTES chemokine has an intermediate charge of +7, which may correlate with the unique receptor use of this chemokine (e.g., RANTES, but not MIP-1α and MIP-1ß, binds the CCR3 receptor [7]). These results are consistent with the idea that positive charges in SDF-1 interact with negative charges in the CXCR4 receptor, and this binding may thus resemble the HIV-1 env-CXCR4 interaction.

Early evidence that both the chemokines and HIV-1 bind to the same domain of the chemokine receptor comes from virus inhibition studies. Several ß-chemokines suppress infection with M-tropic but not T-tropic HIV isolates (8), and SDF-1 specifically blocks entry of T-tropic isolates (6). Although direct blocking of the receptor may explain part of this chemokine-mediated inhibition, it has also been proposed that internalization of the receptor contributes to the antiviral effect (9). Irrespective of the precise antiviral mechanism, the combined results of this analysis

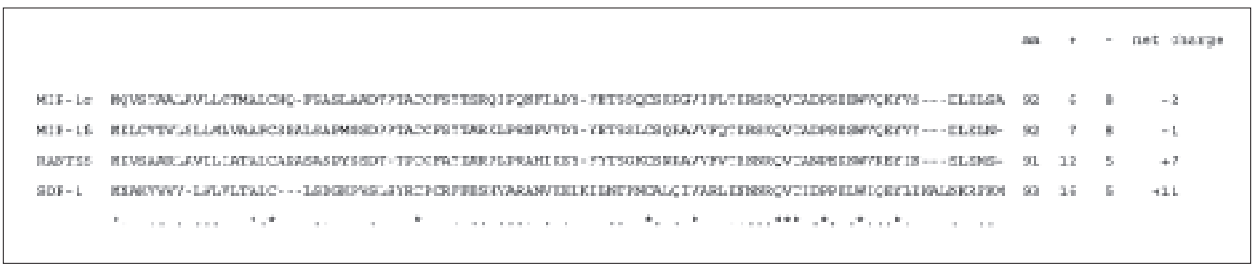

Figure. Amino acid alignment of the four chemokines was performed with the PC/Gene program. An overall identity and similarity of 12.4% and 40.2% was calculated, respectively. The initiator methionine contained within each sequence is removed in the processing of the chemokine. The SDF-1 form shown is the ß-form; the α-form lacks the C-terminal amino acids RFKM, thus reducing the net charge to +9.
* = a perfectly conserved residue.
. = a conservative change.

and the one presented by Jiang (5) suggest that both the SDF-1 chemokine and the V3 loop of T-tropic HIV-1 viruses use positively charged amino acids for an electrostatic interaction with the negatively charged CXCR4 receptor. To examine whether the similarity between the chemokine and env V3 domain is also apparent at the primary sequence level, we performed an amino acid alignment; however, we found no conserved motifs (data not shown). A detailed mutational analysis is required to further our understanding of the env-coreceptor interaction.

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Detection of Glycoprotein of *Burkholderia pseudomallei*

To the Editor: Melioidosis, a potentially fatal disease that is difficult to diagnose and treat, is common in areas with subtropical climate (e.g., Singapore, the southern provinces of China) and is hyperendemic in Thailand. The etiologic agent, *Burkholderia pseudomallei* (*Pseudomonas pseudomallei*), is widely distributed in Southeast Asia and northern Australia. The agent has the potential to become established in regions with similar climate conditions, particularly if animals infected with *B. pseudomallei* are imported from endemic-disease zones (1-3).

Rapid and reliable detection of *B. pseudomallei* and its antigens has many potential applications. Recently, we developed a monoclonal antibody immunoenzyme test system for the detection of minimal concentrations of a *B. pseudomallei* glycoprotein, which is considered one of the pathogenicity factors for this microorganism. This glycoprotein, called Ag8 by N.N. Piven and V.I. Ilyukhin (4), is present in different strains of *B. pseudomallei* and *B. mallei* but not in other *Burkholderia* spp. (*B. aeruginosa, B. putida, B. cepacia, B. malthophilia, B. fluorescens, B. pseudoalcaligenes*). Ag8 is composed of 10% protein and 90% carbohydrate, has molecular mass 800 kDa, and is localized in an extracellular capsulelike substance surrounding *B. pseudomallei* cells (5).

We developed an immunoenzyme test system with three monoclonal antibodies (Mab) to different epitopes of Ag8 (Mab 2A6-IgG3, Mab 2H7-IgG1, Mab 1G2-IgG2b) and one antibody to epitopes common for Ag8 and LPS of *B. pseudomallei* (mab 1ES-IgG2b). A sandwich enzyme-linked immunosorbent assay (ELISA) was used for the detection of Ag8 in different test samples (6). The sensitivity of the immunoenzyme test system was determined with a standard antigen sample. Minimal sensitivity (37 ng/ml of carbohydrate) was observed when polyclonal immunoglobulins were used as “catching” antibodies. Maximal sensitivity (0.37 ng/ml of carbohydrate) was noted when either Mabs 2A6 or mixtures of Mabs were used as catching antibodies.

The test system was further evaluated with samples of extracellular antigens (extracts of cultural media, fractions after gel chromatography of extracellular antigens) and bacterial suspensions of *B. pseudomallei* and *B. mallei* strains isolated in different regions of the world.

Levels of Ag8 in cultural media varied considerably depending on periods of cultivation of bacteria. Additionally, the level of Ag8 varied among strains of *B. pseudomallei* and *B. mallei*. Among 61 strains of *B. pseudomallei* from the museum collection (most of which were isolated in Southeast Asia and northern Australia), three had increased ability to produce Ag8. These strains had been isolated from clinical specimens (blood, abscesses of hospitalized melioidosis patients) in Vietnam. The strains gave results typical of *B. pseudomallei* species in all routine serologic tests (agglutination test, immunofluorescence assay, immunodiffusion test). In contrast, the *B. pseudomallei* glanders agent (16 strains from the museum collection) had reduced ability for Ag8 production; ELISA titers of Ag8 were a thousandfold less in culture fluids in these strains.

The ELISA technique not only facilitates diagnosis of disease but also provides a rational basis for selecting strains for vaccine production. It also has considerable utility for studying the pathogenicity of *B. pseudomallei*.

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Forging New Perspectives on Disease Surveillance

To the Editor: Recognizing disease emergence as a paradigm uniquely influenced by human activity demands a reevaluation of traditional disease surveillance systems. Part of a surveillance program should be focused on the areas of human activity where disease emergence is most likely to occur. A system that monitors areas known to be involved in disease emergence, such as development projects, agriculture, climate, and refugee movements, may greatly increase our ability to detect and prevent outbreaks.

Large development projects entail ecologic upheavals that can facilitate disease emergence. Construction of a dam in 1987 in Mauritania resulted in increased mosquito breeding sites and in an explosion in the mosquito population; epidemics of Rift Valley fever quickly followed (1). The Southeastern Anatolia Irrigation Project on the Euphrates and Tigris Rivers in Turkey, which will provide irrigation for 1.7 million hectares, has already increased malaria and leishmaniasis cases in the local population (2). The massive Three Gorges Dam Project on the Yangtze River in China, which will create a reservoir 760 km long, must be evaluated for its impact on local disease. With knowledge of endemic diseases and their reservoirs and vectors in these areas of ecologic change, public health workers can anticipate disease epidemics and implement prevention measures.

Incorporating climate predictions into a disease surveillance system would supplement resources in an area known to affect disease emergence. The U.S. Agency for International Development's (USAID) Famine Early Warning System monitors the African continent for two major factors implicated in emergence: temperature and precipitation. Focused on countries at high risk for food shortages and famine, the early warning system is an example of a predictive and preventative surveillance system. Precipitation, temperature, and plant health data from satellites are evaluated as indicators of crop failure. These data are supplemented by information from field representatives who directly observe agricultural production. USAID's system and other global monitoring systems can provide a base level of surveillance that can add to our knowledge of climatologic influence on disease emergence.

The beginning of the Zairian refugee crisis in 1994 illustrates the need for surveillance among refugee populations. In July 1994, 500,000 to 800,000 Rwandan Hutus fled into the North Kivu region of Zaire. In the month between July 14 and August 14, 48,347 of these refugees died,

predominantly of infectious diseases (3). Similar infectious disease epidemics occurred among Kurdish refugees in 1991 (4), Somali refugees in 1992 (5), and Burundian refugees in Rwanda in 1993 (6). In the wake of intense political, social, or physical disruption, the movement of large numbers of people creates ideal conditions for disease outbreaks. When moving into new areas, refugees may not be equipped immunologically against endemic diseases. Most refugee camps are overcrowded, with inadequate sanitation or medical care (7). Refugees often have severe shock or stress, which in combination with poor nutrition, weakens immune defenses. Therefore, refugee populations are extremely vulnerable and should be closely monitored for infectious disease outbreaks. The United Nations High Commissioner for Refugees, the International Rescue Committee, and the Human Rights Watch can provide rapid notification about disease emergence in refugee populations.

Recognizing human involvement as a common critical factor in emergence creates the possibility of refining international disease surveillance. The Centers for Disease Control and Prevention, the World Health Organization, and national governments should foster relationships with organizations already placed to provide disease emergence information in populations and locations implicated in disease emergence. These relationships will increase the scope and efficiency of our efforts to prevent human disease.

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Provide a Context for Disease Emergence

To the Editor: When a disease emerges, the trend is to assume that another important and spreading infection is about to devastate humans or animals. Some qualification of the term "emergence" is needed to put emerging diseases into a context for each target species. There may be a cause for alarm and further action or, alternatively, no real change except in knowledge. In Australia, for example, an old disease "emerged" in a new area, while in another, a disease new to the continent emerged. The two disease agents were Ross River virus (which causes fever and polyarthritis in humans) and bluetongue virus (which often causes fatal disease in sheep). Both causative viruses are insect-borne.

Ross River virus is probably of very ancient lineage as an infection transmitted between marsupials and indigenous mosquitoes and was on the Australian continent long before humans first entered (some tens of thousands of years ago). In 1975, the infection was not known to occur in Tasmania, the state separated from the Australian mainland by a wide stretch of sea. In that year, my group detected a clear-cut seroconversion to Ross River virus in sentinel cows in northern Tasmania (1). Cooperative investigations found antibody-positive sera first in marsupials and then in persons who had never left Tasmania. The existing clinical condition of polyarthritis was linked to Ross River virus only after the causative virus was recognized indirectly (2). The marsupial populations of mainland Australia and Tasmania were continuous until the seas rose at the end of the last ice age. Ross River disease had emerged in Tasmania, but only from obscurity.

Bluetongue viruses occur widely in southern and eastern Asia (3). This general picture has been established only since the discovery of bluetongue virus in Australia. Overt disease occurs in sheep on the fringes of the endemic-disease region and in susceptible sheep imported into various countries within the region (3). In

contrast, at least eight members of the bluetongue group of viruses have entered Australia and some of these the Pacific countries; they must have arrived after ruminant populations were introduced in New Guinea, Australia, and the Pacific islands. They spread through the ruminant-dependent *Culicoides* species vectors, also introduced (4). Emergence of bluetongue in Australia has so far meant one sheep dead of bluetongue disease in 1989 (in a population of 100 million sheep) since the discovery of the presence of bluetongue virus in Australia in 1977 (4). However, the potential for a major epidemic remains and the discovery caused major trade difficulties. In both these situations the disease was emergent but its potential was very different.

Since the latter part of 1994, two newly recognized zoonotic viruses have been reported from Australia: paramyxovirus (equine morbillivirus), which caused deaths in horses and humans (5), and lyssavirus (closely related to rabies virus), which has also caused a human death in Australia (6). In both instances, strong evidence indicates that bats are the maintenance hosts. Bats are probably the oldest form of placental mammal in Australia, with fossil evidence from the Middle Miocene era, circa 15 million years ago (7). Some species of bats migrate between various countries of southern Asia and the Pacific; probably they migrated more in the ice ages when sea distances were shorter. This past continuity of the bat populations and the inadequate study of the Microchiroptera in Asia for rabies viruses led me to forecast in 1989 that rabies (or a rabieslike virus) was established in bats in Australia (8). These recently recognized disease agents are both emergent but have not become important for humans or animals.

As a hypothetical example, Ebola virus has been shown by Swanepoel and others to multiply well in bats of three species (9). Two of the species used experimentally belong to the genus *Tadarida*, which is well represented in Africa, Asia, and Australia (10). Evidence that bats are the reservoir hosts of Ebola virus (Reston), which is known to infect monkeys in the Philippines should be sought in the Philippines and for its silent presence in bats in Australia. If such evidence were found, Ebola would be labeled as emerging in Australia and other countries between Australia and the Philippines, although no cases of disease might ever occur east of the line where monkeys are indigenous.

The scientist who looks for evidence of a disease agent in a country or region and the regulator who has to deal with the public health or economic consequences of a new or newly recognized disease have conflicting interests. The national and international reaction to the discovery of many agents in countries where they had not been found does not take into account the measure of risk for the disease. The term "emergent" disease needs some qualifiers to diminish fear and overreaction.

One way would be to rate the risk for the disease on a scale of 1 to 5; another would be to provide a context that notes the capacity of the agent to spread and cause illness and death.

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Resistance to Dryness of *Escherichia coli* O157:H7 Strains from Outbreak in Sakai City, Japan, 1996

To the Editor: A large outbreak of *Escherichia coli* O157:H7 with more than 6,000 cases occurred in Sakai City, Osaka Prefecture, Japan, in July 1996 (1); after the outbreak, more than 1,000 secondary infections occurred in the families of the patients (2). We studied the resistance of *E. coli* O157:H7 to dryness because the survival on surfaces of inert materials under dry conditions may be related to the transmissibility of the strains.

E. coli O157:H7 strains grown on 3.0% nutrient broth with 1.5% agar for 20 to 22 hours at 37°C were suspended at a concentration of approximately 5 x 10^8 cfu/ml in a 10% skim milk, 0.5% NaCl solution. Aliquots (10 µl) of bacterial suspensions were spread to approximately 10 cm^2 on plastic petri plates for bacterial culture and dried under the air flow of a clean bench until no aliquots were evident. After storage in the dark at room temperature, bacteria were harvested with saline and gauze and the viable number was counted (n = 3).

The log reductions 12 hours after drying were employed to show the resistance levels of *E. coli* O157:H7 to dry stress. The log reductions of the three strains from the Sakai City outbreak (RIMD0509950, RIMD0509894, and RIMD0509951) were 0.04 ± 0.34, 0.14 ± 0.06, and 0.20 ± 0.60, respectively (mean = 0.13), whereas those of the *E. coli* O157:H7 strains from the other cases varied from 0.71 ± 0.18 to 4.57 ± 1.02. (RIMD0509861, 0.71 ± 0.18; ATCC35150, 1.15 ± 0.35; RIMD0509826, 1.23 ± 0.60; RIMD0509742, 1.28 ± 0.18; ATCC43890, 1.31 ± 0.17; RIMD0509933, 1.48 ± 0.19; RIMD0509932, 1.48 ± 0.22; RIMD0509765, 3.60 ± 0.24; RIMD0509764, 4.57 ± 1.02; mean = 1.87). Although the strain from the Sakai City outbreak (RIMD0509950) survived for at least 35 days under the dry conditions, the strains from the other cases had no viable cells after 7 days. The log reductions of *E. coli* JM109 and DH10B strains were more than 10, and no viable cells were detected in 12 hours.

The strains from the Sakai City outbreak also showed marked acid resistance. Acid resistance of *E. coli* O157:H7 has been reported to depend on *rpoS*, which is induced in a stationary phase (3). Since the *E. coli* O157:H7 strains in a stationary phase were more resistant to dry and acid stresses than those in a log phase, *rpoS* may also be associated with resistance to dry stress. However, no deletions of *rpoS* were detected by polymerase chain reaction analysis in the *E. coli* O157:H7 strains used in this experiment. Further study on the mechanism of resistance will be needed to establish new strategies for eradicating the bacteria.

A case-control study by the Ministry of Health and Welfare of Japan showed that uncooked radish sprouts were the vehicle of the largest outbreak in Sakai City (4). In two small outbreaks of *E. coli* O157:H7 in March 1997, the vehicle of infection might have been radish sprouts; therefore, the possible contamination of white radish seeds with *E. coli* O157:H7 has been discussed (5). If such contamination was present, dry resistance might be involved in the survival on or in white radish seeds because the bacteria were exposed to dry conditions for a long period before sprouting. We propose that dry resistance be considered an important factor in infection.

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Irradiation Pasteurization of Solid Foods

To the Editor: Osterholm and Potter have made a strong case for irradiation pasteurization of solid foods that enter kitchens as raw agricultural commodities, such as meat, poultry, and seafood (1). Irradiation pasteurization was advocated to protect against foodborne diseases caused by common pathogens such as *Campylobacter*, *Cryptosporidium*, *Escherichia coli*, *Listeria*, *Salmonella*, and *Toxoplasma* (2). An additional rationale for irradiation pasteurization is bacterial resistance to antimicrobial drugs, a major health concern, which will undoubtedly increase in magnitude unless new approaches become available (3). The widespread use of antibiotics in animal husbandry may be the cause of some of this resistance, for example, in vancomycin-resistant enterococci associated with the agricultural use of glycopeptide antibiotics (4,5). Furthermore, resistance to glycopeptide antibitiotics can be transferred from enterococci to other gram-positive organisms, at least in the laboratory (6). Thus, resistant bacterial strains from animal sources may enter the human population through contaminated food without necessarily causing immediate disease but resulting in expanded human reservoirs of antimicrobial resistance through horizontal gene transfer (7). When such bacterial strains are subsequently transmitted to a susceptible person, serious disease could result, which would be exceedingly difficult to treat (8). Irradiation pasteurization of solid foods could reduce the magnitude of transfer of resistance genes through contaminated foods.

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Emerging Infectious Diseases in Brazil

To the Editor: Hooman Momen's update on emerging infectious diseases in Brazil (1) appears to be based solely on notifiable disease data, which cannot adequately describe the current situation. Additional data in several areas may be useful.

Parasitic diseases: Dr. Momen's update restricts itself to protozoal diseases and does not distinguish between mucocutaneous and visceral leishmaniasis. Visceral leishmaniasis is in fact expanding in many suburban and urban areas in the northeast. Mucocutaneous leishmaniasis, after a small retreat following extensive deforestation, has made a comeback; and in many suburban areas in Rio de Janeiro and São Paulo, in the southeast, transmission is occurring, probably because of changes in sandfly ecology (1).

A helminthic disease of interest is mansoni schistosomiasis, which has been expanding its area of transmission, reaching over to Santa Catarina, in the south, to Pará in the north, expanding also westward, to Mato Grosso and Mato Grosso do Sul. The number of cases, as well as the associated illness, has possibly been reduced, but there is no doubt that the disease can be found in a much larger area than 20 years ago. Other emerging helminthiases of interest, albeit not of public health concern, are

onchocerciasis, still restricted to the Yanomami group in Roraima, bordering Venezuela; *Angiostrongylus costaricensis* infection (2), found in the south, Rio Grande do Sul; and some cases of lagochilascariasis, reported from Pará.

Viral diseases: As Dr. Momen pointed out, dengue is by far the most serious emerging viral disease in Brazil, and the area occupied by *Aedes aegypti* is expanding. Dengue hemorrhagic fever has occurred occasionally, but no outbreaks have been recorded. However, measles is no longer a problem; the outbreaks have been controlled.

There is no evidence to support that hepatitis B is declining because of vaccination. Vaccination is still restricted to areas of high prevalence. Other states are beginning vaccination programs in newborns, but it will be some time before these programs have any effect on prevalence. As to hepatitis C, because diagnostic testing is only recently becoming widespread, we are probably experiencing an increase in detection rather than in incidence.

Other notable agents are Mayaro and Oropouche viruses, which are arthropod-borne and among the most common causes of febrile illness in the Amazon region. *Aedes albopictus*, found all over the country, could be a potential vector (3). Apart from HIV, other retroviruses are cause for concern: HTLV-I and HTLV-II screening is recommended for blood banks, and enough data exist to conclude that the infection is widespread in the country but with a low prevalence (0.4% and 0.1%, respectively). Clusters of disease have not been identified, but adult T-cell leukemia/lymphoma is far from a curiosity (4).

Bacterial diseases: Brazilian purpuric fever, caused by *Haemophilus influenzae* biogroup aegyptius, was first reported in outbreaks in the central-south part of the country (western São Paulo, eastern Mato Grosso do Sul, and northwestern Paraná) about 10 years ago, causing a syndrome much like meningococcemia (5). For enteric infections, the limited data available present interesting trends. *Salmonella* Enteritidis is rising and *S.* Typhimurium is declining in São Paulo and the southern states. These trends may reflect improved sanitation and increased use of industrialized foods and contaminated animal feeds (6).

Fungal diseases are not reportable, but many epidemiologic studies have been conducted. Paracoccidioidomycosis (South American blastomycosis) was unheard of in the Amazon region, never being found in native habitants; however, because of environmental and socioeconomic changes, the infection is now being identified (7).

Antimicrobial resistance is a serious problem, not only within hospitals, but also in the community. Penicillin-resistant pneumococcus is not yet a widespread problem, but it has been detected (8); the same situation exists with regard to *Mycobacterium tuberculosis* (9).

The problem of emerging infectious disease is gaining increasing attention in Brazil, and published reports together with notifiable disease data underline the main points of concern.

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Reply to L.J. da Silva

To the Editor: Dr. da Silva's letter raises several important points. My article, however, was never intended to be comprehensive. The choice of which emerging infectious diseases to include was difficult, especially in a country where many endemic infections continue at a high prevalence and others, thought to be controlled, are reemerging.

As Dr. da Silva states, many reports (in Portuguese and English) discuss infectious diseases in Brazil; however, this information is rarely current. The information about measles in my article is a case in point. At the time of my article, an outbreak causing national concern was occurring in Brazil; it has since been controlled. A further problem is that the most detailed and reliable studies are generally of only a regional or local nature, for example, the recent excellent report by Merchan-Hamann (1) on the situation of endemic diseases in north and northeastern Brazil and other references cited by Dr. da Silva. To obtain current information at the national level and provide numerical data rather than merely discuss current trends, I focused on notifiable diseases.

As Dr. da Silva states, schistosomiasis has continued to decrease both in the number of cases and associated illness. Onchocerciasis has been restricted to a small focus in northern Brazil for many years, and a recent report of a new focus in the state of Goias has yet to be confirmed. In my opinion, neither infection could be considered emerging. An important helminthiasis that perhaps should be mentioned is Bancroftian filariasis with a main focus in Recife and minor foci in Belem and Alagoas. Because of traditional and novel control strategies, the number of cases is declining in all foci.

The information I used about hepatitis is confirmed by the National Reference Center on Viral Hepatitis of the Ministry of Health. Febrile illnesses in the Amazon are the great enigma and probably provide the cover for many new diseases that may still emerge. For example, only approximately 20% of blood slides taken from suspected malaria patients in the Amazon are confirmed as positive, which leaves at least one million cases of febrile illness per year undiagnosed. I am unaware of any data that show Mayaro and Oropouche viruses as the most common cause of these illnesses. Dr. da Silva's letter provides useful additional information on bacterial diseases, antimicrobial resistance, and a number of low-prevalence diseases that may in time prove to be important emerging infections.

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Reference:

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A Brief Update on Rabbit Hemorrhagic Disease Virus

To the Editor: We read with interest the paper by A. Smith et al. (Emerg Infect Dis 1998; 1:13-20) on calicivirus emergence from ocean reservoirs. Our attention was drawn particularly to the data and comments regarding rabbit hemorrhagic disease (RHD), a recently emerged and devastating disease of just one rabbit species, *Oryctolagus cuniculus*. We have been involved in RHD research and diagnosis since 1989. Like D. Gregg's laboratory at the Foreign Animal Diseases, U.S. Department of Agriculture, Greenport, USA, our laboratory at the Istituto Zooprofilatico Sperimentale della Lombardia e dell'Emilia, Brescia, Italy, was in 1991 designated a Reference Laboratory for RHD by the International Office of Epizootics (OIE), Paris, France. Although other aspects of the article by Dr. Smith and colleagues appear unclear (e.g., the fact that feline calicivirus is classified among human pathogens like Norwalk virus), we will confine our comments to a few main points concerning RHD virus (RHDV).

Is RHDV a calicivirus or a parvovirus? RHD is caused by a calicivirus (1-3). The articles cited by Dr. Smith date back to 1991 and are part of a book review promoted and edited by OIE (4). This landmark review includes papers from China and the United States supporting the parvovirus hypothesis and papers from Europe concluding that RHDV is a calicivirus. A retrospective reading of those articles may explain the reasons for the misinterpretation of some results. However, this occurred in 1991 and, after 7 years, more than 50 published articles consider RHDV a

calicivirus. Actually, RHDV is one of the best characterized caliciviruses, and the publication of its full genome sequence in 1991 was the first of a *Caliciviridae* member (5).

Diagnostic tools have been developed by our and other laboratories (3,4,6). Thanks also to specific monoclonal antibodies produced towards RHDV and European brown hare syndrome virus (EBHSV) by our colleague E. Brocchi, we standardized different enzyme-linked immunosorbent assays (ELISAs) for the diagnosis of related diseases (4,6-8). In particular, we developed five different ELISAs for serology that allow the detection of antibodies specific for RHDV or EBHSV or that are cross-reactive. In addition, we can define the antibody response in rabbits and hares in terms of isotype-involved immunoglobulin M (IgM), IgA, and IgG (9). Today the main difficulty is the qualitative distinction between RHDV and rabbit calicivirus (RCV, a recently identified nonpathogenic calicivirus) antibodies because of the close antigenic profiles of these viruses (6). Finally, RHDV- and EBHSV-specific polymerase chain reaction has been developed in at least five laboratories besides ours. We have sent these reagents and/or diagnostic methods to at least 19 laboratories outside Italy, including Australia, New Zealand, and the United States.

Does RHDV infect humans? This question has arisen together with the prospect of using RHDV as a biologic control agent in countries like Australia and New Zealand, when they were free of RHDV. In Europe, where the disease naturally occurred and quickly spread, no particular control on human health was planned. In Italy only, between 1987 and 1990, hundreds of millions of rabbits died of RHD in regions where the average density of humans is very high. As a consequence of the use of the vaccine since 1991, the incidence of RHD among breeding rabbits decreased drastically and quickly. Nevertheless, the disease is still endemic, mainly in small farms and among wild rabbits. EBHS also is endemic in wild hares, and hunters are highly exposed to the virus since hares are their main target. However, neither in humans nor in animal species other than rabbits and hares have any diseases similar to RHD ever been reported. In relation to the likelihood of mild or inapparent infections, we used 100 human sera randomly selected from blood donors to carry out a preliminary standardization of an RHD-ELISA that has been periodically used to control the sera of the RHD laboratory staff. Very recently, we tested nine sera from laboratory personnel exposed to RHDV; again no positive result was noted by RHD-ELISA. These findings have limited epidemiologic value, but considering the high level of exposure of part of the sample, it is evident that RHDV infection in humans is unlikely to be the rule.

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Rabbit Hemorrhagic Disease

To the Editor: The recent article on calicivirus by Smith et al. (1) is misleading in its use of the study concerning human health aspects of rabbit hemorrhagic disease (RHD) by Mead et al. (2).

The RHD exposure categories of "low" and "high" used by Mead et al. and mentioned in the first column of page 18 (1) are not related to the categories of "low" and "high" given in the same paragraph at the top of the second column. The reader might easily assume that it was Mead et al. who considered that Jul–Dec 1995 was "a low exposure period." This is not so—such a classification is made by Smith et al.

Further, the reader might assume that it was the study by Mead et al. that concluded "that exposure to RHD virus remains a plausible explanation for increased disease incidence." Again this is an inference drawn by Smith et al. and is the opposite of the conclusion of Mead et al.

The basis of exposure in the study by Mead et al. is at an individual level—the respondents were chosen either because they had been handling rabbits or as controls in determining the level of disease. In contrast, Smith et al. consider exposure at a broad environmental level and disregard whether the respondents had been handling infected rabbits or not. Actually, more contact with rabbits occurred during the first half of the study than during the second.

Smith et al. do not mention the conclusions of Mead et al.: These neither showed any significant difference between levels or types of illness in those exposed and those not exposed to RHD virus nor demonstrated any association between the exposure to RHD and number of episodes of illness in the subsequent 1 to 2 months.

The results of the study by Mead et al. may be summarized by noting that the average number of episodes of illness over the 13-month reporting period was 2.6 for respondents who had not been exposed to RHD virus, 2.2 for those classified as having a low level of exposure, and 2.3 for those classified as having a high level.

The study by Mead et al. concluded that, on the basis of the health survey and the lack of any serologic reaction of the respondents, there was considerable support to the view that RHD virus is not associated with infection or disease in humans. The results of the study have been submitted for publication in a scientific journal.

Reference 31 should refer to the Bureau of Resource Sciences (not Studies).

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Reply to Drs. Capucci, Lavazza, and Mead

To the Editor: We are aware of Capucci and Lavazza's excellent work. Indeed, one of the best characterized calicivirus genomes is that detected in rabbit hemorrhagic disease (RHD); however, the virus' infectivity, pathogenesis, modes of transmission, reservoirs, survival in nature, host of origin, virulence factors, number of neutralization serotypes, and multispecies infectivity are poorly characterized. Propagating this virus in vitro could provide insight for addressing questions relevant to caliciviruses that cannot be propagated in vitro.

We are unclear about the confusion regarding Norwalk virus and feline calicivirus (FCV). Both are caliciviruses. Norwalk virus is a human pathogen. FCV is in a different genus (1) that includes strains infecting humans (2). We know of no documented FCV infections in humans nor of detailed studies to search for such occurrences, although some evidence suggests the possibility (3).

Capucci and Lavazza's remaining questions address the etiology of RHD, diagnostic reagents, and possible human infection. They report nine laboratory workers as antibody negative but do not report test results on persons at high risk, such as rabbit farm workers, nor do they mention having positive control human or primate sera. Koch's postulates have been fulfilled for RHD: a parvovirus was isolated in vitro and was cell-passaged 15 times; at a second laboratory, the parvovirus was identified in materials causing RHD (4,5). In Europe the parvovirus etiology for RHD was deemed hypothetical but has not been refuted on a scientific basis. The calicivirus consistently identified in European materials has not been isolated in vitro, and Koch's postulates have not been fulfilled. Are the parvovirus-associated outbreaks of RHD in Mexico and China (4,5) and the calicivirus-associated RHD

outbreaks in Europe identical disease manifestations of two different viruses? Is RHD multifactorial requiring two or more agents? Is RHD caused by only a calicivirus or only a parvovirus? A calicivirus and a parvovirus can be isolated in vitro from the same fecal sample of a sick rabbit (N. Keefer, D.E. Skilling, A.W. Smith, unpub. data).

Our comments on RHD diagnostic assays referred to those used in Australia (6,7) to screen humans and experimentally infected animals to support legalizing the spread of RHD in Australia and New Zealand.

Public health protection requires prudent avoidance of pathogens associated with risk of adverse outcome, not necessarily proof of causation (8). In this context, human health risk for RHD goes largely unaddressed. The deliberate introduction of a new disease agent (RHD) known to cause death in mammals requires prudence rather than proof of human illness, especially when the scientific literature includes reports that the agent has induced antibody reactions in a wide range of mammalian and avian species (6).

Mead et al. (9) conclude, "No significant association between exposure to RCV and subsequent bouts of sickness could be demonstrated." Their recorded data do not support a statistically significant risk of illness because sample sizes in the monthly groups were too small for any meaningful interpretation. Mead et al. (9) state a "lack of any serologic reaction of the respondents," but a 50% cut-point was used for the competitive ELISA test, and some individual sera were repeated up to six times with percent inhibition reactions ranging from approximately 1% to 44% in one instance and 12% to 100% in another. Results were selected from these laboratory data and reported "lack of serologic reaction."

We derived our findings from data obtained under a freedom of information request. Mead et al. used the same data to support an opposite conclusion. Opposing conclusions "red flag" the quality of the study. In summary, the reporting of negative results of such a study cannot be used to support the important biologic, health, and political conclusion that humans are not at risk from infection with RHD.

We encourage a well-designed longitudinal study of persons at high risk of RHD exposure to answer conclusively whether RHD has infected humans. If "the rule" means that most humans exposed to RHD would become infected, we agree with Dr. Capucci "that infection is unlikely to be the rule," but transmission of equine morbillivirus, Rift Valley fever, and H5N1 influenza to humans is also "unlikely to be the rule" (10).

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The National Food Safety Initiative

For many years, the Centers for Disease Control and Prevention (CDC) and state and local health departments have investigated and controlled outbreaks of foodborne diseases and conducted limited passive surveillance for specific foodborne pathogens. However, recent changes in the food supply and in the consuming public have changed the type and range of infections caused by food and have made traditional approaches to disease surveillance increasingly inadequate. In particular, new technology which facilitates the national and international distribution of food to a population of consumers increasingly vulnerable to infection by new microorganisms has changed the character of foodborne disease in the United States in ways that demand more and better information for decision making.

Foodborne Disease Surveillance

In 1997, President Clinton announced an administration initiative to create a nationwide early warning system for foodborne diseases, enhanced food safety inspection, and expanded food safety research, training, and education. The National Food Safety Initiative will meet the challenges presented by the changing nature of our food safety concerns by strengthening and broadening surveillance and response systems at CDC and the state and local health departments. The following areas of national surveillance for foodborne diseases are addressed by the initiative: rapid identification of and effective response to foodborne hazards; identification of large, diffuse outbreaks that have a low attack rate within one health jurisdiction but affect the health of many people within a larger geographic area; and evaluation of the nature and scope of food safety problems. In addition, the National Food Safety Initiative will 1) link CDC, state and local health departments, and other federal agencies within a powerful electronic network developed by CDC for rapid sharing of microbial subtyping information, including the digitalized images of subtyping patterns needed for molecular epidemiology, and 2) will enhance and expand the activities of FoodNet, the interagency foodborne disease component of CDC's emerging infectious disease active surveillance program to improve the quantitative base for foodborne disease.

Strengthening and Broadening the Base of Surveillance

Under the National Food Safety Initiative, CDC is building epidemiologic and laboratory capacity in state and local health departments so they can quickly and effectively identify foodborne hazards, investigate and control outbreaks, and develop effective programs to prevent additional illnesses from the same source. Routine surveillance and outbreak response, critical to the proper functioning of health departments, have been neglected at the state and local levels because of limited resources and competing priorities. Therefore, CDC is providing training and equipment needed by the health departments to update surveillance systems and diagnostic capability for bacterial, parasitic, and viral foodborne diseases. In addition, CDC, state epidemiologists, and state public health laboratory directors are working to define the elements of core capacity for effective response to food safety problems. The results of this evaluation will provide guideposts for efficient allocation of initiative resources in 1999 and beyond to construct a truly effective national early warning system for foodborne diseases.

CDC's response to foodborne hepatitis A virus infections, supported in 1998 by the National Food Safety Initiative, illustrates the value of better surveillance. Existing hepatitis A surveillance data document food as a vehicle of transmission in only 2% to 5% of cases. On the basis of these data, economic analysis suggests that routine immunization of food workers to prevent foodborne transmission of hepatitis A virus (with a vaccine already available) is not a cost-effective nationwide public health response. However, existing surveillance data document a source of exposure (food or otherwise) for only half of the reported cases of hepatitis A. Therefore, CDC is collaborating with health departments in three states to more fully describe the epidemiology of hepatitis A infections for which routine investigation fails to determine a source. In addition, CDC will evaluate the effectiveness of the algorithm used to determine which patrons of food service establishments with infected food workers will receive immune globulin for postexposure prophylaxis and will establish a genetic sequence-based library of hepatitis A virus strains circulating in the United States and other

countries to permit the effective use of molecular epidemiology in investigations of hepatitis A outbreaks and sporadic cases.

Diarrheal disease results in large numbers of physician visits and hospitalizations each year in the United States. However, the infectious cause of the illness is frequently not determined, which prevents good surveillance and impedes development of effective prevention strategies. Therefore, CDC is embarking on studies of mild and severe diarrhea to determine to the greatest extent possible the bacterial, parasitic, and viral agents involved. Similarly, applied research at CDC that is improving diagnostic and subtyping methods for parasitic foodborne diseases and caliciviruses will support future surveillance and outbreak response for these foodborne pathogens, as well as help define the extent of their contribution as causes of sporadic foodborne disease.

Establishing Electronic Networks and Generating Quantitative Data

Another aspect of CDC's response to foodborne disease under the National Food Safety Initiative is the development of an electronic network that links public health laboratories for rapid data sharing, including the digitalized images of pulsed-field gel electrophoresis (PFGE) patterns. These patterns can be used to distinguish related cases that may be part of an outbreak from background disease so that risk factors can be identified and control measures can be established. By the end of 1997, CDC had laboratories in 16 state health departments trained and equipped to perform PFGE subtyping of *Escherichia coli* serotype O157:H7 with standardized techniques so the results could be compared among laboratories. Microbiologists in federal regulatory laboratories were also trained in these standardized techniques so *E. coli* O157:H7 isolated from food and animal samples could be directly compared with clinical isolates from human cases. Laboratories in four states were established as regional resources to make PFGE subtyping available to surrounding states, and one was directly linked to CDC's PFGE library of strains so it could query CDC's database in addition to being able to submit electronic data for analysis. The other three regional laboratories and laboratories in USDA and FDA will be directly linked to CDC's database early in 1998; the remaining six FoodNet sites will be linked later in the year. In addition, in 1998 six more states will be trained and equipped to perform PFGE subtyping of *E. coli* O157:H7, training will be provided to health department laboratories in two foreign countries, PFGE subtyping of *Salmonella* Typhimurium will begin, and methods for subtyping *Salmonella* Enteritidis and other foodborne pathogens will be developed.

Changes in food safety regulations (e.g., regulatory use of Hazard Analysis and Critical Control Points systems and international requirements for quantitative risk assessment) and increasing emphasis on the cost-effectiveness of prevention programs require epidemiologic data on foodborne disease that cannot be provided by routine passive surveillance systems based on laboratory and physician diagnoses. In response, CDC collaborated with U.S. Department of Agriculgure, Federal Drug Administration, and the five state health departments enrolled in CDC's emerging infections active surveillance program in 1995 to create FoodNet. FoodNet expands the quantitative database on foodborne diseases, expedites response to certain food safety concerns, and provides a test bed for evaluating new prevention strategies. FoodNet activities include 1) comprehensive documentation of foodborne infections through active laboratory-based surveillance, 2) determination of health and food-handling practices of the population under surveillance, 3) determination of health-care practices by surveys of the physicians and diagnostic laboratories in the surveillance areas, and 4) implication of specific foods as vehicles for various foodborne pathogens by conducting case-control studies of sporadic infections.

These efforts have shown that *Campylobacter* is the most commonly diagnosed cause of diarrheal disease, that great geographic variations in the frequency of *E. coli* O157:H7 infections and other foodborne pathogens suggest a need for targeted control measures, and that approximately 1.4 diarrheal illness episodes occur per person per year in the United States.

FoodNet began with active surveillance of sporadic infections with seven bacterial pathogens in the five sites. In 1997, two new sites were added, and in 1998 an eighth active surveillance site will be added; active surveillance for *Cryptosporidium*, *Cyclospora*, and *Calicivirus* infections will be included, and intensive

investigation of foodborne disease outbreaks will be initiated. Many bacterial pathogens, including those that are foodborne, are becoming resistant to antibiotics. To measure this change in resistance, CDC, in collaboration with FDA, USDA, and the states, began monitoring resistance levels of several foodborne bacteria. This information is being used to promote the prudent use of antibiotics in human health and in agriculture.

Other Food Safety Initiative Projects

In 1999, in addition to continuing and extending surveillance activities, CDC plans to support the following projects through the National Food Safety Initiative: school-based health education to teach foodborne disease prevention to a new generation of consumers; a foodborne disease diagnosis and surveillance component to CDC's Field Epidemiology Training Program, which helps Ministries of Health in foreign countries (many of which are major food exporters to the United States) improve their surveillance and diagnostic capabilities; and new methods of determining the risk for exposure to pesticide residues in food. The National Food Safety Initiative is building the public health framework for an effective and efficient response when novel foods from new areas that contain unfamiliar foodborne hazards are introduced into the marketplace.

Sue Binder, Rima Khabbaz, Bala Swaminathan, Robert Tauxe, and Morris Potter
Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Meeting Summary

Conference on Global Disease Elimination and Eradication as Public Health Strategies, February 26, 1998

The goals of the conference were to assess the role of elimination and eradication in decreasing global disease and in using health resources more effectively. Two hundred invited representatives from 81 organizations and 34 countries participated in the multidisciplinary conference; the proceedings will be published in late 1998 in a supplement to the Bulletin of the World Health Organization.

The working definitions during the conference were those developed at the Dahlem Workshop on the Eradication of Infectious Diseases, March 1997. Disease control: reduction of disease incidence, prevalence, illness, or death to a locally acceptable level as a result of deliberate efforts; continued intervention measures are required to maintain the reduction. Disease elimination: reduction to zero of the incidence of a specific disease in a defined geographic area as a result of deliberate efforts; continued intervention measures are required. Disease eradication: 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.

The successful smallpox program and the ongoing poliomyelitis and dracunculiasis (Guinea worm disease) programs served as models in the discussions on eradication. The malaria, yellow fever, and yaws programs of earlier years were recognized as unsuccessful but to have contributed to better understanding of the biologic, social, political, and financial complexities and responsibilities of disease eradication. The conference addressed five major areas: sustainable development, noncommunicable diseases, bacterial diseases, parasitic diseases, and viral diseases. The following is a summary of the conclusions.

Sustainable Development

Eradication programs should have two objectives: 1) eradication of the disease and 2) strengthening and further development of health systems, especially functions such as monitoring and surveillance, supervision, and program management. Potential risks of eradication to the health system and health development include the diversion of resources from basic services and other priorities in countries where the disease being eradicated is perceived to be of lower priority. An additional concern is the failure to accurately estimate the human and financial needs of the eradication efforts. Potential benefits for health development should be identified and delineated at the start of any eradication initiative. Measurable targets should be set, and the program should be held accountable for achieving them. Resources for

eradication activities should be supplementary to those available for basic health-care services.

Successful eradication programs are powerful examples of effective management and can build management capacities that can be carried to other health programs. Therefore, eradication programs should incorporate efforts to design program activities that enhance leadership development and managerial skills among health personnel. These programs also should actively aid in the development and implementation of surveillance systems that can be readily adapted to meet the needs of other national priority programs after eradication goals have been achieved.

Noncommunicable Diseases

The conference concluded that better control was achievable for micronutrient deficiencies (iodine, vitamin A, iron, and folic acid) and lead intoxication, even though none of these conditions meet the requirements for eradication. Recommendations were made for reducing protein/energy malnutrition and lead intoxication and for accelerating attainment of global goals for control of micronutrient deficiencies. Micronutrient supplementation should be enhanced by food fortification and the opportunities presented by existing health infrastructure and immunization programs.

Bacterial Diseases

No bacterial diseases were judged to be candidates for eradication within the next 10 to 15 years. *Haemophilus influenzae* type b (Hib) and congenital syphilis are candidates for regional elimination, and trachoma is a candidate for regional elimination over a somewhat longer term. By the strict definition, neonatal tetanus elimination cannot be guaranteed, although the World Health Organization "elimination goal" of <1 case/1,000 live births in every district is attainable.

Eradication was deemed to be a long-term vision for tuberculosis and Hib. Each of the bacterial diseases considered at the conference represents a major disease, and each has substantial research needs before long-term goals can be achieved. Aggressive action was strongly recommended to improve global control of these conditions.

Parasitic Diseases

The availability of potent, long-acting drugs makes possible the control of onchocerciasis (river blindness) and lymphatic filariasis on a scale heretofore unconsidered. Even though no parasitic diseases were candidates for eradication within the next 10 to 15 years, the group recommended onchocerciasis as a candidate for elimination. Lymphatic filariasis caused by *Wucheraria bancrofti* also could be eliminated and possibly eradicated at some time in the future.

Filariasis caused by *Brugei malayi* could be eliminated from many areas, as could American trypanosomiasis (Chagas disease).

Viral Diseases

The group urged stronger international efforts to control rabies, yellow fever, and Japanese encephalitis by using existing tools. None of the three was considered a candidate for eradication. Hepatitis A eradication was deemed biologically feasible, but further demonstration of sustainable elimination is a prerequisite.

Rubella and measles were considered possible candidates for eradication within the next 10 to 15 years. The eradication of rubella as an add-on to measles eradication was thought biologically plausible, but several issues first needed to be addressed: the human and financial cost of rubella disease, the human and marginal costs of adding rubella to a measles eradication effort, and demonstration that elimination is programmatically feasible and sustainable in a large geographic area.

Measles eradication was concluded to be biologically plausible with the present vaccine. In the Americas, measles transmission appears to have been interrupted for variable time intervals in many countries. Elimination has yet to be demonstrated in other regional settings. The group recommended that industrialized countries proceed with elimination of measles as a step toward eradication. In other countries, accelerating measles control should be the priority, especially in areas with high death rates. Developing countries should proceed cautiously to more costly measles elimination programs to avoid undermining the polio eradication effort. Experience gained from regional and country interventions should be used to refine the strategies for eventual eradication.

General Comments

The short list of conditions for elimination and eradication within the next 10 to 15 years was concluded to be a reflection of current

limitations in our knowledge. Additional basic and operational research was strongly urged. Some participants expressed concern over the use of the term "elimination," on the grounds that the distinctions between elimination and control and elimination and eradication were unclear. Further discussion and possible revision of these terms were recommended.

In summary, the conference provided a multidisciplinary forum for addressing issues around elimination and eradication and their relationship to sustainable health development. There was widespread agreement that an eradication program could have many positive effects on systems development and that explicit efforts should be made to maximize these positive effects as well as to minimize any negative effects. Community mobilization and organization should be seen as a component of sustainable health development, with the additional potential for disease control and eradication. The conclusions and recommendations of the conference should be brought to other forums to expand international health goals and strengthen the mutual ties between sustainable health development and disease control, elimination, and eradication.

For further information, contact Dr. Walter R. Dowdle, 404-371-0466, e-mail: wdowdle@taskforce.org; or Dr. Richard Goodman, 404-639-7400, e-mail: rag4@cdc.gov.

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Conference sponsors included Burroughs Wellcome Fund; CARE; The Carter Center; Centers for Disease Control and Prevention (CDC); CDC Foundation; Children's Vaccine Initiative; The Edna McConnell Clark Foundation; The Fogarty International Center; Glaxo Wellcome; International Life Sciences Institute (ILSI); International Union of Microbiological Societies (IUMS); Merck & Co., Inc. Vaccine Division; National Countil for International Health (NCIH); National Institute of Allergy and Infectious Diseases (NIAID); Pan American Health Organization (PAHO); Pasteur Merieux Connaught USA; The Rockefeller Foundation; Rollins School of Public Health of Emory University; The Task Force for Child Survival and Development; United National Children's Fund; United National Development Programme (UNDP); The World Bank; World Federation of PUblic Health Associations (WFPHA); World Health Organization; and Wyeth-Lederle Vaccines and Pediatrics.

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Erratum

Vol. 4, No. 1

In the article, "Emerging Infectious Diseases—Brazil," by Hooman Momen, on page 3 the last sentence of the next-to-the-last paragraph should read, "The existing, generally passive epidemiologic surveillance system produces information that arrives too late to be effective; however, a number of measures, if implemented immediately, can mitigate the impact of any future epidemic: a containment laboratory (biosafety level 4) that can handle known and unknown microbes of high virulence; at least one infirmary, properly designed and fully equipped, to treat highly contagious and virulent diseases (the current lack of this facility poses a great danger to the population should an outbreak of such a disease occur); and a mobile multidisciplinary task force, including epidemiologists, microbiologists, entomologists, and clinicians, ready to investigate suspected disease outbreaks on short notice."

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Abbreviations. Use abbreviations sparingly. Spell out a term the first time it is used.

Manuscript Submission

Include a cover letter verifying that the final manuscript has been seen and approved by all authors.

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS C-12, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov.

Types of Articles

Perspectives: Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases or related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change; human demographics and behavior; technology and industry; economic development and land use; international travel and commerce; and the breakdown of public health measures. Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of additional subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Synopses: This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. Synopses should be approximately 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Dispatches: These brief articles are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome. Dispatches (1,000 to 1,500 words of text) need not be divided into sections. Provide a short abstract (50 words); references, not to exceed 10; figures or illustrations, not to exceed two; and a brief biographical sketch.

Letters: This section includes letters that give preliminary data or comment on published articles. Letters (500 to 1,000 words of text) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.