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On the Cover

Jack Shadbolt (1909–1998)
Toward Totem (1983–1986)
Acrylic paint on canvas (177.7 cm × 127.4 cm)
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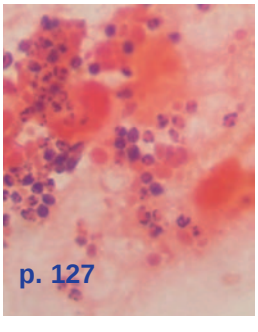
About the Cover p. 185

Perspective

Synopsis

Research



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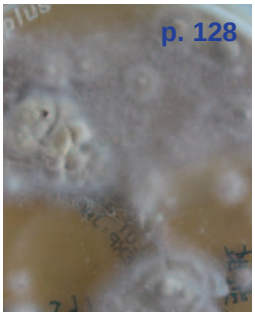
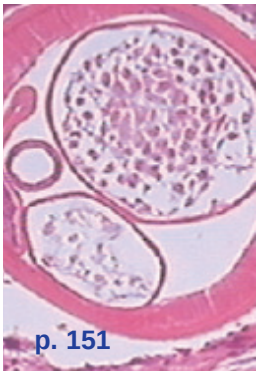


Dispatches



Commentary

Letters

Media Review

News and Notes

Epidemics after Natural Disasters

John T. Watson,* Michelle Gayer,* and Maire A. Connolly*

The relationship between natural disasters and communicable diseases is frequently misconstrued. The risk for outbreaks is often presumed to be very high in the chaos that follows natural disasters, a fear likely derived from a perceived association between dead bodies and epidemics. However, the risk factors for outbreaks after disasters are associated primarily with population displacement. The availability of safe water and sanitation facilities, the degree of crowding, the underlying health status of the population, and the availability of healthcare services all interact within the context of the local disease ecology to influence the risk for communicable diseases and death in the affected population. We outline the risk factors for outbreaks after a disaster, review the communicable diseases likely to be important, and establish priorities to address communicable diseases in disaster settings.

Natural disasters are catastrophic events with atmospheric, geologic, and hydrologic origins. Disasters include earthquakes, volcanic eruptions, landslides, tsunamis, floods, and drought. Natural disasters can have rapid or slow onset, with serious health, social, and economic consequences. During the past 2 decades, natural disasters have killed millions of people, adversely affected the lives of at least 1 billion more people, and resulted in substantial economic damages (*1*). Developing countries are disproportionately affected because they may lack resources, infrastructure, and disaster-preparedness systems.

Deaths associated with natural disasters, particularly rapid-onset disasters, are overwhelmingly due to blunt trauma, crush-related injuries, or drowning. Deaths from communicable diseases after natural disasters are less common.

Dead Bodies and Disease

The sudden presence of large numbers of dead bodies in the disaster-affected area may heighten concerns of disease outbreaks (*2*), despite the absence of evidence that dead bodies pose a risk for epidemics after natural disasters (*3*). When death is directly due to the natural disaster, human remains do not pose a risk for outbreaks (*4*). Dead bodies only pose health risks in a few situations that require specific precautions, such as deaths from cholera (*5*) or hemorrhagic fevers (*6*). Recommendations for management of dead bodies are summarized in the Table.

Despite these facts, the risk for outbreaks after disasters is frequently exaggerated by both health officials and the media. Imminent threats of epidemics remain a recurring theme of media reports from areas recently affected by disasters, despite attempts to dispel these myths (*2*,*3*,*7*).

Displacement: Primary Concern

The risk for communicable disease transmission after disasters is associated primarily with the size and characteristics of the population displaced, specifically the proximity of safe water and functioning latrines, the nutritional status of the displaced population, the level of immunity to vaccine-preventable diseases such as measles, and the access to healthcare services (*8*). Outbreaks are less frequently reported in disaster-affected populations than in conflict-affected populations, where two thirds of deaths may be from communicable diseases (*9*). Malnutrition increases the risk for death from communicable diseases and is more common in conflict-affected populations, particularly if their displacement is related to long-term conflict (*10*).

Although outbreaks after flooding (*11*) have been better documented than those after earthquakes, volcanic eruptions, or tsunamis (*12*), natural disasters (regardless of type) that do not result in population displacement are rarely associated with outbreaks (*8*). Historically, the large-scale displacement of populations as a result of natural disasters is not common (*8*), which likely contributes to the low risk for outbreaks overall and to the variability in risk among disasters of different types.

Risk Factors for Communicable Disease Transmission

Responding effectively to the needs of the disaster-affected population requires an accurate communicable disease risk assessment. The efficient use of humanitarian funds depends on implementing priority interventions on the basis of this risk assessment.

*World Health Organization, Geneva, Switzerland

Table. Principles for management of dead bodies*

- Mass management of dead bodies is often based on the false belief that they represent an epidemic hazard if not buried or burned immediately.
- Burial is preferable to cremation in mass casualty situations.
- Every effort should be made to identify the bodies. Mass burial should be avoided if at all possible.
- Families should have the opportunity (and access to materials) to conduct culturally appropriate funerals and burials according to social custom.
- Where existing facilities such as graveyards or crematoria are inadequate, alternative locations or facilities should be provided.
- For workers routinely handling bodies, ensure
  - Universal precautions for blood and body fluids
  - Use and correct disposal of gloves
  - Use of body bags if available
  - Hand-washing with soap after handling bodies and before eating
  - Disinfection of vehicles and equipment
  - Bodies do not need disinfection before disposal (except in cases of cholera, shigellosis, or hemorrhagic fever)
  - Bottom of any grave is ≥1.5 m above the water table, with a 0.7-m unsaturated zone

*Adapted from Morgan (*3*).

A systematic and comprehensive evaluation should identify 1) endemic and epidemic diseases that are common in the affected area; 2) living conditions of the affected population, including number, size, location, and density of settlements; 3) availability of safe water and adequate sanitation facilities; 4) underlying nutritional status and immunization coverage among the population; and 5) degree of access to healthcare and to effective case management.

Communicable Diseases Associated with Natural Disasters

The following types of communicable diseases have been associated with populations displaced by natural disasters. These diseases should be considered when postdisaster risk assessments are performed.

Water-related Communicable Diseases

Access to safe water can be jeopardized by a natural disaster. Diarrheal disease outbreaks can occur after drinking water has been contaminated and have been reported after flooding and related displacement. An outbreak of diarrheal disease after flooding in Bangladesh in 2004 involved >17,000 cases; *Vibrio cholerae* (O1 Ogawa and O1 Inaba) and enterotoxigenic *Escherichia coli* were isolated (*13*). A large (>16,000 cases) cholera epidemic (O1 Ogawa) in West Bengal in 1998 was attributed to preceding floods (*14*), and floods in Mozambique in January–March 2000 led to an increase in the incidence of diarrhea (*15*).

In a large study undertaken in Indonesia in 1992–1993, flooding was identified as a significant risk factor for diarrheal illnesses caused by *Salmonella enterica* serotype Paratyphi A (paratyphoid fever) (*16*). In a separate evaluation of risk factors for infection with *Cryptosporidium parvum* in Indonesia in 2001–2003, case-patients were >4× more likely than controls to have been exposed to flooding (*17*).

The risk for diarrheal disease outbreaks following natural disasters is higher in developing countries than in industrialized countries (*8*,*11*). In Aceh Province, Indonesia, a rapid health assessment in the town of Calang 2 weeks after the December 2004 tsunami found that 100% of the survivors drank from unprotected wells and that 85% of residents reported diarrhea in the previous 2 weeks (*18*). In Muzaffarabad, Pakistan, an outbreak of acute watery diarrhea occurred in an unplanned, poorly equipped camp of 1,800 persons after the 2005 earthquake. The outbreak involved >750 cases, mostly in adults, and was controlled after adequate water and sanitation facilities were provided (*19*). In the United States, diarrheal illness was noted after Hurricanes Allison (*20*) and Katrina (*21–23*), and norovirus, *Salmonella*, and toxigenic and nontoxigenic *V. cholerae* were confirmed among Katrina evacuees.

Hepatitis A and E are also transmitted by the fecal-oral route, in association with lack of access to safe water and sanitation. Hepatitis A is endemic in most developing countries, and most children are exposed and develop immunity at an early age. As a result, the risk for large outbreaks is usually low in these settings. In hepatitis E–endemic areas, outbreaks frequently follow heavy rains and floods; the illness is generally mild and self-limited, but for pregnant women case-fatality rates can reach 25% (*24*). After the 2005 earthquake in Pakistan, sporadic hepatitis E cases and clusters were common in areas with poor access to safe water. Over 1,200 cases of acute jaundice, many confirmed as hepatitis E, occurred among the displaced (*25*). Clusters of both hepatitis A and hepatitis E were noted in Aceh after the December 2004 tsunami (*26*).

Leptospirosis is an epidemic-prone zoonotic bacterial disease that can be transmitted by direct contact with contaminated water. Rodents shed large amounts of leptospires in their urine, and transmission occurs through contact of the skin and mucous membranes with water, damp soil or vegetation (such as sugar cane), or mud contaminated with rodent urine. Flooding facilitates spread of the organism because of the proliferation of rodents and the proximity

of rodents to humans on shared high ground. Outbreaks of leptospirosis occurred in Taiwan, Republic of China, associated with Typhoon Nali in 2001 (*27*); in Mumbai, India, after flooding in 2000 (*28*); in Argentina after flooding in 1998 (*29*); and in the Krasnodar region of the Russian Federation in 1997 (*30*). After a flooding-related outbreak of leptospirosis in Brazil in 1996, spatial analysis indicated that incidence rates of leptospirosis doubled inside the flood-prone areas of Rio de Janeiro (*31*).

Diseases Associated with Crowding

Crowding is common in populations displaced by natural disasters and can facilitate the transmission of communicable diseases. Measles and the risk for transmission after a natural disaster are dependent on baseline immunization coverage among the affected population, and in particular among children <15 years of age. Crowded living conditions facilitate measles transmission and necessitate even higher immunization coverage levels to prevent outbreaks (*32*). A measles outbreak in the Philippines in 1991 among persons displaced by the eruption of Mt. Pinatubo involved >18,000 cases (*33*). After the tsunami in Aceh, a cluster of measles involving 35 cases occurred in Aceh Utara district, and continuing sporadic cases and clusters were common despite mass vaccination campaigns (*26*). In Pakistan, after the 2005 South Asia earthquake, sporadic cases and clusters of measles (>400 clinical cases in the 6 months after the earthquake) also occurred (*25*).

Neisseria meningitidis meningitis is transmitted from person to person, particularly in situations of crowding. Cases and deaths from meningitis among those displaced in Aceh and Pakistan have been documented (*25*,*26*). Prompt response with antimicrobial prophylaxis, as occurred in Aceh and Pakistan, can interrupt transmission. Large outbreaks have not been recently reported in disaster-affected populations but are well-documented in populations displaced by conflict (*34*).

Acute respiratory infections (ARI) are a major cause of illness and death among displaced populations, particularly in children <5 years of age. Lack of access to health services and to antimicrobial agents for treatment further increases the risk for death from ARI. Risk factors among displaced persons include crowding, exposure to indoor cooking using open flame, and poor nutrition. The reported incidence of ARI increased 4-fold in Nicaragua in the 30 days after Hurricane Mitch in 1998 (*35*), and ARI accounted for the highest number of cases and deaths among those displaced by the tsunami in Aceh in 2004 (*26*) and by the 2005 earthquake in Pakistan (*25*).

Vectorborne Diseases

Natural disasters, particularly meteorologic events such as cyclones, hurricanes, and flooding, can affect vector-breeding sites and vectorborne disease transmission. While initial flooding may wash away existing mosquito-breeding sites, standing water caused by heavy rainfall or overflow of rivers can create new breeding sites. This situation can result (with typically some weeks' delay) in an increase of the vector population and potential for disease transmission, depending on the local mosquito vector species and its preferred habitat. The crowding of infected and susceptible hosts, a weakened public health infrastructure, and interruptions of ongoing control programs are all risk factors for vectorborne disease transmission (*36*).

Malaria outbreaks in the wake of flooding are a well-known phenomenon. An earthquake in Costa Rica's Atlantic Region in 1991 was associated with changes in habitat that were beneficial for breeding and preceded an extreme rise in malaria cases (*37*). Additionally, periodic flooding linked to El Niño–Southern Oscillation has been associated with malaria epidemics in the dry coastal region of northern Peru (*38*).

Dengue transmission is influenced by meteorologic conditions, including rainfall and humidity, and often exhibits strong seasonality. However, transmission is not directly associated with flooding. Such events may coincide with periods of high risk for transmission and may be exacerbated by increased availability of the vector's breeding sites (mostly artificial containers) caused by disruption of basic water supply and solid waste disposal services. The risk for outbreaks can be influenced by other complicating factors, such as changes in human behavior (increased exposure to mosquitoes while sleeping outside, movement from dengue-nonendemic to -endemic areas, a pause in disease control activities, overcrowding) or changes in the habitat that promote mosquito breeding (landslide, deforestation, river damming, and rerouting of water).

Other Diseases Associated with Natural Disasters

Tetanus is not transmitted person to person but is caused by a toxin released by the anaerobic tetanus bacillus *Clostridium tetani*. Contaminated wounds, particularly in populations where vaccination coverage levels are low, are associated with illness and death from tetanus. A cluster of 106 cases of tetanus, including 20 deaths, occurred in Aceh and peaked 2 ½ weeks after the tsunami (*26*). Cases were also reported in Pakistan following the 2005 earthquake (*25*).

An unusual outbreak of coccidiomycosis occurred after the January 1994 Southern California earthquake. The infection is not transmitted person to person and is caused by the fungus *Coccidioides immitis*, which is found in soil in certain semiarid areas of North and South America. This outbreak was associated with exposure to increased levels of airborne dust subsequent to landslides in the aftermath of the earthquake (*39*).

Disaster-related Interruption of Services

Power cuts related to disasters may disrupt water treatment and supply plants, thereby increasing the risk for waterborne diseases. Lack of power may also affect proper functioning of health facilities, including preservation of the vaccine cold chain. An increase in diarrheal illness in New York City followed a massive power outage in 2003. The blackout left 9 million people in the area without power for several hours to 2 days. Diarrhea cases were widely dispersed and detected by using nontraditional surveillance techniques. A case-control study performed as part of the outbreak investigation linked diarrheal illness with the consumption of meat and seafood after the onset of the power outage, when refrigeration facilities were widely interrupted (*40*).

Discussion

Historically, fears of major disease outbreaks in the aftermath of natural disasters have shaped the perceptions of the public and policymakers. These expectations, misinformed by associations of disease with dead bodies, can create fear and panic in the affected population and lead to confusion in the media and elsewhere.

The risk for outbreaks after natural disasters is low, particularly when the disaster does not result in substantial population displacement. Communicable diseases are common in displaced populations that have poor access to basic needs such as safe water and sanitation, adequate shelter, and primary healthcare services. These conditions, many favorable for disease transmission, must be addressed immediately with the rapid reinstatement of basic services. Assuring access to safe water and primary healthcare services is crucial, as are surveillance and early warning to detect epidemic-prone diseases known to occur in the disaster-affected area. A comprehensive communicable disease risk assessment can determine priority diseases for inclusion in the surveillance system and prioritize the need for immunization and vector-control campaigns. Five basic steps that can reduce the risk for communicable disease transmission in populations affected by natural disasters are summarized in an online table (Appendix Table, available from www.cdc.gov/ncidod/EID/13/1/1-appT.htm).

Disaster-related deaths are overwhelmingly caused by the initial traumatic impact of the event. Disaster-preparedness plans, appropriately focused on trauma and mass casualty management, should also take into account the health needs of the surviving disaster-affected populations. The health effects associated with the sudden crowding of large numbers of survivors, often with inadequate access to safe water and sanitation facilities, will require planning for both therapeutic and preventive interventions, such as the rapid delivery of safe water and the provision of rehydration materials, antimicrobial agents, and measles vaccination materials.

Surveillance in areas affected by disasters is fundamental to understanding the impact of natural disasters on communicable disease illness and death. Obtaining relevant surveillance information in these contexts, however, is frequently challenging. The destruction of the preexisting public health infrastructure can aggravate (or eliminate) what may have been weak predisaster systems of surveillance and response. Surveillance officers and public health workers may be killed or missing, as in Aceh in 2004. Population displacement can distort census information, which makes the calculation of rates for comparison difficult. Healthcare during the emergency phase is often delivered by a wide range of national and international actors, which creates coordination challenges. Also, a lack of predisaster baseline surveillance information can lead to difficulties in accurately differentiating epidemic from background endemic disease transmission.

Although postdisaster surveillance systems are designed to rapidly detect cases of epidemic-prone diseases, interpreting this information can be hampered by the absence of baseline surveillance data and accurate denominator values. Detecting cases of diseases that occur endemically may be interpreted (because of absence of background data) as an early epidemic. The priority in these settings, however, is rapid implementation of control measures when cases of epidemic-prone diseases are detected. Despite these challenges, continued detection of and response to communicable diseases are essential to monitor the incidence of diseases, to document their effect, to respond with control measures when needed, and to better quantify the risk for outbreaks after disasters.

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Dr Watson is a medical epidemiologist with the Disease Control in Humanitarian Emergencies Program at the World Health Organization in Geneva. The program provides technical and operational support for control of communicable diseases in humanitarian emergencies.

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Wildlife, Exotic Pets, and Emerging Zoonoses[1]

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Most emerging infectious diseases are zoonotic; wildlife constitutes a large and often unknown reservoir. Wildlife can also be a source for reemergence of previously controlled zoonoses. Although the discovery of such zoonoses is often related to better diagnostic tools, the leading causes of their emergence are human behavior and modifications to natural habitats (expansion of human populations and their encroachment on wildlife habitat), changes in agricultural practices, and globalization of trade. However, other factors include wildlife trade and translocation, live animal and bushmeat markets, consumption of exotic foods, development of ecotourism, access to petting zoos, and ownership of exotic pets. To reduce risk for emerging zoonoses, the public should be educated about the risks associated with wildlife, bushmeat, and exotic pet trades; and proper surveillance systems should be implemented.

Emerging and reemerging infectious diseases have received increasing attention since the end of the 20th century. An estimated 75% of emerging infectious diseases are zoonotic, mainly of viral origin, and likely to be vectorborne (*1*). The emergence and rapid spread of West Nile virus in North America and the monkeypox outbreak in pet prairie dogs have been major awakening public health events that underscored the need for closer collaboration between the veterinary profession, wildlife specialists, and public health personnel (*2*,*3*). These events emphasized the role that veterinarians and other wildlife specialists can play in surveillance, control, and prevention of emerging zoonoses, as their training in disease recognition and population medicine makes them well suited for early detection networks (*4*).

Infectious pathogens of wildlife affect not only human health and agricultural production but also wildlife-based economies and wildlife conservation. Zoonotic pathogens that infect domestic animals and wildlife hosts are more likely to emerge (*5*). Furthermore, our quest for close contact with wild animals and for exotic pets puts us at risk for exposure to zoonoses.

Economic Effects of Wildlife

Wildlife is a major source of income, either directly for consumptive or productive use value or indirectly for touristic and scientific values (*6*). For instance, wildlife tourism is among the top exporting activities of Tanzania and Kenya and generates an annual income of approximately half a billion US dollars (*6*). Even in industrialized countries, wildlife-related activities can generate major income. In the United States, the total expenditure for wildlife-related activities was $101 billion in 1996, ≈1.4% of the national economy (*6*). Hunting activities for the 10 million hunters in Europe generate a financial flux of almost 10 billion euros and ≈100,000 jobs. Europe is also the world's largest importer of venison (>50,000 tons/year). Similarly, in the United States, hunting activities generate >700,000 jobs (*6*). In Africa, the bushmeat trade is generating hundreds of millions of dollars (*7*). In the Congo Basin, trade and regional consumption of wild animal meat could reach 4.5 million tons annually; the demand for bushmeat in western and Central Africa could reach up to 4× the demand for bushmeat in the Amazon Basin (*8*). Worldwide, deer farming has been developing dramatically. In New Zealand, ≈2 million farmed deer, half of the world's farmed deer population, generate an annual income of NZ $200 million (*6*).

Human Population Expansion and Encroachment on Wildlife Habitat

The exponential growth of the human population, from ≈1 billion in 1900 to 6.5 billion in 2006, has led to major ecologic changes and drastic wildlife habitat reduction. Many examples of the emergence or reemergence of zoonoses related to human encroachment on wildlife habitats exist.

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[1]Presented at the International Symposium on Emerging Zoonoses, March 22–24, 2006, Atlanta, Georgia, USA.

Deforestation, development of human habitat, and mining activities have been suggested as risk factors associated with the reemergence of vampire bat rabies in humans in the Amazon Basin. In 2004, 46 persons died of rabies transmitted by vampire bats, mainly in Brazil (22 cases) and Colombia (14 cases); only 20 human cases of rabies were transmitted by dogs in all Latin America (*9*). A similar trend was again observed for 2005.

When first described in 1957, Kyasanur Forest disease was restricted to a much smaller area (300 square miles) in India than the actual 2,000 square miles of endemic zone (*10*). This tickborne disease occurs in evergreen rain forests interspersed with deciduous patches and clearings for rice cultivation and human habitations. Forest workers are particularly at risk; their mortality rates may reach 10%. In 1983, a major epidemic occurred during which several monkeys died, 1,555 humans were infected, and 150 humans died. The outbreak occurred in previously undisturbed forest where some 400 ha were clearcut to establish a cashew tree plantation. Most of the human patients were immigrant laborers employed to clear the forest (*10*). As many as 1,000 human cases occur each year, and this number has increased in the past 5 years. Most cases occur during the dry season (January–May), when nymphal activity is maximal. Such a zoonosis is a good example of deforestation and agricultural development leading to human habitat expansion into natural foci of a viral infection. Because cleared areas were widely used for grazing of cattle, a major host for adult ticks, these areas favored the proliferation of the tick *Haemaphysalis spinigera*.

Conversely, the reduction of traditional agricultural land and its replacement with forested areas, home to the main reservoirs and hosts of *Borrelia burgdorferi*, in association with the settlement of persons in periurban areas, led to a considerable increase in human cases of Lyme disease in the United States (*11*). An estimated 32.4 million wild ruminants, major amplifiers for adult *Ixodes scapularis* ticks, live in North America (*12*). From an estimated 23–40 million white-tailed deer inhabited North America before the arrival of Europeans, the deer population was greatly reduced by habitat loss and unrestricted hunting. However, by the mid-20th century, the population was restored throughout North America, and an estimated 14–20 million white-tailed deer are believed to inhabit the United States alone. In many areas of the eastern United States, populations have soared to previously unattained levels (www.aphis.usda.gov/ws/nwrc/is/living/deer.pdf).

Human activities may also be a source of wildlife infection, which could create new reservoirs of human pathogens. The recent outbreak of tuberculosis caused by *Mycobacterium tuberculosis* in suricats and mongooses was one of the first documented spillovers of a human disease within a wildlife population (*13*). Banded mongooses were observed feeding regularly at garbage pits and were therefore exposed to human excretions and any infectious material from tuberculosis-infected humans.

Changes in Agricultural Practices and Emergence of Wildlife Zoonoses

The emergence of Argentine hemorrhagic fever in east-central Argentina during the 1950s, and its expansion to north-central Argentina, has been directly linked to development of agricultural activities (mainly corn growing) that sustain the virus's main reservoir, the corn mouse (*Calomys musculinus*). Caused by the Junin virus, Argentine hemorrhagic fever affects primarily adult male agricultural workers, mainly during the harvest season (*14*).

In the late 1970s and early 1980s, a rabies epidemic occurred in free-ranging greater kudus (*Tragelaphus strepsiceros*) in Namibia (*15*). The kudu population had increased considerably in response to favorable conditions and human-made environmental changes. Suitable conditions for transmission in the kudu population after initial infection by rabid carnivores are provided by the social behavior of kudus, such as browsing on thorny acacia trees and resultant lesions in the kudus' oral cavity, and excretion of relatively high titers of virus in the saliva of infected animals (*15*).

The outbreak of Nipah virus infection in Malaysia during 1998–1999, which caused 265 human cases of viral encephalitis and a 38% mortality rate, was also the result of several major ecologic and environmental changes associated with deforestation and expansion of nonindustrial pig farming in association with production of fruit-bearing trees (*16*). Such combination led to infection of pigs, which developed respiratory and neurologic symptoms after indirect exposure to infected fruit bats that shed the virus. The sick pigs were a subsequent source of human infection (*16*).

Farming of wild animal species led to reemergence of zoonoses such as bovine tuberculosis in captive deer populations. Deer at low population densities on natural range are less likely to be affected to any major extent by disease. However, disease becomes a factor in intensive management of deer (*17*). Reemergence of zoonotic diseases that had been controlled from their domestic animal reservoirs is also of major concern. Wildlife may become new reservoirs of infection and may recontaminate domestic animals; examples include bovine tuberculosis in the United Kingdom associated with *Mycobacterium bovis* infection in badgers (*Meles meles*) (*18*) and brucellosis in outdoor-reared swine in Europe that resulted from spillover from the wild boar brucellosis (*Brucella suis* biovar 2) reservoir (*19*).

Wildlife Trade and Translocation

Wildlife trade provides mechanisms for disease transmission at levels that not only cause human disease out-

breaks but also threaten livestock, international trade, rural livelihoods, native wildlife populations, and ecosystem health (*7*). Worldwide, an estimated 40,000 primates, 4 million birds, 640,000 reptiles, and 350 million tropical fish are traded live each year (*7*). International wildlife trade is estimated to be a US $6-billion industry (*20*).

Translocation of wild animals is associated with the spread of several zoonoses. Rabies was introduced in the mid-Atlantic states in the 1970s when hunting pens were repopulated with raccoons trapped in rabies-endemic zones of the southern United States (*21*). In Eastern Europe, raccoon dogs (*Nyctereutes procyonoides*) are becoming a new reservoir for rabies, in addition to the established red fox reservoir, as raccoon dogs have spread into new habitats from accidental release of animals raised for fur trade (*22*). Brush-tailed possums (*Trichosurus vulpecula*) from Tasmania were introduced into New Zealand to establish a new species of fur-bearing animals. The translocated population proliferated and is now estimated to be >70 million, of which 3%–30% are possibly infected by *M. bovis*, a permanent threat to the cattle- and deer-farming industries (*21*). Translocation of hares from central and Eastern Europe for sporting purposes led to several outbreaks of tularemia, introduction of *B. suis* biovar 2 to western Europe, and subsequent encroachment of this brucellosis strain into the wild boar population of western Europe (*19*). During 1993–2003, *B. suis* biovar 2 infections were reported in >40 outdoor-rearing pig farms in France (*19*).

Illegal trade can also be a possible source of human infection. In March 1994, psittacosis developed in several customs officers in Antwerp, Belgium (*23*). A customs officer had been hospitalized with pneumonia 10 days after exposure to parakeets illegally imported by an Indian sailor. The risk of contracting psittacosis was 2.8× higher for officers exposed to parakeets >2 hours than for those exposed only briefly. Similarly, a highly pathogenic avian influenza A H5N1 virus from crested hawk eagles smuggled into Europe by air travel has been isolated and characterized (*24*); fortunately, however, screening of human and avian contacts indicated that no dissemination had occurred.

Bushmeat, Wet Markets, Exotic Foods, and Zoonotic Diseases

Another risk factor related to the emergence of zoonotic diseases from wildlife has been the considerable increase in consumption of bushmeat in many parts of the world, especially Central Africa and the Amazon Basin, where 1–3.4 million tons and 67–164 million kilograms, respectively, are consumed each year (*7*). The simian foamy virus has been identified as a zoonotic retrovirus that infects people who have direct contact with fresh nonhuman primate bushmeat; this finding indicates that such zoonoses are more frequent, widespread, and contemporary than previously appreciated. Similarly, new retroviruses, human T-lymphotropic virus types 3 and 4 were found in persons who hunt, butcher, or keep monkeys or apes as pets in southern Cameroon (*25*). The combination of urban demand for bushmeat (a multibillion-dollar business) and greater access to primate habitats provided by logging roads has increased the amount of hunting in Africa, which has increased the frequency of human exposure to primate retroviruses and other disease-causing agents. Similarly, several outbreaks of Ebola virus in western Africa have been associated with consumption of bushmeat, mainly chimpanzees that were found dead (*26*).

Traditional and local food markets in many parts of the world can be associated with emergence of new zoonotic diseases. Live animal markets, also known as wet markets, have always been the principal mode of commercialization of poultry and many other animal species. Such markets, quite uncommon in the United States and, until recently, in California, are emerging as a new mode of commercialization within specific ethnic groups for whom this type of trade assures freshness of the product but raises major public health concerns. The avian influenza epidemic, which began in Southeast Asia in 2003 and recently spread to other parts of the world, is directly related to infected birds sold live in traditional markets. Live bird markets facilitate the spread of this avian H5N1 virus by wild birds (*27*). Similarly, the newly discovered severe acute respiratory syndrome–associated coronavirus was linked to trade of live, wild carnivores, especially civets, in the People's Republic of China (*2*). However, recent data suggest that civets may be only amplifiers of a natural cycle involving trade and consumption of bats (*28*). Trichinellosis has long been associated with consumption of undercooked meat from wild animals, such as bears, and now consumption of uncooked meat from deer and wild boar has recently been associated with emergence of severe cases of hepatitis E in hunters in Japan (*29*). Industrialized nations' new taste for exotic food has also been linked with various zoonotic pathogens or parasites, such as protozoa (*Toxoplasma*), trematodes (*Fasciola* sp., *Paragonimus* spp.), cestodes (*Taenia* spp., *Diphyllobothrium* sp.), and nematodes (*Trichinella* spp., *Anisakis* sp., *Parastrongylus* spp.).

Ecotourism

Adventure travel is the largest growing segment of the leisure travel industry; growth rate has been 10% per year since 1985 (Adventure Travel Society, pers. comm.). This type of travel increases the risk that tourists participating in activities such as safaris, tours, adventure sports, and extreme travel will contact pathogens uncommon in industrialized countries. The most commonly encountered rickettsial infection in travel medicine is African tick bite fever, caused by *Rickettsia africae* and transmitted in rural sub-

Saharan Africa by ungulate ticks of the *Amblyomma* genus; >350 imported cases have been reported from several continents during the past few years (*30*). Most patients are infected during wild game safaris and bush walks. Moreover, because ecotourism is becoming increasingly popular with international travelers, more cases of imported rickettsioses are likely to occur in Europe, North America, and elsewhere in years to come.

Cercopithecine herpesvirus 1 (herpes B virus) is an alpha herpesvirus endemic to Asian macaques, which mostly carry this virus without overt signs of disease. However, zoonotic infection with herpes B virus in humans usually results in fatal encephalomyelitis or severe neurologic impairment (*31*). Herpes B virus has been implicated as the cause of ≈40 cases of meningoencephalitis in persons who had direct or indirect contact with laboratory macaques. A survey of workers at a Balinese Hindu temple, a major tourist attraction where macaques roam free, showed that contact sufficient to transmit B virus occurred commonly between humans and macaques. Furthermore, 31 (81.6%) of 38 macaques at that location had antibodies to herpes B virus (*32*).

Petting Zoos and Exotic Pets

Petting zoos, where children are allowed to approach and feed captive wildlife and domestic animals, have been linked to several zoonotic outbreaks, including infections caused by *Escherichia coli* O157:H7, salmonellae, and *Coxiella burnetii* (*33*). More than 25 outbreaks of human infectious diseases associated with visitors to animal exhibits were identified during 1990–2000 (*33*). In an outbreak of salmonellosis at a Colorado zoo, 65 cases (most of them in children) were associated with touching a wooden barrier around the Komodo dragon exhibit. *Salmonella* organisms were isolated from 39 case-patients, a Komodo dragon, and the wooden barrier. Children who did not become infected were more likely to have washed their hands after visiting the exhibit (*34*).

Exposure to captive wild animals at circuses or zoos can also be a source of zoonotic infection. Twelve circus elephant handlers at an exotic animal farm in Illinois were infected with *M. tuberculosis*, and 1 had signs consistent with active disease after 3 elephants died of tuberculosis. Medical history and testing of the handlers indicated that the elephants had been a probable source of exposure for most of the infected persons (*35*). After an *M. bovis* outbreak in rhinoceroses and monkeys at a zoo in Louisiana, 7 animal handlers, previously negative for tuberculosis, had positive test results (*36*).

Exotic pets are also a source of several human infections that vary from severe monkeypox related to pet prairie dogs or lyssaviruses in pet bats to less severe but more common ringworm infections acquired from African pygmy hedgehogs or chinchillas. Epidemiologic and animal traceback investigations confirmed that the first community-acquired cases of monkeypox in humans in the United States (71 cases) resulted from contact with infected prairie dogs that had been housed or transported with African rodents imported from Ghana (*3*). Similarly, an outbreak caused by *Francisella tularensis* type B occurred among wild-caught, commercially traded prairie dogs; *F. tularensis* antibodies in 1 exposed person documented the first evidence of tularemia transmission from prairie dog to human (*37*). African pygmy hedgehogs have been implicated in human salmonellosis cases in the United States and Canada (*38*). In the United States, the number of commercialized reptiles, especially iguanas, imported per year has increased considerably to ≈1 million. The number of human cases of salmonellosis, especially in very young children, increased dramatically in parallel with iguana pet ownership. The Centers for Disease Control and Prevention estimates that ≈7% of human infections with salmonellae in the United States are associated with having handled a reptile. Most iguanas have a stable mixture of *Salmonella* serotypes in their intestinal tract and intermittently or continuously shed *Salmonella* organisms in their feces (*39*).

Eight cases of rabies caused by a new rabies virus variant were reported in the state of Ceará, Brazil, from 1991 through 1998. Marmosets (*Callithrix jacchus jacchus*) were determined to be the source of exposure. These primates are common pets; most cases occurred in persons who had tried to capture them, and 1 case was transmitted by a pet marmoset (*40*). In 1999, encephalitis was diagnosed in an Egyptian rousette bat (*Rousettus egyptiacus*) that had been imported from Belgium and sold in a pet shop in southwestern France. The pet bat was infected with a Lagos bat lyssavirus and resulted in the treatment of 120 exposed persons (Y. Rotivel, pers. comm.).

Conclusion

Emerging infectious diseases have a major effect on human health and can create tremendous economic losses. Animals, particularly wild animals, are thought to be the source of >70% of all emerging infections (*41*). Leading factors for emergence of zoonoses are unbalanced and selective forest exploitation and aggressive agricultural development associated with an exponential increase in the bushmeat trade (*8*). Similarly, the increase of ecotourism, often in primitive settings with limited hygiene, can be associated with the acquisition of zoonotic agents. Therefore, development of appropriate programs for surveillance and for monitoring emerging diseases in their wildlife reservoirs is essential. Most animal pathogens for which surveillance programs exist relate to farm animals, and few or no programs are specifically aimed at wildlife. Two different but complementary approaches are 1) to monitor the pres-

ence of specifically identified pathogens that have emerged as human pathogens and 2) to investigate in a given wildlife species the presence of known or unknown infectious agents. Furthermore, conservation of habitat biodiversity is critical for preventing emergence of new reservoirs or amplifier species. Key measures for reducing the dispersion of emerging zoonoses include sustainable agricultural development, proper education of tourists about the risks of outdoor activities, and better control of the live animal trade (exotic pets, wet markets, bushmeat). Public health services and clinical practitioners (physicians, veterinarians) need to more actively educate the public about the risks of owning exotic pets and adopting wild animals.

As suggested by Kuiken et al. (*41*), it is time to form "a joint expert working group to design and implement a global animal surveillance system for zoonotic pathogens that gives early warning of pathogen emergence, is closely integrated to public health surveillance and provides opportunities to control such pathogens before they can affect human health, food supply, economics or biodiversity." Major tasks that should be taken by the international community include better integration and coordination of national surveillance systems in industrialized and developing countries; improved reporting systems and international sharing of information; active surveillance at the interface of rural populations and wildlife habitats, especially where poverty and low income increase risks for pathogen transmission; training of professionals, such as veterinarians and biologists, in wildlife health management; and establishment of collaborative multidisciplinary teams ready to intervene when outbreaks occur.

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Vaccine Effectiveness Estimates, 2004–2005 Mumps Outbreak, England

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The United Kingdom and United States have recently experienced large outbreaks of mumps, which raises concerns about vaccine effectiveness. The effectiveness of the mumps component of the measles, mumps, rubella (MMR) vaccine was estimated using the screening method. In England from January 2004 through March 2005, 312 cases of mumps were reported in children eligible to have received 2 doses of MMR vaccine. Of these children, 52 (16.7%) had received 1 dose of MMR vaccine, and 97 (31.1%) had received 2 doses. Vaccine effectiveness was 88% (95% confidence interval [CI] 83%–91%) for 1 dose and 95% (95% CI 93%–96%) for 2 doses. The effectiveness of 1 dose declined from 96% (95% CI 81%–99%) in 2-year-olds to 66% (95% CI 30%–83%) in 11- to 12-year-olds, and the effectiveness of 2 doses declined from 99% (95% CI 97%–99.5%) in 5- to 6-year-olds to 86% (95% CI 74%–93%) in 11- to 12-year-olds (p<0.001 for 1 or 2 doses). Waning immunity may contribute to mumps outbreaks in older vaccinated populations.

In October 1988, immunization against mumps was introduced in the United Kingdom as a single dose of measles, mumps, rubella (MMR) vaccine offered to all children 12–15 months of age (*1*). Reports of disease declined 79% in the first year, from 20,713 in 1989 to 4,277 in 1990. In 1996, a second dose of MMR was introduced at school entry (*2*). From 1990 through 2003, the number of reported cases remained <5,000 per year (*3*), and since 1995, a high proportion of clinically diagnosed cases were shown by laboratory investigation not to be genuine mumps (*4*).

During 2004–2005, a major increase in reported and confirmed cases occurred in all regions of England and Wales. In 2005, >56,000 clinical cases were reported, most in patients 19–23 years of age (*5*). Of the confirmed cases, <3% occurred in children eligible to have received 2 doses of MMR vaccine routinely (i.e., those born from 1993 through 1999) (*5*,*6*).

During 2005–2006, a large outbreak of mumps involving >2,500 possible cases from 11 states was reported in the United States (*7*). The reemergence of mumps in countries that had high levels of vaccine coverage for many years raises questions about the effectiveness of the mumps component of the MMR vaccine and the possible contribution of waning immunity.

In early clinical trials, the efficacy of a single dose of mumps vaccine was >95%, but estimates of the effectiveness in field evaluations have been 62%–85% (*8*–*18*). The possibility of waning immunity has been suggested in several studies but not conclusively demonstrated (*11*–*13*,*19*). The UK outbreak offered the opportunity to evaluate the effectiveness of 1 or 2 doses of MMR vaccine and to investigate the presence of waning immunity by using routinely collected data.

Methods

We reviewed clinically reported mumps cases in England from January 1, 2004, through March 31, 2005, that were confirmed by oral fluid mumps-specific immunoglobulin M testing within 6 weeks of symptom onset (*20*). Only case-patients eligible to have received 2 doses of MMR vaccine through the routine program (i.e., those born after 1992) were included. Case-patients 3–5 years of age were excluded because children receive the second dose of MMR between these ages and, therefore, reliable population coverage data are not available for comparison. Pos-

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sible vaccine-associated cases (i.e., symptom onset within 6 weeks after vaccination) were also excluded. Vaccination histories were obtained from physician records or child health computerized recordkeeping systems.

Quarterly population vaccine coverage data for children 2 and 5 years of age were obtained from the Cover of Vaccination Evaluated Rapidly program (*21*,*22*). Because of changes in health service boundaries and child health computerized recordkeeping systems, data for a small number of areas were missing for some quarterly periods. In addition, 5-year coverage data have been shown to underestimate the true coverage in some areas (*23*). To compensate for this, coverage data were adjusted. Missing values were estimated using linear interpolation from the values submitted in previous and subsequent quarters. When coverage of first-dose MMR for the same birth cohort was lower at 5 than at 2 years of age, the coverage at 5 years of age was assumed to be 3% higher than the value at 2 years of age (based on data from a sentinel surveillance scheme) (*22*). The adjusted data were used to approximate population coverage for school-year cohorts (born from October 1 through September 30 of the following year) to allow for similar levels of exposure within school years. Coverage data at 5 years of age for children born in 2000 were not yet available, so values were estimated from the previous cohort and data from the first 2 quarters. Coverage data were divided into 2 categories, within and outside London, because coverage estimates are lower in London but fairly similar in the rest of the country (*24*).

Statistical Analysis

Vaccine effectiveness was calculated from the proportion of confirmed mumps cases vaccinated (PCV) and the proportion of the population vaccinated (PPV) using the following formula (*25*):

$$\text{Vaccine effectiveness} = \frac{1 - (\text{PCV}(1 - \text{PPV}))}{(1 - \text{PCV})\text{PPV}}$$

The data were grouped by school year, age, sex, and area of residence (within or outside London) and were analyzed by using logistic regression with an offset (incorporating expected PPV by area and birth cohort) for vaccine coverage. When estimating vaccine effectiveness for 1 dose, those who had received 2 doses were excluded from the calculation of PPV. Similarly, those with only 1 dose were excluded when estimating vaccine effectiveness for 2 doses. To estimate overall effectiveness, a model including only a constant was fitted. Effectiveness in different subgroups was estimated from univariable models. If >1 variable was statistically significant on univariable analysis, multivariable analysis was performed.

Data from computerized child health systems, used for scheduling and recording vaccinations given, have consistently been shown to underestimate vaccine coverage (*26*,*27*). Sensitivity analyses explored the effects of possible underestimation of vaccine coverage by repeating calculations assuming that PPV was 1%, 2%, and 5% higher than that reported. Vaccine effectiveness estimates were also calculated using the unadjusted coverage data to examine the effect of data cleaning.

Estimating coverage in older children from a measurement at 5 years of age could lead to an underestimate of effectiveness if there were a subsequent increase in coverage. Therefore, we obtained data on children vaccinated with MMR after 5 years of age from the child health computer system in the former South Thames region in 2000. Analyses explored the effect of an increase in coverage of 0.04%–0.4% per year of age after 5 years of age. The proportion of persons predicted to be susceptible to mumps, by age in England in 2005, was calculated by multiplying age-specific estimates of vaccine effectiveness by annual birth cohort coverage data.

Results

Vaccine Effectiveness

We found 312 confirmed cases of mumps that were eligible for inclusion. Vaccination history was obtained for all case-patients. The proportion of unvaccinated case-patients decreased with increasing age and was higher in younger birth cohorts (Table 1). The proportion of vaccinated case-patients did not differ according to area of residence or sex.

Age at first dose of MMR vaccine ranged from 10 months to 5 years 9 months and at second dose from 16 months to 6 years. Of those who had received MMR, >90% received the first dose at 12–24 months of age and the second dose at 3–5 years of age.

Adjusted population vaccine coverage declined during the study period (Table 2). Outside London, the percentage of children who received 1 dose of MMR by their second birthday declined from 92.7% to 82.7% from the 1993 to the 2002 birth cohort. The percentage who received 2 doses by their fifth birthday was more stable, declining from 78.1% to 76.4% from 1993 to 2000. In London, estimates of coverage were lower and also declined over time.

Overall estimates of vaccine effectiveness were 87.8% (95% confidence interval [CI] 83.1%–91.1%) for 1 dose and 94.6% (95% CI 92.9%–95.9%) for 2 doses. There was no statistically significant variation in vaccine effectiveness for 1 or 2 doses by area of residence ($p = 0.3$ for 1 dose, $p = 0.7$ for 2 doses) or sex ($p = 0.7$ for 1 dose, $p = 0.2$ for 2 doses). Vaccine effectiveness decreased with increasing age for those who received either 1 or 2 doses ($p<0.001$) (Table 3). Vaccine effectiveness also varied with birth cohort ($p = 0.02$ for 1 dose, $p = 0.003$ for 2 doses). As birth cohort and age were correlated, it was not possible

Table 1. Characteristics of reviewed mumps case-patients by number of doses of measles, mumps, rubella vaccine received

Characteristic	No. doses received (%)			p value*
	0	1	2	
Age group, y				
2	11 (84.6)	2 (15.4)	–	
5–6	51 (83.6)	5 (8.2)	5 (8.2)	<0.001†
7–8	52 (61.2)	11 (12.9)	22 (25.9)	
9–10	35 (39.8)	16 (18.2)	37 (42.1)	
11–12	14 (21.5)	18 (27.7)	33 (50.8)	
Birth cohort‡				
1993–1995	31 (30.1)	25 (24.3)	47 (45.6)	<0.001†
1995–1997	42 (48.8)	12 (14.0)	32 (37.2)	
1997–1999	45 (66.2)	8 (11.8)	15 (22.1)	
1999–2001	34 (81.0)	5 (11.9)	3 (7.1)	
2001–2003	11 (84.6)	2 (15.4)	–	
Area of residence				
Outside London	143 (51.3)	46 (16.5)	90 (32.3)	0.1
London	18 (64.3)	6 (21.4)	4 (14.3)	
Data not available	2 (40.0)	–	3 (60.0)	
Sex				
Female	63 (48.8)	19 (14.7)	47 (36.4)	0.2
Male	100 (55.0)	32 (17.6)	50 (27.5)	
Data not available	–	1 (100.0)	–	
Total	163 (52.2)	52 (16.7)	97 (31.1)	

*Fisher exact test.
†Children <2 years of age and 2001–2003 birth cohorts excluded for age group and birth cohort, respectively.
‡Birth cohorts for 2 consecutive school years (i.e., children born October 1–September 30 of the following year).

to separate the effects of age group and birth cohort for 1-dose effectiveness; the effect of birth cohort on 2-dose effectiveness was lost when adjusted for age. Application of our estimates of vaccine effectiveness to UK coverage data (Figure 1) predicts that >20% of children 11–12 years of age are not protected against mumps.

Sensitivity Analysis

Estimates of vaccine effectiveness using raw coverage data were 86.5% (95% CI 81.5%–90.3%) for 1 dose and 96.8% (95% CI 95.6%–97.7%) for 2 doses. Estimates of vaccine effectiveness increased from 87.8% to 90.8% for 1 dose and from 94.6% to 99.3% for 2 doses, for an increase in PPV of 1%–5% (Table 4).

Age at first MMR vaccination was available for 148,525 children registered on the South Thames child health computer system. In each birth cohort, 3–45 children per year received the first MMR dose at >5 years of age, most between 5 and 6 years of age. On average, an additional 0.04% (95% CI 0.036%–0.045%) of children received MMR vaccine per year of age after their fifth birthday. In the sensitivity analysis (Figure 2), a fixed increase in coverage per year of age did not abolish the statistically significant decline in vaccine effectiveness until coverage increased by at least 0.4% per year, 10× greater than that estimated from children in the South Thames region.

Table 2. Estimated coverage of measles, mumps, rubella vaccine at 2 and 5 years of age by area and birth cohort

Birth cohort*	% Coverage at 2 years of age		% Coverage at 5 years of age			
	Outside London	London	Outside London		London	
	1 dose	1 dose	≥1 dose	2 doses	≥1 dose	2 doses
1993–1994	92.7	77.1	95.7	78.1	88.6	57.1
1994–1995	92.0	76.6	95.0	78.3	87.9	56.3
1995–1996	91.9	76.0	94.9	76.4	85.0	59.6
1996–1997	90.4	73.3	93.4	76.7	84.5	58.0
1997–1998	88.8	69.7	92.0	76.8	82.9	56.8
1998–1999	89.0	69.5	92.3	77.6	81.3	56.3
1999–2000	86.9	65.8	91.3	77.3	80.1	56.8
2000–2001	85.3	64.5	90.1	76.4	78.9	55.8
2001–2002	80.6	59.4	–	–	–	–
2002–2003	82.7	60.3	–	–	–	–

*Birth cohorts for school year (i.e., children born October 1–September 30 of the following year).

Table 3. Age-specific estimates of vaccine effectiveness for 1 and 2 doses of measles, mumps, rubella vaccine

	Effectiveness	
Age group, y	1 dose, % (95% CI*)	2 doses, % (95% CI*)
2	95.9 (81.1–99.1)	–
5–6	93.8 (84.1–97.6)	98.8 (97.0–99.5)
7–8	90.3 (81.2–95.0)	95.8 (92.9–97.5)
9–10	86.5 (75.3–92.6)	92.4 (87.7–95.3)
11–12	65.9 (30.3–83.3)	86.4 (74.1–92.9)

*CI, confidence interval; p for linear trend <0.001 for 1 and 2 doses.

Discussion

Our estimate of 87.8% effectiveness for 1-dose mumps vaccine is lower than efficacy estimates from clinical trials (*8*,*9*) but higher than those from most published field evaluations (*10*–*14*). Lower estimates in field studies could result from problems with vaccine storage or administration, errors in case definition (i.e., clinical instead of laboratory-confirmed cases) or ascertainment, inaccurate determination of vaccination status, and bias from conducting studies during outbreaks (*10*,*28*,*29*). Because clinical trials have relatively short follow-up periods, waning immunity may also produce lower observed effectiveness in field evaluations. We observed a decline in protection with increasing age, which suggests that waning immunity may occur. After 2 doses, the magnitude of this decline is small, and effectiveness remains above >85% even 6–7 years after the second vaccination.

Research on whether protection from mumps vaccine declines with time since vaccination is contradictory. In 2 US outbreaks, children vaccinated ≥5 (*12*) or ≥3 (*19*) years before each outbreak were at higher risk for mumps. In 2 other US outbreaks, no evidence was found for increased infection rate with time since vaccination (*11*,*16*). A study from Belgium found increasing risk for disease with time since vaccination (*13*). In the presence of natural boosting, neutralizing antibodies have been demonstrated up to 12 years after vaccination (*30*). However, duration of antibody persistence in a high-coverage setting where mumps circulation has declined is not known. In Finland, a decline in mumps antibody titers was demonstrated in vaccinated children (*31*), and the proportion of children seropositive for mumps antibodies some years after MMR vaccination was lower than expected in Sweden and the United Kingdom (*32*,*33*).

Our estimates may be affected by several biases. Unvaccinated children may mix with other unvaccinated children and exposure may be more common than in vaccinated children, which would lead to an overestimation of vaccine effectiveness. Because most cases occurred in age groups not eligible for vaccination, however, it is likely that exposure to mumps was fairly uniform.

In addition, those who do not access healthcare for vaccination may be less likely to seek care for mumps disease. Consequently, these cases may be less likely to be reported, which would lead to an underestimation of vaccine effectiveness. The availability of free universal primary care, however, means that persons with mumps should not have difficulty accessing medical care in the United Kingdom. Studies during local outbreaks may also underestimate vaccine effectiveness, as a chance cluster of cases is more likely to be reported (*29*). The 2004–2005 outbreak of mumps was a national outbreak affecting all regions of the United Kingdom and is therefore unlikely to be subject to this bias (*6*).

The screening method relies on accurate estimates of population vaccine coverage (*25*). Estimates of vaccine effectiveness were similar using raw and adjusted coverage values, which suggests that our adjustments had not introduced any major bias. Sensitivity analyses that explore the effects of underestimation of vaccine coverage demonstrate that overall effectiveness could be as high as 90% for 1 dose, but this would require true coverage to be ≥5% higher than recorded.

If mumps cases had occurred before the period of our investigation, a false impression of waning immunity could have resulted because these cases would have affected proportionately more unvaccinated than vaccinated children (*34*). Immunity develops in children who have had mumps, so unvaccinated children would be preferentially removed from the population at risk. Therefore, with increasing age, the proportion of mumps cases diagnosed in vaccinated children would increase. From 1990 through 2003, however, UK mumps incidence was extremely low, so past infection is unlikely to be responsible for the observed decline in vaccine effectiveness with increasing age.

The possible effect of migration to and from the United Kingdom on these estimates is difficult to assess. Of immigrants to the United Kingdom, ≈80% arrive from com-

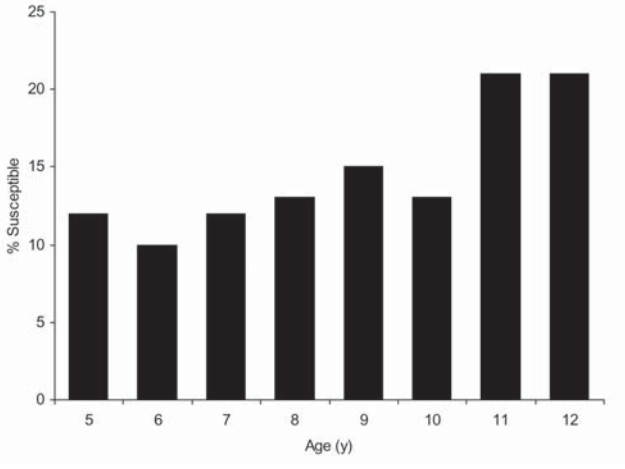

Figure 1. Estimates of the proportion of the population susceptible to mumps by age in 2005, applying study estimates of vaccine effectiveness to population coverage data.

Table 4. Estimates of vaccine effectiveness assuming that the proportion of the population vaccinated (PPV) was 1%, 2%, or 5% higher than reported

Coverage	Effectiveness	
	1 dose, % (95% CI*)	2 doses, % (95% CI)
Baseline	87.8 (83.1–91.1)	94.6 (93.0–95.9)
PPV + 1%	88.4 (84.0–91.6)	97.5 (96.6–98.2)
PPV + 2%	89.0 (84.8–92.1)	98.0 (97.2–98.6)
PPV + 5%	90.8 (87.3–93.4)	99.3 (99.0–99.5)

*CI, confidence interval.

monwealth countries or the European Union each year, and ≈40,000 are children <15 years of age (*35*). Some of these countries do not use routine mumps vaccine; therefore, our estimates of vaccine coverage in older children may be too high. This would lead to an apparent increase in effectiveness with age, rather than the effect observed.

The use of vaccine coverage at the fifth birthday assumes that no further mumps vaccines are given after this age. If coverage gradually increased after this age, effectiveness would be underestimated for older age groups. Children 5–12 years of age, however, have generally not been targeted by MMR catch-up campaigns, and the target payment incentive in primary care does not apply after 6 years of age (*36*). From the South Thames data, most MMR given after the fifth birthday is given to children 5–6 years of age, and the numbers vaccinated are too low to produce the observed decline in effectiveness. We therefore conclude that an increase in coverage after 5 years of age is unlikely to fully explain the decline.

No evidence has shown that vaccine quality and handling changed over time. All cohorts >6 years of age were only eligible for MMR-II (Sanofi Pasteur MSD, Berkshire, UK) for the first dose, so a change in product cannot be responsible for the decline in first-dose effectiveness after this age. Priorix (GlaxoSmithKline, Uxbridge, UK) became available in 1998, but as population coverage by vaccine manufacturer is not available, we are unable to show any differences between vaccines.

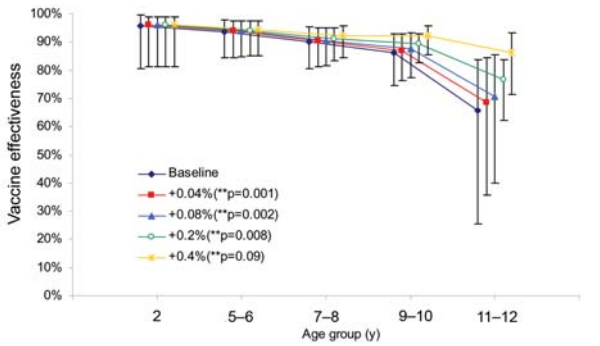

Figure 2. Estimates of 1-dose vaccine effectiveness for mumps cases in 2004–05, assuming an increase in coverage of 0.04%–0.4% per year of age, which represents vaccination of approximately 1%–10% of unvaccinated persons per year of age. Values are offset on the x-axis so that 95% confidence intervals are visible.

According to this study, <80% of children 11–12 years of age are protected against mumps, less than the suggested threshold of 90%–92% to interrupt transmission (*37*). The true proportion of those with immunity may now be higher than this because many unvaccinated persons will have acquired natural infection and vaccinated persons may have been boosted by exposure during the outbreak.

Our estimates of vaccine effectiveness suggest that the mumps component of MMR provides excellent protection through routine administration in the United Kingdom. The observation of waning immunity is, however, a cause for concern because the proportion of susceptible adolescents may increase, which could lead to future outbreaks in this age group. Because most recent cases have occurred in persons too old to have been vaccinated, the contribution of waning immunity to the current UK outbreak is small (*6*). To minimize the risk of future outbreaks, MMR vaccine coverage with both doses must be improved and maintained.

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Prevalence of G2P[4] and G12P[6] Rotavirus, Bangladesh

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Approximately 20,000 stool specimens from patients with diarrhea visiting 1 urban and 1 rural hospital in Bangladesh during January 2001–May 2006 were tested for group A rotavirus antigen, and 4,712 (24.0%) were positive. G and P genotyping was performed on a subset of 10% of the positive samples (n = 471). During the 2001–2005 rotavirus seasons, G1P[8] (36.4%) and G9P[8] (27.7%) were the dominant strains, but G2[4] and G12P[6] were present in 15.4% and 3.1% of the rotavirus-positive patients, respectively. During the 2005–06 rotavirus season, G2P[4] (43.2%) appeared as the most prevalent strain, and G12P[6] became a more prevalent strain (11.1%) during this season. Because recently licensed rotavirus vaccines include only the P[8] specificity, it is unknown how the vaccines will perform in settings where non-P[8] types are prevalent.

Group A rotaviruses are the major etiologic agents of severe infantile diarrhea. Worldwide, >125 million infants and young children develop rotavirus-associated diarrhea each year, resulting in 440,000 infant and child deaths, mostly in developing countries (*1*). In Bangladesh, rotaviruses cause 6,000–14,000 deaths each year in children <5 years of age (*2*).

The tremendous incidence of rotavirus disease underscores the urgent need for interventions such as vaccines, particularly to prevent childhood deaths in developing nations. Fortunately, 2 rotavirus vaccines, RotaTeq (Merck and Co., Whitehouse Station, NJ, USA) and RotaRix (GlaxoSmithKline, Research Triangle Park, NC, USA) passed a large safety trial, showed high efficacy against the major rotavirus G types, and have been approved by the Food and Drug Administration (*3,4*). The efficacy trials of these vaccines have been conducted in the United States, Latin America, and Europe but not in developing countries in Africa and Asia.

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Rotavirus infection shows a characteristic seasonal pattern that is not clearly understood. In developed countries with temperate climates, peak incidence is in winter; however, in developing countries with tropical or subtropical climates, the virus circulates year-round (*5–8*). The temperature in Bangladesh is usually high from April through October and relatively low from December through February. In addition, previous studies have indicated that rotavirus in Bangladesh is affected by floods, which increase opportunities for transmission of the virus (*8,9*). Bangladesh lies on the confluence of hundreds of rivers and is inundated with water every year due to enhanced rainfall during the monsoon season, starting in June (*10*).

Rotaviruses belong to the genus *Reoviridae* and consist of 11 segments of double-stranded RNA. Two outer capsid proteins, VP7 (defining G genotypes) and VP4 (defining P genotypes), independently elicit neutralizing responses. Based on these proteins, a dual classification system of group A rotaviruses has been introduced (*5*). Rotaviruses can be serotyped by using neutralization assays with panels of antisera and genotyped by type-specific primer-dependent reverse transcription–PCR (RT-PCR) and nucleotide sequence analysis (*11–13*). So far, ≥15 G and 26 P genotypes have been described in humans and a variety of animals (*6,14,15*). The major human G types are G1, G2, G3, G4, and G9, which, combined with the P types P[8], P[4], and P[6], account for >80% of rotavirus-associated gastroenteritis episodes worldwide (*16,17*).

Rotaviruses show great genomic diversity, and several studies in different regions of Bangladesh have identified types not targeted by candidate rotavirus vaccines (*18–21*). Unicomb et al. (*8*) showed that frequent genomic reassortment among different rotavirus types was accelerated by mixed infection and generated huge genomic diversity. Although the importance of type-specific immunologic protection against rotavirus disease is still under discussion, many investigators suggest that genomic characterization of rotaviruses is needed to assess whether vaccine efficacy

might be altered by the changing pattern in the distribution of different G and P genotypes (*17*,*22*,*23*).

In our study, stool samples from gastroenteritis patients admitted to 2 hospitals, 1 urban and 1 rural, within the hospital surveillance system of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), from January 2001 through May 2006 were tested for the rotavirus VP6 antigen. The study objective was to clarify the genomic diversity of rotavirus in urban and rural areas in Bangladesh, with a goal of providing information for rotavirus vaccine development programs.

Materials and Methods

Study Population

The ICDDR,B runs an urban hospital situated in Dhaka, the capital city of Bangladesh, which has a population of ≈10 million, and a rural hospital at Matlab, 45 km southeast of Dhaka, which has ≈300,000 inhabitants. Each year, >100,000 patients are treated for diarrhea at the Dhaka hospital and ≈15,000 at the Matlab hospital. At the Dhaka hospital, diarrhea surveillance is conducted in a systematic manner; stool samples are collected to determine the presence of enteric pathogens in every 50th (2%) patient attending the hospital for treatment of diarrhea. In Matlab hospital, stool samples are collected from all patients from the community, which is under active rotavirus surveillance.

Rotavirus Antigen Detection

As part of the surveillance system, rotavirus antigens (group A rotavirus-specific VP6 proteins) were detected in the stool specimens using a solid-phase sandwich-type enzyme immunoassay modeled after the Dakopatts commercial kit (Dakopatts, Copenhagen, Denmark), incorporating rabbit hyperimmune antisera produced at ICDDR,B and an anti-human rotavirus–horseradish peroxidase conjugate. The same criteria as those used by the Dakopatts kit were used for determination of positivity (*8*).

RNA Extraction

Rotavirus RNA was extracted from the stool samples. The QIAamp Viral RNA mini kit (Qiagen/Westburg, Leusden, the Netherlands) was used according to the manufacturer's instructions

RT-PCR

A multiplex RT-PCR was performed by using the Qiagen OneStep RT-PCR Kit (Qiagen/Westburg) for rotavirus G and P genotypes using type-specific oligonucleotide primers as previously described (Table 1) (*11*,*13*,*24*). The reaction was carried out with an initial reverse-transcription step at 45°C for 30 min, followed by 35 cycles of amplification (30 sec at 94°C, 30 sec at 48°C, 1 min at 72°C), and a final extension of 7 min at 72°C in a thermal cycler (Eppendorf, Hamburg, Germany). PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide, and observed under ultraviolet light. Specific segment sizes for different G and P genotypes were observed on the stained gel.

Nucleotide Sequencing

The PCR products were purified with the QIAquick PCR purification kit (Qiagen/Westburg) and sequenced by using the dideoxy-nucleotide chain termination method with the ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer. The consensus forward primer Beg9 and reverse primer End9 were used to amplify and sequence the VP7 gene. For the VP4 gene, the forward primer Con3 and reverse primer Con2 were used as described previously (*11*).

Table 1. Oligonucleotide primers used in the study for PCR amplification

Primer	Type	Position (nt)	Strand	Sequence (5′–3′)	Reference
Beg9	VP7	1–28	Plus	GGCTTTAAAAGAGAGAATTTCCGTCTGG	(*13*)
End9	VP7	1062–1036	Minus	GGTCACATCATACAATTCTAATCTAAG	(*13*)
RVG9	VP7	1062–1044	Minus	GGTCACATCATACAATTCT	(*13*)
Con2	VP4	868–887	Minus	ATTTCGGACCATTTATAACC	(*11*)
Con3	VP4	11–32	Plus	TGGCTTCGCCATTTTATAGACA	(*11*)
MR-G1	G1	314–335	Plus	CAAGTACTCAAATCAGTGATGG	Present study
MR-G2	G2	436–459	Plus	CTATGAATCCACAACTGTATTGTG	Present study
aET3	G3	689–709	Plus	CGTTTGAAGAAGTTGCAACAG	(*13*)
MR-G4	G4	480–499	Plus	GCTTCTGGTGAAGAGTTG	Present study
aAT8	G8	178–198	Plus	GTCACACCATTTGTAAATTCG	(*13*)
MR-G9	G9	757–776	Plus	GAACCATAAACTTGATGTG	Present study
MR-P8	P[8]	314–335	Minus	TCTACTGGATCGACGTGC	Present study
MR-P4	P[4]	474–494	Minus	CTATTATTAGAGGTTAAAGTC	Present study
3T-1	P[6]	259–278	Minus	TGTTGATTAGTTGGATTCAA	(*11*)
4T-1	P[9]	385–402	Minus	TGAGACATGCAATTGGAC	(*11*)
ND2	P[11]	116–133	Minus	AGCGAACTCACCAATCTG	(*11*)

Data Analysis and DNA Sequence Submission

Data were analyzed by SPSS for Windows, release 11.5.1 (SPSS Inc., Chicago, IL, USA). The nucleotide sequence data of the rotavirus strains were submitted to the GenBank under the accession nos. DQ482712, DQ482718, DQ482725, DQ146652, DQ146653, DQ146654, DQ146658, DQ146663, DQ146664, DQ146665, DQ146669, EF033338, EF033339, and EF033340.

Results

Detection of Rotavirus Antigen

From January 2001 through May 2006, 19,039 stool specimens were tested for group A rotavirus VP6 antigen; 4,644 (24.4%) samples had positive results. Table 2 shows the distribution of rotavirus-positive patients in the hospital surveillance systems in Dhaka and Matlab. The average detection rate of rotavirus was 25.2% in Dhaka and 23.3% in Matlab.

Quality Control

Stool specimens obtained from 311 patients with diarrhea were tested for the presence of rotavirus particles using the IDEIA rotavirus kit (DAKO Ltd., Cambridgeshire, UK). By using the IDEIA kit, 234 samples were found to be positive and 77 negative. By comparison, our in-house ELISA kit could detect rotavirus antigen in 232 of the IDEIA-positive samples. Among the IDEIA-negative samples, 74 were negative for rotavirus antigen by our in-house kit. Thus, a comparison of the results indicated that our in-house ELISA kit had an overall sensitivity of 99.1% and specificity of 96.1% compared with the IDEIA rotavirus kit.

Age of the Rotavirus-positive Patients

The age range of the rotavirus diarrhea patients (2001–2005) was 1 month–63.2 years, median age 10 months, and mean age 22.8 months. Most of the rotavirus-positive patients (91%) were <2 years of age (Figure 1). Infection rates were lowest in patients <3 months and >5 years of age.

Seasonal Pattern of Rotavirus Infection

Figure 2 shows the monthly distribution of rotavirus diarrhea in Dhaka and Matlab. Rotaviruses were detected throughout the year in both settings, even though 2 clear seasonal rotavirus peaks were observed each year: a sharp winter peak in January and February, and a monsoon peak in July and August. Taking the average for each setting into account, our model suggests that the rotavirus season in Bangladesh usually starts in June and ends in May (year-round).

Air temperature records for Dhaka and water level data for the Buriganga River (Sadarghat point, Dhaka) for the 5 years of the study (2001–2005) were obtained from the Institute of Water Modelling, Dhaka, Bangladesh (www.iwmbd.org). These data and the number of rotavirus patients admitted to the Dhaka hospital by month are shown in Figure 3. The temperature was lowest from December through February each year, which coincided with the increased number of rotavirus diarrhea cases. On the other hand, the monsoon peaks of rotavirus diarrhea were correlated with the water level. The water level reached the highest mark during July–August each year, which corresponded to the increase of the proportion of rotavirus diarrhea. The meteorologic data from Matlab also correlated to the increased incidence of rotavirus infection, as was seen for Dhaka (data not shown).

Distribution of G and P Types

G and P genotyping were carried out on 471 rotavirus antigen–positive stool samples (10% of all rotavirus-positive patients) by using a type-specific primer–based multiplex RT-PCR that could detect 6 G genotypes (G1, G2, G3, G4, G8, G9) and 5 P genotypes (P[8], P[4], P[6], P[9], P[11]). The untypeable and suspicious samples with lower amounts of PCR products were successfully typed and confirmed by using nucleotide sequencing. Table 3 shows the distribution of G and P types of rotavirus strains detected in Dhaka and Matlab. No significant difference was observed between the distribution of rotavirus strains in Dhaka and Matlab ($p>0.05$). Overall, the most prevalent genotype was G1P[8] (33.8%), which was followed by G9P[8] (25.3%),

Table 2. Distribution of specimens positive for rotavirus, Bangladesh, January 2001–May 2006

	Dhaka		Matlab	
Rotavirus season*	No. tested	Rotavirus positive (%)	No. tested	Rotavirus positive (%)
2000–01†	879	214 (24.3)	715	202 (28.3)
2001–02	1,824	563 (30.9)	1,665	428 (25.7)
2002–03	1,806	458 (25.4)	1,583	338 (21.4)
2003–04	1,786	458 (25.6)	1,425	281 (19.7)
2004–05	2,374	521 (21.9)	1,547	350 (22.6)
2005–06	2,070	492 (23.8)	1,365	339 (24.8)
Total	10,739	2,706 (25.2)	8,300	1,938 (23.3)

*Each season starts in June and ends in May of the following year.
†Data from January–May 2001 only.

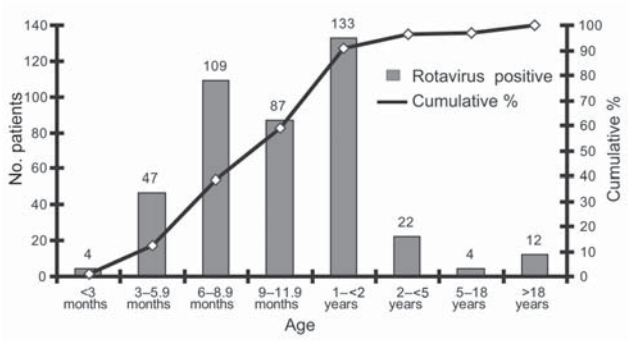

Figure 1. Age distribution for rotavirus-positive patients, Bangladesh, 2001–2005.

G2P[4] (20.2%), and G4P[8] (8.3%). Mixed infections were detected in 3.2% of the samples. Strains with unusual G-P combinations, such as G1P[6], G2P[6], and G2P[8], were also detected.

Unusual porcine-like G11 rotavirus strains were detected in 3 patients (0.6%). These strains were untypeable by multiplex PCR because no G11-specific primer was included in the routine primer set. Therefore, sequencing of the VP7 and VP4 genes was required. The partial VP7 gene sequences of the 3 G11 rotavirus strains (Dhaka22-01, Matlab36-02, and Dhaka13-06) were most similar (>98% similarities at the nucleotide and >97% at the amino acid level) to the porcine-like G11 strain Dhaka6 (*15*). On the other hand, the VP4 genes were most similar to human P[8] or P[6] strains (Malawi strain OP351, Thai strain 15vp4w, and US strain Se585).

Uncommon human G12 rotavirus strains (5.6%), were also detected during our study period. Because the G12 strains were untypeable by using our routine primers, nucleotide sequencing of their VP7 genes was required. All the VP7 gene sequences of the Bangladeshi G12 strains were most similar to the recently isolated G12 strains (Indian strain ISO-2) but distantly related to the prototype G12 strain L26 isolated in the Philippines. The gene segments encoding the VP4, VP6, and NSP4 proteins were sequenced for Bangladeshi G12 strains Dhaka25-02 (G12P[8]) and Dhaka12-03 (G12P[6]). The VP4 gene sequence of strain Dhaka25-02 was most similar to the human P[8] rotavirus strain DRC88 (98% similarity at the amino acid level) isolated in Democratic Republic of the Congo, and strain Dhaka12-03 was most similar to the human P[6] strain US1205 (99% similarity at the amino acid level) isolated in the United States. The VP6 and NSP4 sequences of both strains were also most similar to human rotavirus strains (Indian rotavirus strains RMC100, G25795, and V13520).

Polyacrylamide gel electrophoresis was performed for the 26 G12 strains isolated in our study, and 18 showed a clear RNA migration pattern. Long electropherotypes were detected in 15 (83.3%) samples, which included both G12P[8] and G12P[6] strains. Short electropherotypes were detected in only 3 (16.7%) samples, which also included both G12P[8] and G12P[6] strains.

Fluctuation of the G and P Types

Distribution over Time

Large fluctuations of the rotavirus genotype distribution were observed both in Dhaka and Matlab. However, no significant difference was observed between the urban and rural setting with regard to the yearly distribution of genotypes ($p>0.05$). The overall distribution of the major genotypes over time is shown in Figure 4. The G1P[8] strains were less common in 2001, became the most predominant strains in the following years, but decreased again in 2005–06. G9P[8] strains dominated in the first 2 rotavirus seasons, decreased sharply during 2002–03, dominated again for the

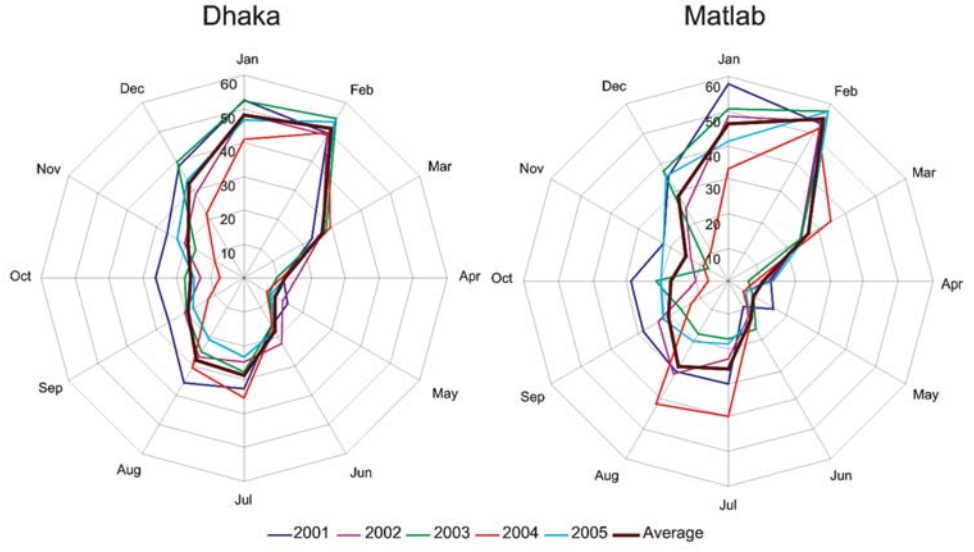

Figure 2. Distribution of rotavirus-positive patients by month, Dhaka and Matlab, Bangladesh. Percentages of positive rotavirus patients were calculated based on all diarrhea patients admitted to the Dhaka and Matlab hospital surveillance system during 2001–2005. The years are shown with different colored lines. The thick brown line represents the average for all years.

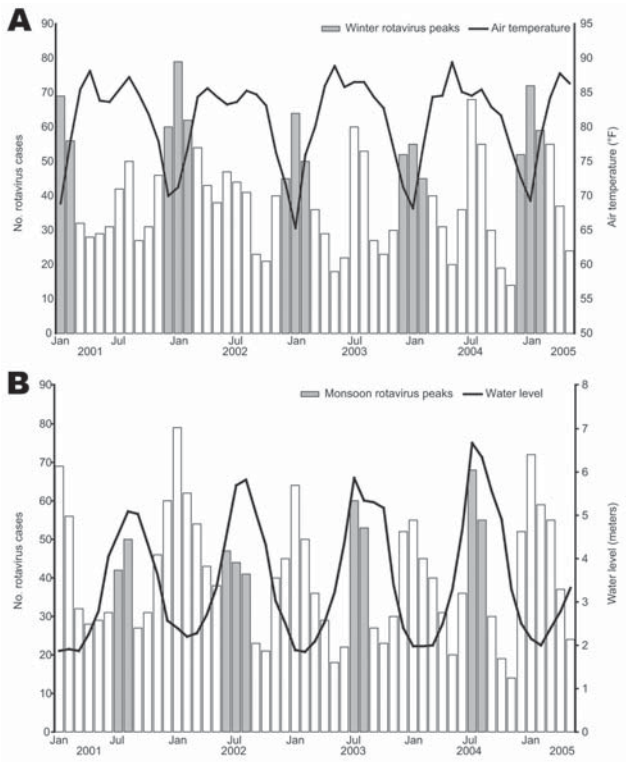

Figure 3. Correlation between cases of rotavirus diarrhea and air temperature (A) and water level (B) in Dhaka, Bangladesh, January 2001–May 2005.

next 2 years, and decreased again during 2005–06. G4P[8], which had been the most prevalent strain in the 1990s in Bangladesh, was found to be less common in our study and constituted only 1.2% during 2005–06. Most interestingly, the strain G2P[4] was the most predominant (43.2%) during the 2005–06 rotavirus season, although it was less common during the previous seasons (15.4% 2001–05). The uncommon strains G12P[6] and G12P[8], introduced in Bangladesh for the first time during the 2000–01 season, became more prevalent (13.6%) in this region by the 2005–06 season.

Discussion

Rotavirus was found in approximately one fourth of all patients with diarrhea who were treated at ICDDR,B hospitals; most rotavirus cases (92.5%) occurred in children during their first 2 years of life. A recent report indicated that 33% of children <5 years age admitted to the ICDDR,B from 1993 through 2004 were rotavirus positive (*2*). Reports from other Asian countries also indicated that rotaviruses were present in 20%–58% of patients with diarrhea who were <5 years of age. Thus, the rotavirus detection rate from our study is comparable to rates from some other Asian countries, including India, South Korea, and Hong Kong (20%–30%), but much lower than those reported in Taiwan, Thailand, China, Japan, Mayanmar, and Vietnam (43%–58%) (*25–34*).

Although rotavirus-associated diarrhea was documented year-round in Dhaka and Matlab, a sharp winter peak and a monsoon peak were observed each year. The winter rotavirus peak is usually observed worldwide, but the monsoon peak is not common in settings with temperate climates. We analyzed environmental data including rainfall and water level of the nearest river and found that the monsoon rotavirus peaks in Bangladesh could be defined by high water levels due to heavy rainfall, which normally starts in the second week of June (*35*). Because of the heavy rainfall, the water level of the rivers begins to increase and reaches its highest level during July–August each year, resulting in inundation of the surrounding areas and increasing the chance of fecal contamination of water. Ahmed and colleagues reported that the number of rotavirus diarrhea cases increased remarkably, and mixed rotavirus types were frequently isolated during the floods in 1988 in Dhaka (*10*). In July and August 2004 in Matlab (Figure 2), a large increase in rotavirus-associated diarrhea was observed. Analysis of the water level of the nearest river (Chandpur point of the Meghna River, data not shown) showed that this increase correlated directly with an increased water level. The water level reached the 5.42 meter mark in July 2004, the highest in that region during our study period.

Our main goal was to characterize the VP7 (G genotype) and VP4 (P genotype) gene segments of the rotavirus strains. We identified most of the globally common rotavirus types (G1, G2, G4, and G9) in our study. Surprisingly, no G3 strain has been detected in Bangladesh since 1993, even though G3 is one of the most prevalent rotavirus types worldwide (*8,25,29*). Results of rotavirus diversity from this

Table 3. Distribution of G and P genotypes of rotavirus strains, Bangladesh, January 2001–May 2006

G type	P type	No. (%) rotavirus strains*		Total no. (%) rotavirus strains
		Dhaka	Matlab	
G1	P[6]	1 (0.4)	2 (1.0)	3 (0.6)
G1	P[8]	85 (31.3)	74 (37.2)	159 (33.8)
G2	P[4]	55 (20.2)	40 (20.1)	95 (20.2)
G2	P[6]	1 (0.4)	0	1 (0.2)
G2	P[8]	2 (0.7)	0	2 (0.4)
G4	P[8]	26 (9.6)	13 (6.5)	39 (8.3)
G9	P[6]	7 (2.6)	2 (1.0)	9 (1.9)
G9	P[8]	67 (24.6)	52 (26.1)	119 (25.3)
G11	P[6]	1 (0.4)	0	1 (0.2)
G11	P[8]	1 (0.4)	1 (0.5)	2 (0.4)
G12	P[6]	16 (5.9)	5 (2.5)	21 (4.5)
G12	P[8]	2 (0.7)	3 (1.5)	5 (1.1)
Mixed G/P		8 (3.0)	7 (3.5)	15 (3.2)
Total		272 (100.1)	199 (99.9)	471 (100.0)

*The percentage of the total for Dhaka is >100% and for Matlab <100% because each number was rounded off to the nearest one tenth of 1%.

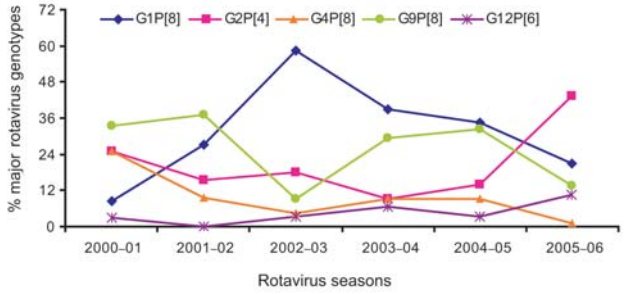

Figure 4. Temporal changes in the distribution of major rotavirus genotypes in Bangladesh, 2001–2006.

study were compared with previous findings in Bangladesh (*8*), and we observed that the distribution of rotavirus genotypes was changing over time. From 1992 through 1997, the most common rotavirus genotype was G4 (47% of the typeable rotavirus strains), but this genotype's prevalence gradually decreased, and it became a less common rotavirus strain over time (1.2% in 2005–06). The distribution of G2 strains, on the other hand, remained nearly unchanged through rotavirus season 2004–05 (19.5% in 1992–1997 and 16.2% in 2001–2005). However, G2 suddenly became the most prevalent genotype in 2005–06 (43.2%).

Three G11 strains, commonly found in pigs, were isolated from humans in the present study. In Bangladesh, pigs are uncommon farm animals, and no genotyping studies on pigs or other animals have been conducted. Therefore, the identification of the strains with an animal-like G11 VP7 specificity and a human P[8] or P[6] specificity raises the question whether these strains are reassortants of human and animal rotavirus strains. This finding underscores the need to include animal rotavirus strains rotavirus surveillance programs. At the same time, water samples, particularly those collected during floods, can be evaluated for the presence of unusual rotavirus strains that might have been introduced from domestic animals.

For the first time in Bangladesh, a very uncommon human rotavirus strain, G12, was detected. The strain was first detected in 1987–1988 in the Philippines, and since then, it has been emerging all over the world (*36–40*). G12 is reported as an important rotavirus strain in India (17.1% in 2003–2005) and in Argentina (6% in 1999–2003) (*17*,*36*). A considerable proportion of G12 was also documented during our study period and reached 13.6% in the latest rotavirus season (2005–06). Thus, the emergence of G12 strains has led to the need for prospective surveillance using new diagnostic RT-PCR primers for G12 strains.

Genetic analysis of the VP4, VP6, and NSP4 gene segments showed that the Bangladeshi G12 strains contained typical human rotavirus gene segments distantly related to the prototype G12 strains L26 and T152. It is possible that the VP7 gene segments from the prototype G12 strains were reassorted with the typical human rotavirus strains. More genetic analyses of complete genome sequences would be helpful to investigate the possible reassortment events and evolution of the recently emerging G12 strains.

P genotype analysis showed that the rotavirus strains with the P[8] specificity made up 76.4% of the circulating strains during 2001–2005; non-P[8] strains constituted 21.9%. The most interesting finding about P types in our study was that the non-P[8] strains represented more than half of the strains (56.8%) during the rotavirus season 2005–06. The currently licensed rotavirus vaccines have shown high efficacy rates in trials and have focused on the role of the major G genotypes, but the role of P genotypes has not been addressed clearly (*3*,*4*). These vaccines include the P[8] specificity, but it is unknown how the vaccines will perform in settings where the non-P[8] types are prevalent. An efficacy trial of the rotavirus vaccine RotaTeq will begin soon in Bangladesh, so the findings of our study regarding rotavirus strain diversity will be important for evaluating the results of this trial.

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Elimination of Arctic Variant Rabies in Red Foxes, Metropolitan Toronto

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To control the Arctic variant of rabies virus in red foxes, 332,257 bait doses containing live, attenuated Evelyn-Rokitnicki-Abelseth rabies vaccine were distributed in greater metropolitan Toronto during 1989–1999. Human and pet contact with bait was minimal, and no adverse reactions to the vaccine were noted. Significantly fewer rabid foxes were found during the 17 years after fox baiting (5 cases during 1990–2006) than in the 17 years before (96 cases during 1973–1989). The last report of a rabid fox in metropolitan Toronto was in 1996 (reporting period through September 2006), which confirms that distributing oral rabies vaccine bait is a feasible tactic for the control of rabies in foxes in urban environments.

The Arctic variant of rabies virus has been present in red fox (*Vulpes vulpes*) populations in Ontario, Canada, since the mid-1950s (*1*,*2*). During 1954–2006, more than 57,000 rabid animals were reported in Ontario, and, on average, 1,000–2,000 humans received rabies postexposure treatment (*3*,*4*). Before rabies control programs were implemented, red foxes accounted for ≈45% of all rabies cases in Ontario (*2*,*5*). In metropolitan Toronto, rabies was cyclic from the 1960s to the 1980s; outbreaks in red foxes and striped skunks (*Mephitis mephitis)* occurred every 2 to 5 years (Figure 1).

Methods

Beginning in 1989, oral vaccination using bait that contained the live, attenuated, Evelyn-Rokitnicki-Abelseth (ERA) (*6*,*7*) strain of rabies virus was distributed in Ontario to control rabies in red foxes in rural and urban habitats (*4*,*8*–*10*). The bait matrix consisted of beef tallow, wax, and attractants such as chicken or cod (*6*). The vaccine was contained in a blister pack, which was embedded in the matrix of the bait (Figure 2). Vaccine-bait components are described in more detail by Bachmann et al. (*6*) and Rosatte et al. (*9*) (Figure 2).

The metropolitan Toronto area (centered at 43°42′N, 79°25′W) was defined as a 638-km^2 urban complex that included the cities of Toronto, North York, Etobicoke, East York, York, and North York. The program in metropolitan Toronto was expanded during 1994–1999 (Figure 3) to include the urban corridor from Oshawa to Hamilton (greater metropolitan Toronto, 1,850 km^2) (Figure 3). During 1998–1999, baiting in rural southwestern Ontario extended into the greater metropolitan Toronto area (Figure 3). During the 1990s, ≈1,000 foxes (about 1.5/km^2) lived in close proximity to ≈3 million people in metropolitan Toronto (*10*,*11*). In addition, during 1987–1996, trap-vaccinate-release programs (vaccination by hand-delivered injection) to control rabies in striped skunks and raccoons (*Procyon lotor*) were conducted in a 60-km^2 portion of metropolitan Toronto (Scarborough) (*9*). From a public health perspective, rabies control was crucial because since the 1950s, >63,000 humans had been treated for exposure to potentially rabid animals in Ontario (*10*,*12*).

During 1989–1999, a total of 332,257 bait doses containing ERA rabies vaccine were distributed in Toronto and the greater metropolitan Toronto area (Table). Bait density was ≈49–69 doses/linear km of ravine (*10*). Bait was distributed primarily by personnel who walked (ground baiting) throughout the ravines and green-belt areas of greater metropolitan Toronto; ecologic studies of red foxes indicated that ravines are used as travel corridors by foxes (*11*). In 1994, 5,500 bait doses were also distributed aerially along the ravine systems from a Turbo Beaver

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[1]Now retired.

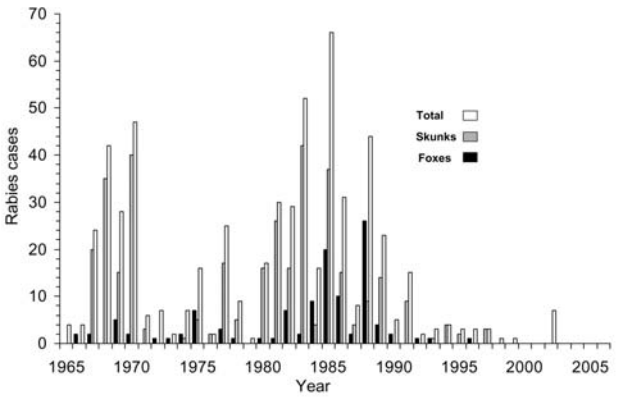

Figure 1. Rabies cases in metropolitan Toronto, 1965–2006. Total includes all species that were reported rabid, most of which were bats.

aircraft flying at an altitude of 150 m with an approximate airspeed of 140 km/hr. In addition, during 1998, about 16,000 doses were aerially distributed in the greater metropolitan Toronto area by Twin-Otter aircraft. Timing of bait placement varied each year but was generally in June and November, from 1989 through 1999. Hand-baiting personnel tried to place 1 vaccine-bait dose every 50 m on both sides of waterways in the ravine systems (*9*). As a modified live virus rabies vaccine was being used in an urban setting, news releases were issued to the media before and during annual baiting operations. The primary objective of the media campaign was to notify the public of the program and ask people not to touch the bait.

Results

We documented that 15 persons found bait (but did not touch the vaccine) and 22 dogs had contact with or consumed the bait during hand-baiting operations. Clinical signs in dogs after bait ingestion sometimes included diarrhea or vomiting (most likely attributable to the tallow and wax in the bait). Three of the dogs had intestinal problems, and 1 had an intestinal obstruction, likely caused by the blister pack. After the 1994 aerial baiting campaign, only 5 persons reported finding bait in their yards. The time needed to hand-distribute ≈28,000 vaccine-bait doses each year in metropolitan Toronto was ≈145 person-days, which is ≈193 bait doses/person/day. The annual cost to hand-distribute these ≈28,000 bait doses was about Can $25,000 for labor, travel expenses, vehicles, and gas plus ≈$30,000 for the bait (total cost of ≈$1.96/dose).

Acceptance of vaccine-bait was determined by the presence of tetracycline in tooth sections (6). Bait acceptance by foxes sampled in metropolitan Toronto during 1989–1991 was 55%–80%, and rabies antibody was detected in 74%–100% of the foxes that consumed the bait (*9*). During this period, 50%–68% of the foxes were vaccinated each year (*9*,*10*). Significantly fewer rabid foxes were reported in metropolitan Toronto during the 17 years after fox baiting began (1990–2006, 5 cases, mean 0.3/yr, standard deviation [SD] 0.6) than during the 17 years before baiting began (1973–1989, 96 cases, mean 5.7/yr, SD 7.3) ($t = 3.01$, $p<0.005$) (Figure 1). On the basis of the cyclic nature of outbreaks in metropolitan Toronto of rabies in foxes (every 2–5 years), as well as in skunks, an outbreak should have occurred during the mid-1990s; but no outbreak occurred. As of September 2006, the last rabid fox in metropolitan Toronto, as well as the greater metropolitan Toronto area, had been reported in 1996. Distribution of vaccine-bait in that urban complex was discontinued in 2000 because metropolitan Toronto had been free from reported rabies in foxes for 3 years.

Discussion

Metropolitan Toronto is connected to rural areas through a series of ravine systems dominated primarily by deciduous trees. These ravines provide a travel corridor through which wildlife, including red foxes, moves into and out of metropolitan Toronto (*11*). The ground and aerial distribution of rabies vaccine bait in metropolitan and greater metropolitan Toronto, which resulted in immunization of a substantial portion of the fox population against rabies, eliminated rabies from that urban complex. Aerial baiting in rural habitats surrounding metropolitan Toronto, as well as greater metropolitan Toronto, after 1995 may have contributed to rabies control in metropolitan Toronto, as few rabid foxes have been available to disperse rabies into that urban complex. As well, one cannot discount the effect that the trap-vaccinate-release programs in Scarborough had on the control of rabies in metropolitan Toronto. However, the trap-vaccinate-release program targeted raccoons and skunks as opposed to foxes (*9*). Greater

Figure 2. Evelyn-Rokitnicki-Abelseth rabies vaccine bait, showing the vaccine container (normally embedded in the matrix of the bait) to the right of the bait. Photograph, Ontario Ministry of Natural Resources Rabies Unit.

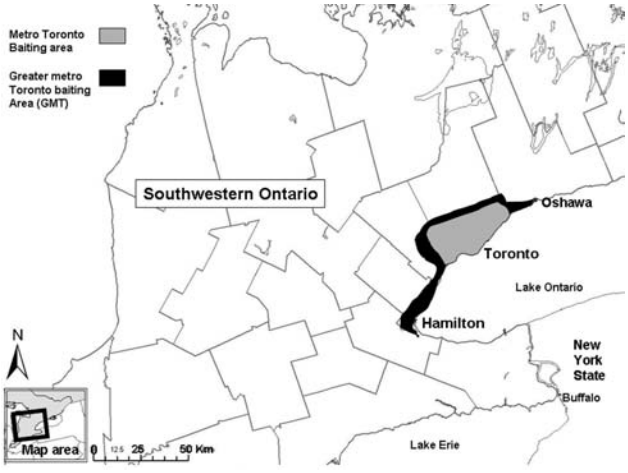

Figure 3. Greater metropolitan Toronto area where rabies vaccine bait doses were distributed during 1989–1999.

metropolitan Toronto has been free of reported cases of rabies in red foxes for a decade (1997–2006) and is a notable success for the Ontario Ministry of Natural Resources rabies control programs. The results of this program confirm that distribution of oral rabies vaccine bait is a feasible tactic for controlling rabies in foxes in urban environments.

Table. Number of rabies vaccine bait doses distributed in metropolitan and greater metropolitan Toronto*

Year	Region	Ground placement	Aerial distribution
1989	Metropolitan Toronto	10,262	
1990	Metropolitan Toronto	27,535	
1991	Metropolitan Toronto	28,371	
1992	Metropolitan Toronto	21,635	
1993	Metropolitan Toronto	24,992	
1994	GMT	40,000	6,000
1995	GMT	45,509	
1996	GMT	41,000	
1997	GMT	40,000	
1998	GMT	15,953	16,000
1999	GMT	15,000	

*Total no. bait doses distributed, 332,257; GMT, greater metropolitan Toronto.

Acknowledgments

We thank the countless technicians from the Ontario Ministry of Natural Resources Rabies Unit and the volunteers from many agencies, including the Metropolitan Toronto Region Conservation Authority and Ontario Correction Services, who participated in hand-baiting the ravine systems of Toronto; the pilots of the aircraft; Dave Johnston and C.D. MacInnes for their support and input to the program; and L. Brown for reviewing the manuscript, providing helpful comments, and assisting with preparation of Figure 1.

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Model for Assessing Human Papillomavirus Vaccination Strategies

Elamin H. Elbasha,* Erik J. Dasbach,* and Ralph P. Insinga*

We present a transmission dynamic model that can assess the epidemiologic consequences and cost-effectiveness of alternative strategies of administering a prophylactic quadrivalent (types 6/11/16/18) human papillomavirus (HPV) vaccine in a setting of organized cervical cancer screening in the United States. Compared with current practice, vaccinating girls before the age of 12 years would reduce the incidence of genital warts (83%) and cervical cancer (78%) due to HPV 6/11/16/18. The incremental cost-effectiveness ratio (ICER) of augmenting this strategy with a temporary catch-up program for 12- to 24-year-olds was US $4,666 per quality-adjusted life year (QALY) gained. Relative to other commonly accepted healthcare programs, vaccinating girls and women appears cost-effective. Including men and boys in the program was the most effective strategy, reducing the incidence of genital warts, cervical intraepithelial neoplasia, and cervical cancer by 97%, 91%, and 91%, respectively. The ICER of this strategy was $45,056 per QALY.

Human papillomavirus (HPV) causes cervical intraepithelial neoplasia (CIN); cervical, anal, penile, vaginal, vulvar, and head/neck cancers; anogenital warts; and recurrent respiratory papillomatoses, resulting in disease and death in both women and men (*1*). Cervical cancer incidence and deaths have substantially decreased in countries with organized cervical cancer screening programs (*2*). However, despite this success, cervical cancer is the second most common malignancy among women and a leading cause of cancer death worldwide, with an estimated 493,000 new cases and 274,000 deaths in 2002 (*3*).

In the United States, public health authorities recommend that girls and women 11–26 years of age be vaccinated with the newly licensed quadrivalent HPV vaccine, Gardasil (Merck & Co., Inc., Whitehouse Station, NJ, USA), to prevent cervical cancer, precancerous and low-grade lesions, and genital warts caused by HPV types 6, 11, 16, or 18. Policymakers will need information on the epidemiologic and economic impact of HPV vaccination to formulate guidelines (*4*,*5*). Cohort models provided some of this information but could not fully assess the impact of HPV vaccination (*6*). In particular, vaccination will not only directly protect through vaccine-derived immunity but also indirectly through herd immunity. To account for these direct and indirect effects, a population dynamic model is necessary (*7*). Moreover, a dynamic model can evaluate a broader range of vaccination strategies (e.g., vaccination of boys and men). A few dynamic models exists (*6*,*8*), but only 1 has examined the cost-effectiveness of bivalent HPV (16/18) vaccination strategies (*9*).

We developed a dynamic model to assess the epidemiologic consequences and cost-effectiveness of alternative quadrivalent HPV (6/11/16/18) vaccination strategies. An online Supplementary Appendix (available from www.cdc.gov/ncidod/EID/13/1/28-app.htm) describes in detail the model structure and inputs. Specifically, we examined 2 questions: What is the potential impact of a quadrivalent HPV vaccine on HPV infection and disease in the US population? What is the cost-effectiveness of a quadrivalent HPV vaccine program when added to the current standard of care from the perspective of the US healthcare system?

Methods

Screening and Vaccination Strategies

We assumed that the vaccine will be combined with current screening and HPV disease treatment practices. We defined the reference vaccination strategy to be routine HPV vaccination of girls by age 12 (F12-only) (*10*). We

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also examined the following strategies: 1) routine vaccination of girls and boys by age 12 (F&M12), 2) routine vaccination of girls by age 12 and catch-up female vaccination for those ages 12–24 (F12-only+CUF-only), 3) routine vaccination of boys and girls by age 12 years and catch-up female vaccination for those ages 12–24 years (F&M12+CUF-only), and 4) routine vaccination of boys and girls by age 12 and catch-up female and male vaccination for those ages 12–24 (F&M12+CUF&M).

Dynamic Model Structure

Our dynamic model has demographic and epidemiologic components ([*11*], Appendix). The demographic model defines the demographic characteristics of the population being simulated and describes how persons enter, age, and exit various categories. The heterosexually mixing population is divided into 17 age groups. Each age group consists of persons with low, medium, or high sexual activity.

Twelve-year-old persons enter the population at a gender-specific and sexual activity–specific rate. Persons then move between successive age groups at an age- and gender-specific rate per year (*11*). Persons exit the model upon death at an age- and gender-specific per capita death rate per year. Cervical cancer patients have an additional age- and stage-dependent death rate. Patients with CIN or genital warts do not face an additional risk for death.

The epidemiologic model simulates HPV transmission and the occurrence of CIN, cervical cancer, and external genital warts in this age-structured population. The acquisition of infection and progression of persons from infection to disease follow a similar natural history structure, as assumed in previous models for HPV 16/18 (*6*). We also incorporated HPV 6/11 infection and genital warts, and grouped infections into HPV 16/18, HPV 6/11, or HPV 6/11/16/18. We divided the population into distinct epidemiologic categories, according to the person's status with respect to infection, disease, screening, and treatment (Appendix, Figure 1A–B).

Parameters for Estimates and Sources

A comprehensive search of the literature was conducted to obtain baseline values for the parameters of the model (Appendix Tables A1–A3). We used age-stratified data to estimate cytology screening rates (*12–14*). Estimates of cytology screening sensitivities and specificities were based on published studies (*15,16*).

The degree of protection from the vaccine (the proportion of challenges against which a recipient is protected) against incident infection (HPV 6/11 or 16/18) was 90%; against associated disease the degree of protection was 100% (*17,18*). We assumed the duration of protection was lifelong for the reference case (*6*) and examined a 10-year duration in sensitivity analyses. We assumed the natural course of disease was unaltered following vaccine failure or loss of vaccine-induced immunity. Because Gardasil is a prophylactic vaccine, we did not include any therapeutic benefits to recipients already infected with the vaccine types. We assumed that up to 70% of 12-year-olds received a 3-dose vaccine (*6*). Coverage increased linearly from 0% up to 70% during the first 5 years of the program (e.g.,

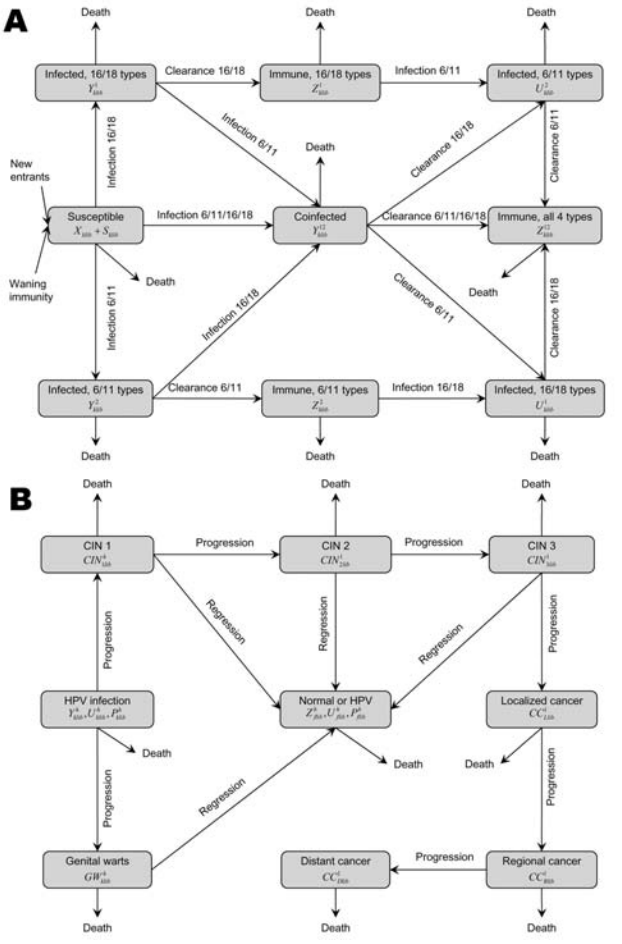

Figure 1. A simplified schematic diagram of human papillomavirus (HPV) infection and disease state transitions, lifetime duration of infection-derived immunity, unvaccinated compartments. A) Persons enter into the susceptible (*X*) compartment and leave all compartments at sex- and age-specific rate. A susceptible host may be infected by either or both HPV types. A host infected with a given type can also be infected with the other type and move into compartment (Y^{12}). An infected person can clear infection with 1 type and can become immune to that type (Z^h) and be infected with the other type (U^h). Infection with and clearance of all types results in lifetime immunity. B) Cervical intraepithelial neoplasia (CIN) develops in females and progresses though several histologic states: infected with a normal cervix; CIN 1; CIN 2; CIN 3; localized, regional, and distant cervical cancer. CIN can regress to normal with or without infection. Genital warts can develop and clear in those infected with HPV 6/11.

14% in year 1, 28% in year 2) and remained at 70% thereafter. Vaccine coverage for the catch-up program increased linearly from 0% up to 50% during the first 5 years (e.g., 10% in year 1, 28% of unvaccinated in year 2), and the program was eliminated after year 5.

We assumed the cost of the HPV vaccine for 3 doses and administration would be US $360 (range $300–$500), consistent with previous analyses (*6*). All costs were updated to 2005 US dollars. Costs and quality-adjusted life years (QALY) were discounted at 3%.

Simulation Method

We assessed the epidemiologic impact and cost-effectiveness of each vaccination strategy over a planning horizon of 100 years. We solved the model for the prevaccination steady-state values of the variables and used them as initial values for the vaccination model. Next, we solved the model for the entire time path of the variables until the system approached a steady-state.

Validation Analyses

We established the face validity of the model by consulting with experts on assumptions regarding the natural history of HPV infection and disease (*19*). The accompanying online Supplementary Appendix allows for further critical review of the model assumptions and provides the mathematical equations necessary to reproduce the results (*19*,*20*). The predictive validity of the model was evaluated by comparing model results with epidemiologic data from unscreened and screened populations in the United States (*2*,*21*–*23*).

Sensitivity Analyses

Because of the large number of equations and inputs, we used a smaller version of the model to determine the most influential inputs. Based on these results, 1-way sensitivity analyses using the full model were performed on vaccine parameters (duration, degree, coverage, cost, target age), quality-of-life weights, discounting, and duration of natural immunity. We also conducted a multivariate sensitivity analysis that examined a pessimistic scenario (i.e., duration of protection = 10 years; vaccine coverage = 50%; health utility for genital warts; CIN 1, 2, 3, and carcinoma in situ (CIS) = 0.97; degree of protection against infection = 75%; and degree of protection against HPV-related disease = 85%). We also examined the role of herd immunity.

Results

Model Validation

Model predictions generally fell within the range of values reported in the literature. Overall, HPV 6/11 steady-state prevalence among females was 0.7%, which is similar to that reported by Giuliano et al. (*24*) for15- to 59-year-old women. The predicted age-specific HPV prevalence curve had a shape and magnitude at peak similar to data reported in the literature (*24*–*28*) (Figure 2). Without screening, the predicted HPV 16/18-attributable cervical cancer incidence curve had a shape and magnitude at peak (39 per 100,000 women-years for ages 45–50) similar to those estimated from unscreened US populations (*22*,*29*). The model predicted that 20% of all cervical cancer cases occurred among women who were never screened, similar to what has been observed in US populations (*30*). Also, the cervical cancer incidence curve (HPV 16/18 attributable) had a shape and magnitude at peak (8.3 per 100,000 women-years for ages 30–39 years) similar to that observed among recent cohorts of US women (*23*). However, the model predicted lower cervical cancer incidence among older cohorts. This approximation may be reasonable given that future cohorts of older women are expected to have lower cervical cancer incidence than women currently in older age groups (fewer women missed screening at younger ages among more recent cohorts [*13*,*14*]). Finally, with screening, the age-specific incidence curves for CIN and genital warts generally had shapes and magnitudes at peak similar to data reported in the literature (*21*,*31*).

Epidemiologic Impact of HPV Vaccination Strategies (Reference Case)

Steady-state HPV prevalence rates were higher for boys or men than for girls or women across all age groups (Figure 2). Overall, HPV 16/18 steady-state prevalence among girls and women ≥12 years of age (2.4%) was higher than that for boys or men (1.7%) and increased with level of sexual activity (data not shown). For both sexes, prevalence increased with age, reached a peak in the 20- to 24-year age group and continuously declined thereafter.

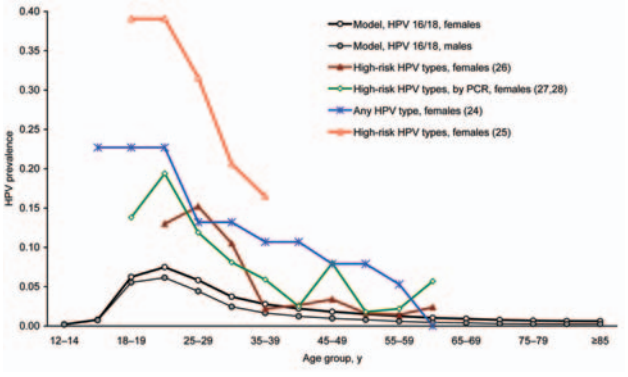

Figure 2. Human papillomavirus (HPV) prevalence by sex and age group, as predicted by the model and reported in selected studies from North America. HPV high risk includes types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82.

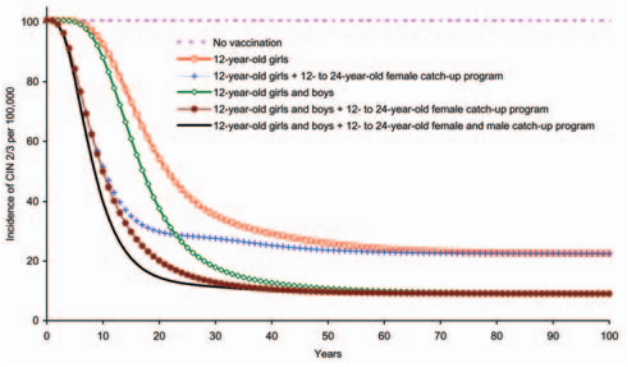

Figure 3. Incidence of cervical intraepithelial neoplasia (CIN) 2/3 due to human papillomavirus 6/11/16/18 infection among girls and women ≥12 years of age, by vaccination strategy.

Across all strategies, the effect of the vaccine was to steadily reduce CIN 2/3 incidence until the system approached a steady state (Figure 3). The largest reduction was accomplished by adopting F&M12+CUF&M. Cervical cancer curves shared the same qualitative features of those of CIN 2/3 (Figure 4). However, because cervical cancer progresses slowly, the effect of vaccination on the reduction in incidence and cancer deaths was more gradual compared with that for CIN 2/3 (Figures 3 and 4).

For genital warts, the reduction occurred sooner (Figure 5A and 5B). Female-only vaccination strategies were effective in reducing genital warts incidence among adolescent girls and women (Figure 5B) and were also effective in reducing the incidence of genital warts among males, but were not as effective as strategies that included male vaccination (Figure 5A).

F&M12+CUF&M had the most effect on the number of cases of genital warts, CIN, and cervical cancer. Compared with screening only, this strategy substantially reduced the long-run, overall number of genital warts (97%), CIN 2/3 (91%), and cervical cancer cases (91%) among adolescent girls and women.

Economic Impact of HPV Vaccination Strategies (Reference Case)

F&M12 was less effective and more costly (dominated) than F12-only+CUF-only (Table 1). The incremental cost-effectiveness ratio (ICER) of F12-only+CUF-only was US $4,666/QALY, and the most effective strategy (F&M12+CUF&M) had an ICER of $45,056/QALY.

Sensitivity Analyses

With 10 years' duration of protection, vaccination reduced disease incidence steadily until ≈10–15 years after vaccination, when the loss of immunity among vaccinated persons and increased numbers of unvaccinated persons reversed these trends and caused the incidence to rise (Figure 6). The rise in incidence continued until years 20–30, after which, it fell steadily until a steady state was approached. The timing and magnitude of the reduction and resurgence in incidence depended on the strategy. The largest reduction and lowest rebound were accomplished by using F&M12+CUF&M. If the duration of protection was only 10 years, long-term reductions in the annual number of cases of genital warts among males, CIN 2/3, and cervical cancer would be 36%, 25%, and 28%, respectively. In addition, ICERs increased by changing the duration of protection from lifelong to 10 years (Table 2).

The long-term cervical cancer incidence and ICER were not very sensitive to changes in the degree of vaccine protection against infection and disease. However, the results were sensitive to varying vaccination coverage. For example, the impact of vaccination on cervical cancer was lower when coverage was 50% compared with 90% (Figure 7). Lower coverage made vaccinating adolescent boys and men more cost-effective (Table 2). Increasing vaccination cost and quality of life weights increased ICERs.

Lower discount rates resulted in higher costs and QALY for each vaccination strategy. Discounting both costs and QALY at 1% decreased ICERs of the nondominated strategies: F12-only+CUF-only had an ICER of $448/QALY, whereas the ICER of F&M12+CUF&M was $28,614 /QALY. With a 5% discount rate, ICERs of these 2 strategies increased to $10,138/QALY and $64,413/QALY, respectively. HPV prevalence and burden of HPV-related diseases increased with shorter duration of natural immunity. A higher background rate of disease made the impact of vaccination look more favorable. For example, with 10-year duration of natural immunity, F12-only+CUF-only was cost-saving, whereas the ICER of F&M12+CUF&M was $11,567/QALY.

When the effects of herd immunity and benefits of prevention of HPV 6/11 were removed, the ICER of F12-

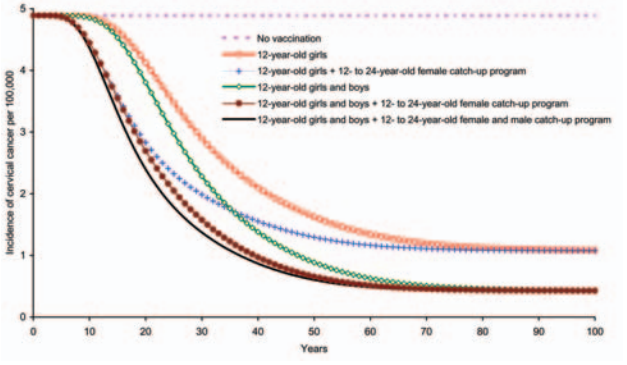

Figure 4. Incidence of cervical cancer due to human papillomavirus 16/18 infection among girls and women ≥12 years of age, by vaccination strategy.

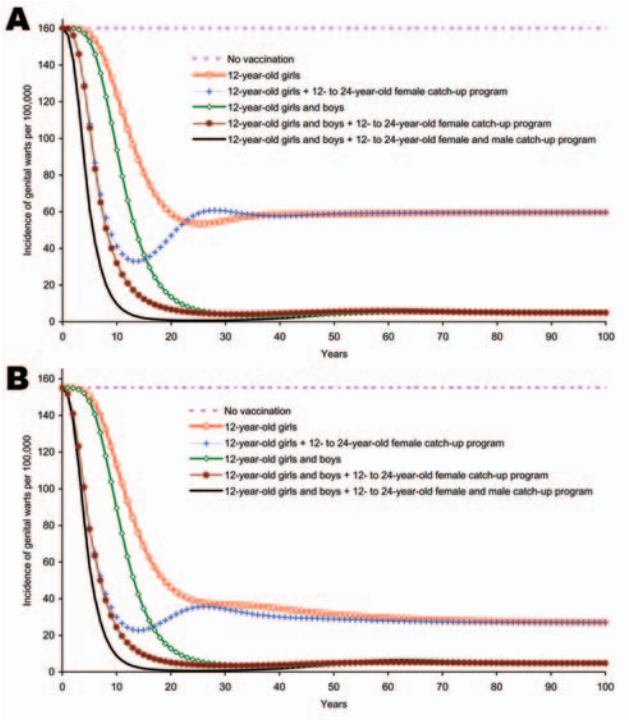

Figure 5. A) Incidence of genital warts due to human papillomavirus (HPV) 6/11 infection among boys and men ≥12 years of age by strategy. B) Incidence of genital warts due to HPV 6/11 infection among girls and women ≥12 years, of age by strategy.

only increased to $21,404. If one assumes a pessimistic scenario, the ICER of the F12-only+CUF-only strategy increased from $4,446/QALY to $29,053/QALY and the ICER of the F&M12+CUF&M increased from $45,056/QALY to $124,063/QALY.

Because vaccination coverage rates are expected to be lower among older age groups, we assumed a rate of 50% among 15- and 18-year-olds. With these rates, F12-only+CUF-only had an ICER of $8,357/QALY compared with delaying age of vaccination to 18 years (Table 3). ICERs of vaccinating by age 12 years increased when coverage rates among persons of ages 15 and 18 years were higher. Increasing the target age of vaccination decreased the benefits of vaccination (Figure 8, Table 3).

Finally, to estimate the additional value of preventing HPV 6/11 infection, we conducted an analysis in which we assumed that persons had no protection against HPV 6/11 infection and related disease. The results of this analysis showed that ICERs of F12-only+CUF-only and F&M12+CUF&M increased to $11,254/QALY and $74,151/QALY, respectively.

Discussion

We developed an integrated transmission dynamic model and economic evaluation to inform HPV vaccine policy recommendations and decisions. We gained valuable insights by comparing various vaccination strategies. In general, the results suggest that a quadrivalent HPV vaccine program that targets female adolescents and women, ages 12–24 years, can be cost-effective ($4,666/QALY) when compared with other commonly accepted medical interventions (*32*). These findings are consistent with other cohort-based cost-effectiveness analyses, which generally show that vaccination of 12-year-old girls can be cost-effective but also illustrate the substantial herd immunity benefits provided by vaccination.

Some results from this model were qualitatively similar to the results of other studies with respect to the finding that male vaccination was more attractive the lower the coverage among girls and women (*9*). However, the results of our base case differ qualitatively from that of Taira et al. (*9*) regarding the conclusion that vaccinating males and females would not be cost-effective. This difference in results may be explained as follows. First, unlike Taira et al., we accounted for the additional benefits conferred by protecting against HPV 6/11 infection among adolescent boys and girls, women, and men. Second, we were able to account for all the benefits and costs of vaccination realized by both those vaccinated and not vaccinated. Third, we assumed lower weights for the quality of life of women

Table 1. Cost-effectiveness analysis of alternative HPV vaccination strategies*

	Discounted total		Incremental		
Strategy	Costs	QALY	Costs	QALY	$/QALY†
No vaccination	72,659,302	2,698,711	—	—	—
12-y-old girls	74,042,990	2,699,178	1,383,687	467	2,964
12-y-old girls and boys	78,707,825	2,699,327	4,664,835	149	Dominated
12-y-old girls plus 12- to 24-y-old females catch-up	74,815,667	2,699,343	–3,892,159	16	4,666
12-y-old girls and boys plus 12– to 24-y-old females catch-up	79,746,357	2,699,461	4,930,690	118	41,803
12-y-old girls and boys plus 12– to 24-y-old females and males catch-up	81,761,210	2,699,506	2,014,853	45	45,056

*Assumes cost of vaccination series is US $360 and duration of protection is lifelong. All costs are measured in 2005 US dollars, and costs and QALY are discounted at 3%. HPV, human papillomavirus; QALY, quality-adjusted life years.
†Compared with the preceding nondominated strategy. Strategy A is dominated if there is another strategy, B, that is more effective and less costly than strategy A.

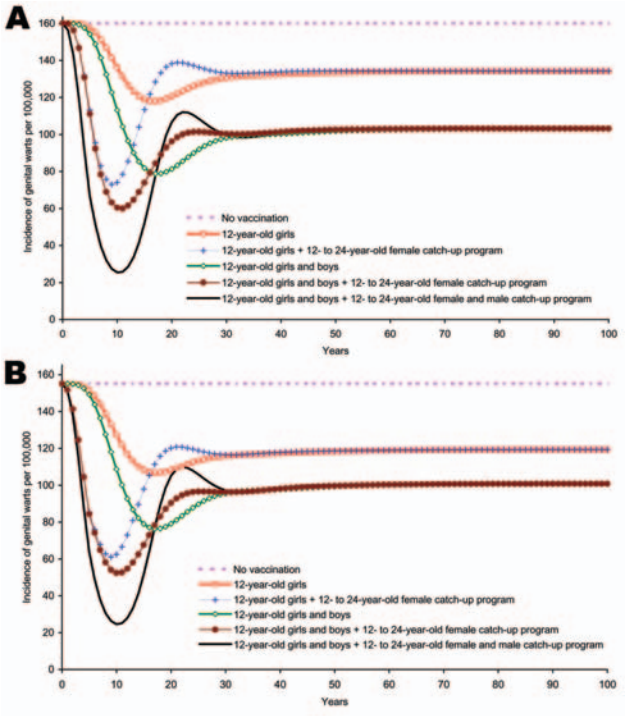

Figure 6. Sensitivity analysis. A) Incidence of genital warts due to human papillomavirus (HPV) 6/11 infection among boys and men ≥12 years of age, by strategy, 10 years' duration of protection. B) Incidence of genital warts due to (HPV 6/11 infection among girls and women ≥12 years of age by strategy, 10 years' duration of protection.

with CIN. However, the comparison is not perfect because our model tracks a population, whereas the model of Taira et al. follows a cohort. Hence, the composition of the numerators and denominators used in the ICERs differs between models. Finally, other methodologic differences occur between the 2 approaches that may explain the differences in results. For example, Taira et al. used steady-state values of HPV infection rates as inputs in their cost-effectiveness model, whereas we measured all outcomes over time, thereby capturing all the effects of transient dynamics generated from widespread vaccination. We also note that the results of the sensitivity analysis, when the effects of herd immunity and benefits of prevention of HPV 6/11 were removed, suggest that the ICER of the female vaccination strategy was $21,404/QALY, which is close to the value of $22,755/QALY reported in another study by Sanders and Taira (*33*).

An important finding from this analysis was that catch-up vaccination can substantially reduce disease in the short term. As a result, the female and male strategy that did not include a catch-up program was less effective and more costly.

One of the influential inputs was vaccine coverage. As female coverage rates decreased, male vaccination became more efficient. Another influential input in the analysis was the quality-of-life weights. The less HPV disease affected quality of life, the more the ICERs increased.

Duration of protection was also an influential parameter. Decreasing duration of vaccine protection to 10 years increased ICERs. However, the impact of this decrease may be mitigated by introducing a booster program. A reasonable approximation for how this program might fare would be to look at the sensitivity of ICERs to changes in vaccination cost. Thus, increasing the cost of the HPV vaccine series to $500 increased ICERs (Table 2). However, all nondominated (i.e., either are less costly or have lower ICERs than more effective strategies) female strategies remained cost-effective. Another influential parameter was the age vaccination was begun. Earlier vaccination resulted in greater benefits. F&M12+CUF&M was cost-effective ($42,697/QALY). However, vaccination by age 12 became less efficient, the higher the vaccination coverage was among older age groups.

Table 2. Sensitivity of incremental cost-effectiveness ratios (US $/QALY) of alternative HPV vaccination strategies to changes in inputs*

Input	F12 only	F12-only+ CUF only	F&M12+ CUF-only	F&M12+ CUF&M†
Baseline	2,964	4,666	41,803	45,056
Cost of vaccination series = $300	997	2,422	33,469	36,161
Cost of vaccination series = $500	7,553	9,900	61,250	65,810
Utility weights for CIN, CIS, GW = 0.97	5,241	7,739	82,700	83,714
Duration of protection = 10 y	Weakly dominated	21,121	54,755	54,928
Degree of protection against HPV 6/11/16/18 = 100%	2,094	4,187	Weakly dominated	51,436
Degree of protection against HPV 6/11/16/18 = 74%	4,273	5,403	39,990	43,930
Degree of protection against disease = 87%	3,116	4,922	40,269	43,974
Coverage with vaccination = 50%	2,636	4,221	23,862	36,235
Coverage with vaccination = 90%	3,449	5,269	Weakly dominated	100,418

*Unless specified otherwise, cost of vaccination series is US $360, and duration of protection is lifelong. QALY, quality-adjusted life years; HPV, human papillomavirus; F12-only, female vaccination by age 12; CUF, catch-up female vaccination for ages 12–24; F&M12+CUF only, female and male vaccination by age 12 and CUF; CIN, cervical intraepithelial neoplasia; CIS, carcinoma in situ; GW, genital warts.
†Compared with the preceding nondominated strategy. Strategy A is dominated if there is another strategy, B, that is more effective and less costly than strategy A. The strategy of female and male vaccination by age 12 that did not include a catch-up program was dominated. A strategy is weakly dominated if there is another more effective program that has a lower incremental cost-effectiveness ratio.

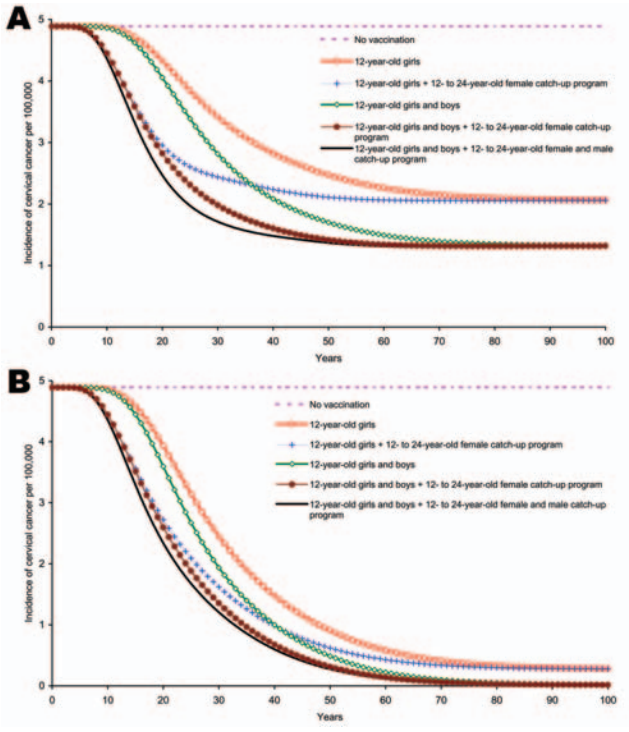

Figure 7. Sensitivity analysis. A) Incidence of cervical cancer due to human papillomavirus (HPV) 16/18 infection among girls and women ≥12 years of age with 50% coverage. B) Incidence of cervical cancer due to HPV 16/18 infection among girls and women >12 years of age with 90% coverage.

Vaccination shifted the age of infection and disease to older age groups. For example, the age of peak cervical cancer incidence increased after introducing vaccination. The upward shifting of age of infection is a common feature of many vaccination programs (*11*).

We believe our modeling approach has several strengths. First, we did extensive validation with existing data. The model is also flexible enough to incorporate better data as they become available. Second, this model accounts for actual screening practices in the United States. Third, because output from this model is population based, the comparison with national registry data is better aligned than comparison of cohort model output with population data (*6*). Finally, all equations and inputs for this model are available to facilitate replication of findings and independent review of the model.

Several enhancements and extensions are desired. First, more relevant data on the natural history of type-specific HPV infection and disease (e.g., HPV transmission probability per sexual contact) are needed. Also, given the influence utility weights have on ICERs, more studies are needed to collect health utilities data on HPV disease states.

Second, we modeled only 4 HPV types and their associated diseases and assumed that HPV types have independent natural histories with no interaction among them. If cross-immunity exists between HPV types, a vaccine that reduces the prevalence of 1 type may promote the prevalence of other types through a process of competitive release. If, however, current or prior infection with 1 HPV type facilitates concurrent or subsequent infection with another HPV type, or if the vaccine provides cross-protection against other types, HPV vaccination could have the additional benefit of reducing the prevalence of HPV infection of types not covered by the vaccine (*34*). The evidence on interaction among HPV types to date is mixed and inconclusive (*35–39*).

Third, we modeled neither coinfection after disease developed in a person nor the coexistence of CIN lesions due to multiple HPV types in the cervix. By accounting for all the cost of vaccinating persons with undetected disease and no benefits for them as a result of the protection against the type that did not cause the disease, our results are biased against the catch-up program.

Fourth, the model assumed that all persons have equal access to healthcare, be it vaccination, screening, or treatment. However, this assumption may not be realistic and may overestimate the benefits of vaccination if women who have limited access to screening are also less likely to get vaccinated. Further studies are required to determine whether those who do not get vaccinated are also likely not to get screened.

Table 3. Incremental cost-effectiveness ratios of alternative HPV vaccination strategies with varying start age of vaccination*

Strategy	Discounted total		Incremental
	Costs	QALY	$/QALY†
18-y-old women plus 18- to 24-y-old female catch-up	73,553,847	2,699,192	1,858
15-y-old female adolescents plus 15- to 24-y-old female catch-up	73,895,046	2,699,214	Weakly dominated
12-y-old girls plus 12- to 24-y-old female catch-up	74,815,667	2,699,343	8,357
18-y-old women and men plus 18- to 24-y-old female and male catch-up	77,535,383	2,699,385	Weakly dominated
15-y-old female and male adolescents plus 15- to 24-y-old female and male catch-up	78,455,750	2,699,404	Weakly dominated
12-y-old female and male adolescents plus 12- to 24-y-old female and male catch-up program	81,761,210	2,699,506	42,697

*Assumes cost of vaccination series is US $360, duration of protection is lifelong, and coverage rate of 50% among age 15- to 24-y-olds. HPV, human papillomavirus; QALY, quality-adjusted life years.
†Compared with the preceding nondominated strategy. Strategy A is dominated if there is another strategy, B, that is more effective and less costly than strategy A. A strategy is weakly dominated if there is another more effective program that has a incremental cost-effectiveness ratio.

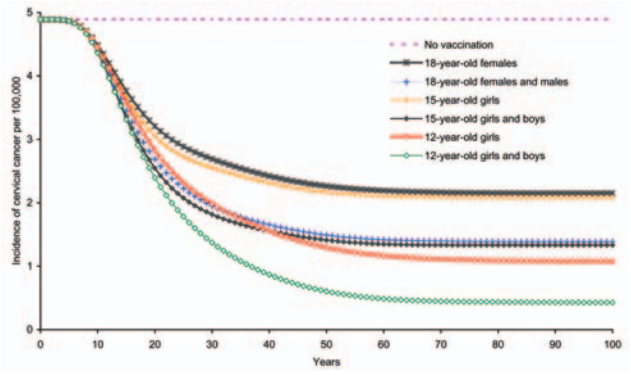

Figure 8. Effect of age that vaccination was begun on cervical cancer incidence due to human papillomavirus 16/18 infection among girls and women ≥12 years of age.

Fifth, the current version of the model focused on heterosexual transmission of HPV and did not incorporate transmission between homosexual and heterosexual persons. Sixth, the scope of the model has been limited to cervical diseases and genital warts. HPV infection has also been associated with recurrent respiratory papillomatoses and cancers of the anus, penis, vagina, vulva, and head and neck. As evidence becomes available, the scope of the model will be broadened to incorporate the potential effects of vaccination on these other HPV conditions. Including these diseases in the model would render more favorable ICERs for vaccination.

Seventh, we did not include death and productivity costs (lost wages), as was done in other analyses (*40*). Including these costs would further reduce ICERs.

Finally, we did not consider vaccination strategies that include infants or mid-adults because current data available on vaccine safety and efficacy are limited to ages 9–26 years (*18*). As data for these other age groups become available, the model can examine these strategies.

In summary, the results from this model suggest that in a setting of organized cervical cancer screening, a prophylactic quadrivalent HPV (16/18/6/11) vaccine can 1) substantially reduce genital warts, CIN, and cervical cancer, 2) improve quality of life and survival, 3) be cost-effective (across a reasonably wide range of assumptions) when administered to girls before age 12 years (with or without a catch-up program), and 4) have a cost-effectiveness ratio near or below (depending on the underlying assumptions of the model) that of several other recommended vaccines, when implemented as a strategy that combines vaccination of both girls and boys before age 12 with a 12–24 years of age catch-up program.

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Dr Elbasha is a director, scientific staff in the Department of Health Economic Statistics at Merck Research Laboratories. His research expertise includes methodologic and applied approaches to economic analysis and modeling of diseases.

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Appendix[1]

Demographic Model

The demographic model stratifies the population by gender and 17 age groups (12–14, 15–17, 18–19, 20–24, 25–29, 30–34, 35–39, 40–44, 45–49, 50–54, 55–59, 60–64, 65–69, 70–74, 75–79, 80–84, and ≥85 years). This age grouping permits age-specific inputs for patterns of sexual activity and cervical cancer screening and allows for age-specific outputs such as rates of cervical human papillomavirus (HPV) disease among girls and women, and genital warts among both males and females. Similar age groupings have been used by other sexually transmitted disease models (*1*,*2*). We further stratified each age group into 3 sexual activity groups (high, medium, low). We defined sexual activity according to the rates of sex partner change per year: low (0–1 per year), medium (2–4 per year), and high (≥5 per year). The number and the initial distribution of new entrants into the population by each gender were chosen to satisfy the Lotka characteristic equation with zero population growth (*3*). This allowed for variation in results across strategies to primarily be due to epidemiologic and program model features and not to changes in the demographic characteristics of the population over time (*3*).

The model starts with 12-year-olds entering the population at a gender-specific and sexual activity–specific rate, and transfers persons between successive age groups at an age- and gender-specific rate per year. The transfer rate depends on the rate of population growth, age- and gender-specific per capita mortality rate, and the number of years within an age group (*3*). We assumed equilibrium in the age distribution with zero population growth.

We set the population size in the model to 100,000 persons divided equally between females and males. Death rates for males and for females without cervical cancer were obtained from Vital Statistics data on gender- and age-specific mortality rates across all races for 2002 (*4*). Death rates among adolescent

[1]Refer to the Appendix References for citations in this Appendix.

girls and women with cervical cancer were obtained from Surveillance Epidemiology and End Results (SEER) Program data for 1997–2002 (*5*). Other demographic data were obtained from US Vital Statistics and the 2000 Census (*4*,*6*).

Epidemiologic Model

The epidemiologic model simulates HPV infection and occurrence of HPV disease (cervical intraepithelial neoplasia [CIN], cervical cancer, and genital warts) in the population. The acquisition of infection and progression from infection to disease follow a similar natural history structure, as assumed in previous models for HPV 16 and 18 (*7*). Building on these previous models, we also incorporated HPV 6 and 11 infection and genital warts and modeled infection by using 3 groups of HPV types (HPV 16/18, HPV 6/11, or HPV 6/11/16/18).

To simulate the occurrence of CIN, genital warts, and cervical cancer among those infected with HPV, we divided the population into distinct epidemiologic categories, according to the population's susceptibility to infection or the population's status with respect to infection, disease, screening, and treatment. These categories were similar to what has previously been defined in other models (*7*). The following, along with Figure 1, describes the movement of the population through these categories.

HPV Infection: Acquisition and Transmission

The epidemiologic model begins with 12-year-olds entering into the susceptible category *X*. Susceptible persons acquire HPV infection with a given type (HPV 16/18 infected only, HPV 6/11 infected only, or HPV 6/11 and HPV 16/18 infected) at a rate dependent upon gender, sexual activity group, age, and time. The rate at which persons of a given gender, sexual activity group, and age class at a given time acquire infection with a certain type (per capita force of infection) depends on the number of sexual partnerships and how these persons form partnerships with persons of the opposite sex, the fraction of infected sex partners, and the transmission probability per partnership. The formation of sexual partnerships is governed by a conditional probability sexual mixing matrix. Each cell in the mixing matrix represents the probability of a person of a given gender, sexual activity group, and age class having a sexual activity group, age-class specific partner from the opposite gender. In generating the mixing matrix, we used 2 parameters to depict the degree of mixing between age and sexual activity groups. This strategy allowed us to represent a wide range of mixing patterns in the matrix, from fully assortative (as for persons with like persons when parameter is zero) to proportionate (random partners when parameter is 1) mixing (*1*,*2*,*8*,*9*). The baseline parameter values for the rate of sexual partner change, stratified by gender, sexual activity, and age, were calculated by using data from the National Health and Social Life Survey (*10*) and methods outlined in Garnett and Anderson (*2*) (Appendix Table 1).

Once HPV transmission occurs, susceptible persons enter the category of infected persons, *Y*. Persons leave this category when the infectious period for HPV ends and enter the category of recovered persons with a fixed duration of immunity, *Z*. In the base case, we assumed that duration of natural immunity is lifelong. Unvaccinated infected persons clear infection at a type-specific per capita rate. Persons in the immune (*Z*) category who are susceptible to only 1 type can be infected with that type and move to another infected/immune category, *U*.

A fraction of susceptible persons are vaccinated and move into the vaccination category *V*. The movement of those vaccinated through the model is similar to the movement of those unvaccinated, shown in Figure 1A. The remaining fraction of persons who are not vaccinated remains in the susceptible category *X*. The vaccine-induced immunity of those in the vaccinated category may wane over time. As a result, persons can eventually move to the susceptible category *S* at an age- and gender-dependent rate. We assumed that when a person loses vaccine-derived immunity, he or she becomes susceptible to infection with any of the types. In the base case, the duration of vaccine-derived immunity is assumed to be lifelong. Vaccinated persons can also expe-

Appendix Table 1. Baseline behavioral parameter values for the sexually active population*

Activity group	Proportion of population, %		Relative partner acquisition rate
	Male	Female	
1 (highest)	2.56	2.56	11.29
2	11.47	11.47	2.96
3 (lowest)	85.97	85.97	1.0
Age group, y	Relative partner acquisition rate		Overall mean partner acquisition rate
12–14	0.11		0.1
15–17	1.18		0.3
18–19	2.42		1.3
20–24	2.61		
25–29	2.55		
30–34	1.72		
35–39	1.65		
40–44	1.53		
45–49	1.38		
50–54	1.25		
55–59	1.00		
60–69	0.61		0.5
≥70	0.44		

*Sources: Lauman et al. (*10*), Abma and Sonenstein (*11*).

Appendix Table 2. Baseline biologic parameter values for HPV disease categories*

Parameter	Base-case estimate	Source†
Progression in the presence of HPV 16/18 per year, %		
Normal to CIN 1	9.4	(RI)
Normal to CIN 1 to CIN 2	5.8	(*17*,RI)
Normal to CIN 1 to CIN 2 to CIN 3	3.5	(*17*,RI)
CIN 1 to CIN 2	13.6	(MRK)
CIN 2 to CIN 3 (severe dysplasia)	14.0	(*26*,*27*)
CIN 3 - severe dysplasia to CIN 3 - CIS 1	42.0	(*26*,*28*)
CIS 1 to CIS 2	5.0	
CIS 2 to LCC	18.0	
LCC to RCC	10.0	(*16*,*24*,*25*,*31*)
RCC to DCC	30.0	(*16*)
Progression in the presence of HPV 6/11 per year, %		
Normal to CIN 1	9.5	(RI)
Normal to CIN 1 to CIN 2	1.9	(RI)
Normal to CIN 1 to CIN 2 to CIN 3	0.0	(RI)
CIN 1 to CIN 2	0.0	(MRK)
Normal to genital warts	57	(*17*)
Mean duration of acute HPV infection, y		
HPV 16/18 infection	1.2	(RI)
HPV 6/11 infection	0.7	(RI)
Regression of HPV 16/18+ disease per year, %		
CIN 1 to normal/HPV	32.9	(MRK,*29*)
CIN 2 to normal/HPV	21.0	(*26*,*27*,*30*)
CIN 2 to CIN 1	13.3	(*27*)
CIN 3 (severe dysplasia) to normal/HPV	11.0	(*26*)
CIN 3 (severe dysplasia) to CIN 1	3.0	(*26*,*27*)
CIN 3 (severe dysplasia) to CIN 2	3.0	(*26*,*27*)
Regression of HPV 6/11+ disease per year, %		
CIN 1 to normal/HPV	55.2	(MRK)
Genital warts to normal/HPV	87.5	(*17*)
Age (y) and stage-specific cervical cancer mortality rates per year, 1997–2002, %		(*5*)
For LCC		
15–29	0.7	
30–39	0.6	
40–49	0.8	
50–59	1.9	
60–69	4.2	
≥70	11.6	
For RCC		
15–29	13.4	
30–39	8.9	
40–49	11.0	
50–59	10.1	
60–69	17.6	
≥70	28.6	
For DCC		
15–29	42.9	
30–39	41.0	
40–49	46.7	
50–59	52.7	
60–69	54.6	
≥70	70.3	

*HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; CIS, carcinoma in situ; LCC, localized cervical cancer; RCC, regional cervical cancer; DCC, distant cervical cancer.
†RI, R. Insinga, unpub. data; MRK, Merck, unpub. data.

rience a breakthrough infection and enter the category of infectious persons, *W*, at a per capita rate that depends on the degree of protection offered by the vaccine. Vaccinated persons can recover from an HPV infection at an age- and gender-specific rate by a factor that is different from the recovery rate for unvaccinated infected persons. Vaccinated persons then move to a category with fixed duration of immunity, *Q*. Persons in this category who are susceptible to 1 type can be infected with that type and move to another vaccinated infected/immune category, *P*.

No epidemiologic studies have estimated the probability of HPV infection transmission per partnership and by type. We assumed that this probability is higher for transmission from males to females (0.8) than that for transmission from females to males (0.7) (*12–15*). Using data on participants in the placebo arm of Merck's HPV vaccine clinical trials, we estimated mean duration of HPV infection before progression to CIN, or regression, at 1.2 years for HPV 16/18 and 0.7 years for HPV 6/11 (R. Insinga, unpub. data).

CIN, Cervical Cancer, and Genital Warts

CIN develops in infected girls and women at a specified rate and moves to the HPV disease categories of the model (Figure 1B). Several categories represent the true histologic health status of a woman: CIN grade 1 (CIN 1), CIN grade 2 (CIN 2), CIN grade 3 (CIN 3), localized cervical cancer (LCC), regional cervical cancer (RCC), distant cervical cancer (DCC), and cervical cancer survivors who are free from cancer. Women with CIN and cancer were further classified into undetected, detected, or treated categories. Two additional absorbing categories are for women who are no longer at risk for cervical cancer (*16*). These include the following: 1) women who have had a benign hysterectomy for reasons other than cervical cancer (at an age-specific rate) and 2) women treated and cured for cervical cancer. Finally, infection with the low-risk type can result in genital warts in females and males and move to the genital warts category, GW (*17*). We assumed women with benign hysterectomies can be infected and are at risk for genital warts (*18*). Women and men recovering from genital warts move to category *Z*.

We assumed all progression and regression rates to HPV and cancer states to be independent of age (*19–23*). Annual transition rates from HPV infection to clinically detectable CIN were calculated from studies by Winer et al. (*17*) and Insinga (R. Insinga, unpub. data). Several published reports were also used to estimate annual rates of CIN regression and progression to cervical cancer (*24–31*) (Merck, unpub. data). Incidence and regression rates for genital warts were obtained from Winer et al. (*17*) (Appendix Table 2). Hysterectomy rates; cervical cancer screening coverage, sensitivity, and specificity; and treatment efficacy were derived from several published studies (*32–40*) (Appendix Table 3).

Appendix Table 3. Hysterectomy, screening, and treatment parameters*

Parameter	Base-case estimate	Source
Hysterectomy rate, % per year		(*32*)
15–24 y	0.02	
25–29 y	0.26	
30–34 y	0.53	
35–39 y	0.89	
40–44 y	1.17	
45–54 y	0.99	
≥55 y	0.36	
Cervical cytology screening, excluding those with hysterectomy, % per year		(*33*)
10–14 y	0.6	
15–19 y	21.0	
20–24 y	44.8	
25–29 y	61.6	
30–34 y	54.9	
35–39 y	50.5	
40–44 y	48.1	
45–49 y	49.1	
50–54 y	51.1	
55–59 y	46.7	
60–64 y	42.5	
65–69 y	38.9	
70–74 y	29.6	
75–79 y	20.1	
80–84 y	11.1	
≥85	5.5	
Females never screened, %	5.0	
Liquid-based cytology specificity, %	94	(*34,35*)
Colposcopy sensitivity, %	96	(*36*)
Colposcopy specificity, %	48	(*36*)
GW patients seeking physician care, %	75	(*37*)
Symptom development, % per year		Assumed
Localized cervical cancer	4	
Regional cervical cancer	18	
Distant cervical cancer	90	
Eradication with treatment, %		
For CIN 1	96	(*38*)
For CIN 2	92	(*38*)
For CIN 3, CIS	92	(*38*)
For localized cervical cancer	92	(*39*)
For regional cervical cancer	55	(*39*)
For distant cervical cancer	17	(*39*)
Persistence of HPV after treatment for CIN or GW, %	34	(*40*)

*HPV, human papillomavirus; GW, genital warts; CIN, cervical intraepithelial neoplasia; CIS, carcinoma in situ.

Economic Parameters

All model costs were updated to 2005 US dollars by using the medical care component of the Consumer Price Index (*41*). The direct medical costs for screening and treatment for CIN, genital warts, and cervical cancer were based on administrative claims data and other sources (*42–44*). We measured the cost of cytology screening per unit time as the product of the cost per test, the test compliance rate, the frequency of administering the test per unit time, and the size of the unidentified population that is eligible for screening. We estimated the cost of following up on false-positive results of the cytology test as a function of the specificities of the cytology test and colposcopy procedure and the costs of colposcopy and biopsy. The cost of the HPV vaccine for 3 doses was assumed to be $360, which was consistent with HPV vaccination costs used in previous cost-effectiveness analy-

Appendix Table 4. Cost and quality-of-life parameters*

Parameter	Base-case estimate		Source
Costs of diagnosing and treating HPV disease			*(42–44)*
Genital warts	$489		
Liquid-based cytology screening	$99		
Colposcopy and biopsy	$318		
CIN 1	$1,554		
CIN 2/3, CIS	$3,483		
Localized cervical cancer	$26,470		
Regional cervical cancer	$28,330		
Distant cervical cancer	$45,376		
Quality-of-life weights (0–1 scale)			
CIN 1	0.91		*(47)*
CIN 2/3, CIS	0.87		*(47)*
Localized cervical cancer	0.76		*(47)*
Regional cervical cancer	0.67		*(47)*
Distant cervical cancer	0.48		*(46)*
Cervical cancer survivor	0.84		*(47,49,50)*
Genital warts	0.91		*(47)*
No condition	F	M	*(46)*
12–17 y	0.93	0.93	
18–34 y	0.91	0.92	
35–44 y	0.89	0.90	
45–54 y	0.86	0.87	
55–64 y	0.80	0.81	
65–74 y	0.78	0.76	
≥75 y	0.70	0.69	

*HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; CIS, carcinoma in situ; F, females; M, males.

ses (*7*). Productivity losses as a result of HPV disease or death were not included in the analyses (*45*).

Quality adjusted life years (QALYs) were measured by weighting survival time by the quality-of-life adjustment weights associated with each health state and integrating the sum of adjusted time in all these health states over the planning horizon. We measured survival time as the total number of years spent alive by the active population during a given period. The health utility values used to estimate QALYs were derived from various sources (*46–48*). Health utility values for diagnosed invasive cancer states were estimated by Myers et al. (*47*) at 0.76 for localized cancer and 0.67 for regional cancer; these values were derived from Gold et al. at 0.48 for distant cancer (*46*). We assumed that the quality of life for cervical cancer survivors after successful treatment would continue to be lower (0.76) than that of healthy women (*49*,*50*). Diagnosed and treated CIN 1 and CIN 2/3 states were assumed to have quality weights of 0.91 and 0.87, respectively (*47*,*48*). We assumed the quality weight for genital warts to be 0.91 (*47*) (Appendix Table 4).

Undiagnosed and asymptomatic HPV, CIN, and cancer states and successfully treated CIN states were assumed to have a quality-of-life weight similar to those of persons without these conditions. Gender- and age-specific quality weights for non-HPV disease states were also derived from Gold et al. (*46*). Time in these states was multiplied by the age- and gender-specific weights to reflect the variation of quality of life by age and gender groups. We assumed that quality of life did not vary by sexual activity groups. Finally, all costs and effects were discounted to present value at a rate of 3%.

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Spread of *Cryptococcus gattii* in British Columbia, Canada, and Detection in the Pacific Northwest, USA

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Cryptococcus gattii, emergent on Vancouver Island, British Columbia (BC), Canada, in 1999, was detected during 2003–2005 in 3 persons and 8 animals that did not travel to Vancouver Island during the incubation period; positive environmental samples were detected in areas outside Vancouver Island. All clinical and environmental isolates found in BC were genotypically consistent with Vancouver Island strains. In addition, local acquisition was detected in 3 cats in Washington and 2 persons in Oregon. The molecular profiles of Oregon isolates differed from those found in BC and Washington. Although some microclimates of the Pacific Northwest are similar to those on Vancouver Island, *C. gattii* concentrations in off-island environments were typically lower, and human cases without Vancouver Island contact have not continued to occur. This suggests that *C. gattii* may not be permanently colonized in off-island locations.

In 1999, *Cryptococcus gattii* emerged on Vancouver Island, British Columbia (BC), Canada, among residents, visitors to the island, and domestic and wild animal populations. Disease incidence on Vancouver Island plateaued at 36 cases/million population/year during 2002–2005, markedly higher than rates reported in other *C. gattii*–endemic areas (*1*,*2*).

Unlike the closely related species *C. neoformans*, a common opportunistic pathogen of immunocompromised hosts, *C. gattii* affects primarily immunocompetent persons. Two *C. gattii* serotypes, B and C, have been described (*3*). The fungus is acquired through inhalation of airborne propagules and may cause pulmonary and central nervous system disease. Activities that disturb colonized soil or trees may increase the likelihood of exposure (*4*). Disease acquisition likely also depends on host factors, including underlying lung conditions and oral steroid use (M. Fyfe, unpub. data).

In a study of Vancouver Island human *C. gattii* serotype B cases from January 1999 through December 2001, infection was most common in men and those >60 years of age. Chest radiograph showed single or multiple pulmonary nodules in 68% of patients. Symptoms included severe cough and shortness of breath, often accompanied by chills, night sweats, and anorexia. Approximately 20% of patients had cryptococcal meningitis (M. Fyfe, unpub. data). The median incubation period was ≈6–7 months (*5*).

C. gattii has been isolated from more than 10 different native tree species on Vancouver Island and from the surrounding soil and air (*6*,*7*; S.E. Kidd et al., in press). Despite sampling in areas both on and off the island, positive environmental isolates have, until recently, been confined to the Coastal Douglas Fir and very dry Coastal Western Hemlock biogeoclimatic zones along the east coast of Vancouver Island (*8*, Figure 1).

VGIIa and VGIIb are the most commonly identified genotypes among human, animal, and environmental isolates from Vancouver Island (*6*). The VGI genotype has been isolated less frequently from clinical and environmental samples (*6*,*9*; S.E. Kidd et al., in press).

The emergence of *C. gattii* infection on Vancouver Island, a temperate climate, was unusual because this

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species previously was associated only with tropical and subtropical climates (*10*,*11*). To facilitate surveillance activities, cryptococcal disease was made provincially notifiable in BC in 2003, formalizing laboratory reporting originally developed in response to disease emergence. A primary goal of surveillance was to monitor fungal spread to other areas of BC.

Vancouver Island is the largest island on the Pacific Coast of North America, covering 32,000 km^2, with a population of ≈700,000. It is separated from the BC mainland by the Strait of Georgia, a body of water ≈50 km wide that contains several smaller islands known as the Gulf Islands (Figure 1). Travel among Vancouver Island, the BC mainland, and the Gulf Islands is very common, with an estimated 17.3 million passengers transported on BC Ferries' Vancouver Island routes annually (*12*).

Until 2004, all human cases of *C. gattii* infection reported to the British Columbia Centre for Disease Control were among those living on or traveling to Vancouver Island during the year before symptoms appeared. In December 2004, the first evidence of disease in humans without exposure to Vancouver Island or other known *C. gattii*–endemic areas was detected. This article summarizes the epidemiologic and environmental support for disease acquisition in parts of the BC lower mainland and focal areas of the US Pacific Northwest.

Methods

Human Surveillance

We interviewed persons from whom *C. gattii* serotype B was cultured through December 31, 2005, and who did not report contact with Vancouver Island or other known disease-endemic areas. We conducted telephone interviews by using a standard questionnaire to assess demographic information, travel history, risk factors for infection, underlying medical conditions, and clinical symptoms. Risk factors and travel exposures were assessed for the 1-year period before the onset of illness (or before diagnosis, in asymptomatic cases). Health authorities in neighboring provinces (Canada) and states (USA), where the disease is not reportable, were provided with case investigation forms and encouraged to investigate cryptococcal disease in immunocompetent persons.

Animal Surveillance

Reports of animal cases were informally collected through veterinary networks in BC. Cases from the United States were reported by state veterinary epidemiologists. Infection in the animals was diagnosed histologically or identified as *C. gattii* serotype B by culture. None of the animals had traveled to Vancouver Island or other disease-endemic areas.

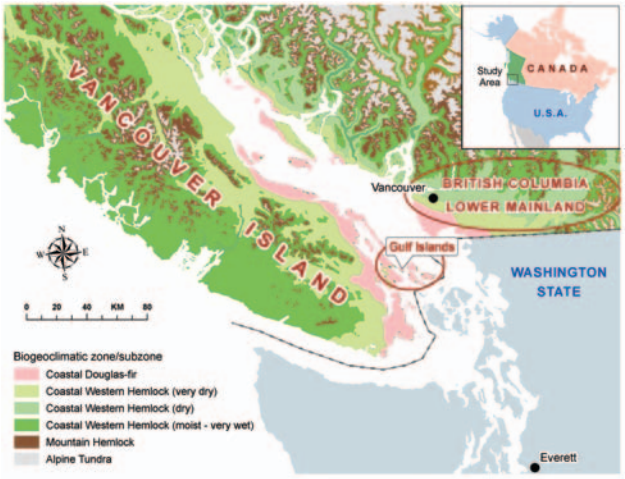

Figure 1. Biogeoclimatic and geopolitical boundaries within British Columbia.

Environmental Surveillance

From October 2001 through December 2005, environmental sampling was undertaken in the BC mainland, the BC Gulf Islands, and the US Pacific Northwest. We sampled 22 map grids as defined by the 1:50,000-scale National Topographic System of Canada (NTS) and US Geological Survey (USGS) mapping system. Geographic data were assembled as described elsewhere (S.E. Kidd et al., in press). Purposive sampling was conducted at selected sites and areas surrounding the homes of persons with *C. gattii* infection, those who reported travel to Vancouver Island and those who did not. Sampled environments included front and back yards, walking trails, public parks, and recreational areas. Trees, small woody debris, soil, air, and water were sampled as described elsewhere (S.E. Kidd et al., in press).

Sample positivity was scored binarily. *C. gattii* concentration was expressed as CFU/gram, CFU/m^3, and CFU/100 mL in soil, air, and water, respectively. The concentrations of multiple samples were described by the geometric mean and geometric standard deviation. When more than 1 sample was taken from a single sampling point (e.g., the same tree), only the first sample was included.

Identification and Genetic Characterization

We initially cultured the samples on Staib media (*13*). Resulting dark brown colonies were grown on canavanine-glycine-bromothymol blue (CGB) agar (*14*) to differentiate *C. gattii* from *C. neoformans* and then serotyped (Crypto-check, Iatron Laboratories, Tokyo, Japan).

Molecular types were identified by a previously described PCR-based restriction fragment length polymorphism (RFLP) method (*15*), which was adapted for further discrimination of variation within the VGII molecular type

(*9*). The *URA5* gene was amplified as previously described (*15*) and then completely digested at 37°C in a 20-μL reaction containing 1× NEB2 buffer, 1× bovine serum albumin, and 4 U each of *Hha* I, *Dde* I, and *BsrG* I (New England Biolabs, Inc., Ipswich, MA, USA). RFLP products were subjected to electrophoresis and visualized on a 3% agarose gel prestained with ethidium bromide. Control strains were used for each possible *C. gattii* RFLP pattern: WM179 (VGI), NIH444 (VGIIa), RB28 (VGIIb), WM161 (VGIII), and WM779 (VGIV). *C. neoformans* strains WM148 (VNI), WM626 (VNII), WM628 (VNIII), and WM629 (VNIV) were also included.

Multilocus sequence typing was performed for selected isolates by using methods previously described (*8*) with the use of 2 additional loci, PLB1 and IGS (*16*). We isolated total DNA from histopathology specimens (n = 3) by using the DNeasy Tissue kit (QIAGEN Inc., Mississauga, Ontario, Canada). Cryptococcal-specific PCR-RFLP was conducted as described above. The internal transcribed spacer region (ITS1-5.8S-ITS2) was amplified and sequenced for identification of *C. gattii*–specific polymorphisms (*17*).

Results

Epidemiology of Human Infection

Five persons with culture-confirmed *C. gattii*, 3 in BC and 2 in Oregon, did not report exposure to Vancouver Island or other cryptococcal disease–endemic areas (Figure 2). Case-patients 1 through 4 received a diagnosis or reported symptom onset from September through December 2004. Case-patient 5, who had a fatal infection, received a diagnosis in December 2005.

Case-patient 1 was a 47-year-old man living in BC who was hospitalized with cough, chills, night sweats, nausea, loss of appetite, muscle pain, headache, and neck stiffness. Both lung and brain cryptococcomas were identified. He had chronic hepatitis C infection and a history of drug addiction. At the time of infection, he smoked 20–40 cigarettes/day. His residence, a farmhouse undergoing significant renovations, was located in NTS grid 092G/05 on the coast north of Vancouver. Environmental exposures included yard and landscaping work at this property.

Case-patient 2 was a 48-year-old woman living in BC who experienced shortness of breath, fever, chills, headache, night sweats, loss of appetite, nausea, and muscle pain. A lung mass was identified by computed tomography. She had no known underlying health conditions. She resided on the BC lower mainland within NTS grid 092G/02; her last visit to Vancouver Island was 4 years before the onset of her illness. In the year before onset, considerable deforestation had occurred near her residence to clear land for housing developments. During this period, she also traveled ≈1 day/week to garden centers and nurseries within NTS 092G/02 to obtain shrubs, trees, and new topsoil for yard landscaping she carried out at her residence.

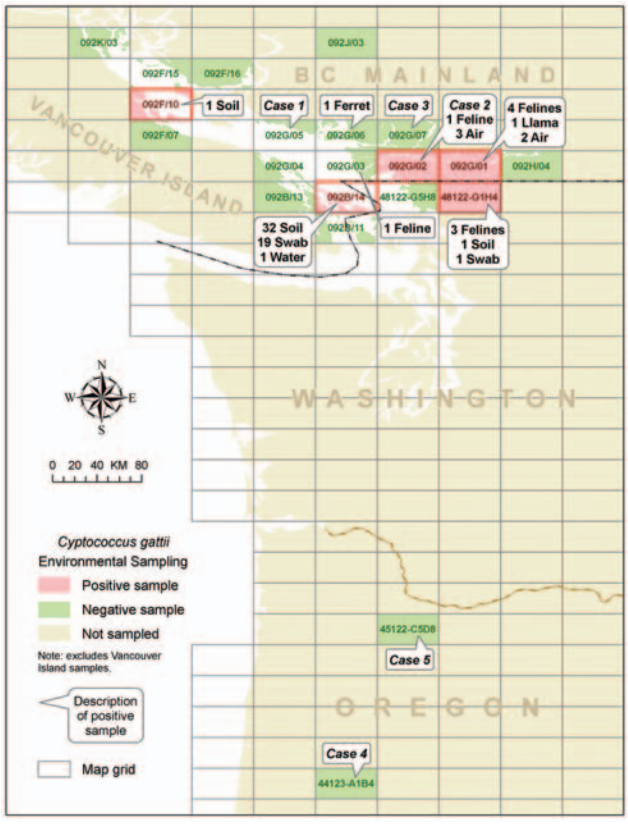

Figure 2. Location of human and animal *Cryptococcus gattii* cases and positive environmental samples found off Vancouver Island.

Case-patient 3 was a 73-year-old woman living in BC who had chronic renal failure requiring dialysis and a history of lung disease and breast cancer. She was asymptomatic; a cryptococcal lung nodule was identified radiographically after she had hip surgery in December 2004. No nodule was apparent on imaging conducted <2 months earlier, which suggests recent acquisition. The patient resided in NTS grid 092G/07. She last visited Vancouver Island 14 years before her diagnosis. She had reduced mobility and consequently little outdoor exposure.

Case-patient 4 was a 59-year-old man living in Oregon who began to experience cough, shortness of breath, fever, chills, weight loss, nausea, and muscle pain in December 2004. He had undergone a kidney transplant in September 2003 and reported scarring of lung tissue due to his occupation. His place of residence was located within USGS grid 44123-A1B4. He had not traveled outside Oregon in the year before symptom onset.

Case-patient 5 was an 87-year-old man living in Oregon (USGS grid 45122-C5D8). He was hospitalized in

December 2005 with meningitis, accompanied by fever, weight loss, and loss of appetite. His medical history included chronic lymphocytic leukemia, and he had taken oral steroids in the year before diagnosis. He had traveled to parts of Oregon, Washington, and Colorado during his exposure period.

Epidemiology of Off-island Animal Cases

In BC, a retrospective review of companion animal cases identified 8 culture-confirmed serotype B cases, which occurred in a ferret, a llama, and 6 cats. Specimens were collected from December 2003 through December 31, 2005; animal residences were located throughout the BC lower mainland (Figure 2).

In Washington, 3 cats with cryptococcal disease residing in USGS 48122-G1H4, close to the BC-USA border, were reported from February through June 2005 (Figure 2). All cases were diagnosed by histopathologic examination, and no cultures were obtained.

Environmental Sampling

From October 2001 through December 2005, 3% of 2,033 off-island environmental samples were positive for *C. gattii* (Table 1). Swab samples included 45% of samples from trees and other structures (n = 925), 38% from soil (n = 781), 15% from air (n = 304), and 1% from water (n = 23).

Five positive air samples were recovered from 2 focal areas of the lower mainland at 2 different times (Figure 3). The first 2 were collected from air in NTS grid 092G/02 on the same day in October 2002. No other positive samples were detected in 2003 despite further sampling at this site and others. In July 2004, a third positive air sample was collected from grid 092G/02 and 2 more from grid 092G/01. Despite extensive sampling in these areas and other parts of the BC lower mainland (n = 1,140), *C. gattii* was not isolated from colonized sources such as trees or soil.

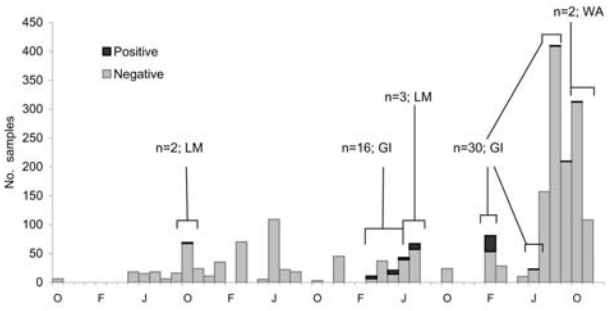

Figure 3. Summary of non–Vancouver Island environmental sampling effort, October 2001–December 2005. LM, lower mainland; GI, Gulf Islands; WA, Washington.

Among environmental samples taken outside Vancouver Island, *C. gattii* was most often recovered from the Gulf Islands (Table 1). NTS grid 092B/14 had the highest proportion of positive samples (52/220; 24%). Colonized sources were identified from a single Gulf Island within this grid in March, May, and June of 2004. In 2005, positive soil and tree samples were obtained in February, August, September, and October from 3 of the Gulf Islands (NTS grids 092B/14 and 092F/01) and Washington (USGS grid 48122-G1H4). From among 50 samples collected a month apart in Washington within 10 km of the BC border (USGS grid 48122-G1H4), a single soil sample and a swab of a fence post were positive. An additional 27 samples from other sites along the US side of the border (USGS grids 48122-G1H4 and 48122-G5H8) were negative, as were all 197 samples collected from several areas of Oregon (USGS grids 44123-A1B4 and 45122-C5D8). The geometric mean concentration of detected *C. gattii* among soil and air samples taken from

Table 1. Summary of sampling results from locations off Vancouver Island*

Sample type	BC mainland	Gulf Islands	Washington, USA	Oregon, USA	Total
Air	196	91	11	6	304
Negative	191	91	11	6	299
Positive (%)	5 (3)	0	0	0	5 (2)
Soil	408	250	28	95	781
Negative	408	217	27	95	747
Positive (%)	0	33 (13)	1 (4)	0	34 (4)
Swab	521	272	38	94	925
Negative	521	253	37	94	905
Positive (%)	0	19 (7)	1 (3)	0	20 (2)
Water	15	6	–	2	23
Negative	15	5	–	2	22
Positive (%)	0	1 (17)	–	0	1 (4)
Total	1,140	619	77	197	2,033
Negative	1,135	566	75	197	1,973
Positive (%)	5 (0)	53 (9)	2 (3)	0	60 (3)

*BC, British Columbia.

various sites outside Vancouver Island is summarized in Table 2.

Molecular Typing Results for Human, Animal, and Environmental Isolates

Table 3 and Figure 4 summarize the molecular subtyping results for human and animal isolates from persons and animals with no recent exposure to Vancouver Island, as well as environmental isolates obtained from off-island locations.

Of the 5 human cases, 3 were attributed to the VGIIa molecular type, 1 to the VGIIb molecular type, and 1 to the VGI molecular type. However, although all 3 molecular types have been identified among clinical and environmental isolates from Vancouver Island, multilocus sequence typing (MLST) results indicated that both the VGIIa and VGIIb strains from Oregon cases were genetically distinct from previously characterized Vancouver Island isolates (*9*,*16*). The Oregon case 4 VGIIa isolate differed from Vancouver Island VGIIa at 1 locus, while Oregon case 5 VGIIb differed from Vancouver Island isolates at 4–5 loci, where it was more similar to Vancouver Island VGIIa than VGIIb (Table 4).

Cryptococcal DNA isolated from the formalin-fixed, paraffin-embedded tissue of 3 cats in Washington belonged to the VGIIa molecular type. MLST profiles could not be determined in these cases because of the relatively poor quality and yield of DNA from the fixed tissue.

Most off-island environmental isolates that were typed belonged to the VGIIa molecular type. These included 4 of the 5 isolates from lower mainland air samples (the fifth could not be separated from contaminants) and 90% of 20 typed isolates from the NTS grid with the highest proportion of positive off-island environmental samples (092B/14). All tested environmental VGIIa isolates from BC and Washington possessed identical MLST profiles to those of representative isolates from Vancouver Island (Table 3).

Discussion

Surveillance for *C. gattii*, conducted in BC since the pathogen emerged on Vancouver Island, identified its spread to off-island locations in 2005. To date, 3 humans and 8 animals residing within the BC lower mainland who had not traveled to Vancouver Island or other known cryptococcal disease–endemic areas within the incubation period for disease have been found to have culture-confirmed *C. gattii* infection. All but 1 case belonged to the VGIIa subtype, the dominant genotype among clinical and environmental isolates from Vancouver Island (*6*,*9*,*16*). Human surveillance findings are supported by a parallel spread of *C. gattii* to animal populations on the BC mainland and positive air samples in this area. In addition, *C. gattii* infections with no recent link to Vancouver Island or other disease-endemic areas have been reported in 3 cats in Washington and 2 persons in Oregon. These cases represent the first evidence of local disease acquisition in this part of the United States. One historic case of VGIIa *C. gattii* (NIH444) was diagnosed in Seattle in the early 1970s; however, residence and travel history for the infected person are unknown (*6*,*9*,*16*).

Before the identification of new disease–endemic areas, all humans with *C. gattii* infection either lived within or traveled to the Coastal Douglas Fir and very dry Coastal Western Hemlock biogeoclimatic zones (Figure 1), located along the eastern edge of Vancouver Island. These zones are characterized by warm, dry summers and mild, wet winters and extend into the southern Gulf Islands and the BC lower mainland. Climates with comparable rainfall and temperature extend south into parts of Washington and Oregon in the United States (*18*). Franklin and Dyrness (*19*) identified plant communities similar to those in BC in the San Juan Islands and Puget Trough of Washington and the Willamette Valley in Oregon. These ecologic likenesses to BC support the idea that *C. gattii* may colonize niche areas of the US Pacific Northwest.

Although microclimate similarities exist, we could not determine whether the isolation of *C. gattii* from areas

Table 2. Detected concentration of *Cryptococcus gattii* in positive soil and air samples from British Columbia and Washington*

Concentration of *C. gattii*	Vancouver Island	BC mainland	Disease-endemic Gulf Island	Other Gulf Islands	Washington, USA
In soil (CFU/g)					
N	143	0	31	2	1
Geometric mean	193.7	–	632	33.9	70.8
Geometric SD	6.5	–	14.2	1.5	–
Range	10–36,350	–	24–192,952	24.8–45.5	–
In air (CFU/m^3) for comparable months of the year					
N	24	5	0	0	0
Geometric mean	43.3	10.8	–	–	–
Geometric SD	4.6	2.7	–	–	–
Range	2–875	5–38	–	–	–

*BC, British Columia; SD, standard deviation.

Table 3. Geographic location and molecular type associated with clinical and environmental isolates from locations off Vancouver Island*

Isolate	Date†	Host	Residence	Geographic grid	Culture/specimen no.	Serotype	Molecular type
Human							
1	Dec 2004	Human	BC mainland	NTS 092G/05	A4MR410	B	VGI
2	Mar 2005	Human	BC mainland	NTS 092G/07	A5MF738	B	VGIIa
3	Mar 2005	Human	BC mainland	NTS 092G/02	A5MR57	B	VGIIa
4	2005‡	Human	Oregon	USGS 44123-A1B4	KB11632	B	VGIIa§
5	Dec 2005	Human	Oregon	USGS 45122-C5D8	A6MR38	B	VGIIb§
Animal							
1	Nov 2003	Llama	BC mainland	NTS 092G/01	KB7092	B	VGIIa
2	Mar 2004	Cat	BC mainland	NTS 092G/01	KB8174	B	VGIIa
3	May 2004	Cat	BC mainland	NTS 092G/01	KB8686	B	VGIIa
4	Aug 2004	Cat	BC mainland	NTS 092G/01	KB10645	B	VGIIa
5	Nov 2004	Cat	BC mainland	NTS 092G/01	KB11242	B	VGIIa
6	Mar 2005	Cat	BC mainland	NTS 092G/03	KB11765	B	VGIIa
7	Sep 2005	Ferret	BC mainland	NTS 092G/06	KB14724	B	VGIIa
8	2005‡	Cat	BC mainland	NTS 092G/01	KB15181	B	VGIIa
9	Jul 2004	Cat	Washington	USGS 48122-G1H4	2004-7975¶	B	VGIIa
10	Jan 2005	Cat	Washington	USGS 48122-G1H4	2005-0550¶	B	VGIIa
11	Apr 2005	Cat	Washington	USGS 48122-G1H4	2005-4659¶	B	VGIIa
Representative environmental isolates (of 60 total)							
–	Oct 2002	Air	BC mainland	NTS 092G/02	KB2045	B	VGIIa
–	Oct 2002	Air	BC mainland	NTS 092G/02	KB2241	B	VGIIa
–	Jul 2004	Air	BC mainland	NTS 092G/02	KB9057	B	VGIIa
–	Jul 2004	Air	BC mainland	NTS 092G/01	KB9101	B	VGIIa
–	Jul 2004	Air	BC mainland	NTS 092G/01	KB9091#	B	–
–	Mar 2004	Swab, tree	Gulf Islands	092B/14	KB7892	B	VGI
–	Mar 2004	Soil	Gulf Islands	092B/14	KB7893	B	VGIIa
–	Feb 2005	Swab, tree	Gulf Islands	092B/14	KB11363	B	VGI
–	Jun 2005	Soil	Gulf Islands	092B/10	KB12611	B	VGIIa
–	Aug 2005	Soil	Gulf Islands	092B/14	KB13866	B	VGIIa
–	Sep 2005	Swab, fence post	Washington	USGS 48122-G1H4	KB14489	B	VGIIa
–	Oct 2005	Soil	Washington	USGS 48122-G1H4	KB14735	B	VGIIa

*BC, British Columbia; NTS, National Topographic System of Canada; USGS, US Geological Survey.
†Date of diagnosis for human and animal case-patients; date of sample for environmental isolates.
‡Month unknown.
§Multilocus sequence typing analyses showed differences between these isolates and characterized VGIIa and VGIIb strains from British Columbia.
¶DNA isolated from formalin-fixed, paraffin-embedded tissue.
#Isolate could not be cleaned from contaminants. Not retained.

outside Vancouver Island represents true colonization or transient dispersal of the organism at the time of sampling, such as through wind flow or mechanical vectors/fomites. Despite repeated sampling, no environmental source (e.g., tree, soil) of the VGIIa isolates detected in air on the BC mainland has been found. Either an undiscovered reservoir exists on the BC mainland or detectable airborne *C. gattii* was aerosolized and dispersed from known colonized sources, such as Vancouver Island or the Gulf Islands. Washington VGIIa environmental isolates, identical by MLST to those from BC, may represent recent dispersal from BC or independent foci of colonization.

Sampling sites on Vancouver Island have shown different patterns of *C. gattii* colonization over time (*4*). Transiently positive sites are characterized by a positive *C. gattii* isolation, followed by a series of negative samples over a period of months or years. Permanently colonized sites consistently yield positive samples. Intermittently colonized sites yield cycles of positive and negative samples over time, perhaps the result of population fluctuation above and below the limits of detection as the organism competes with local microbiota, while it adjusts to a new ecologic niche. Repeated sampling of non–Vancouver Island sites previously positive for *C. gattii* may show the extent of colonization and the likelihood of these areas becoming *C. gattii*–endemic.

The detected concentration of *C. gattii* in air and soil samples from the BC lower mainland and northern Washington was lower than in samples from Vancouver Island. Based on a comparison of geometric means, the detected *C. gattii* concentration in air samples from the mainland was 4-fold lower than in Vancouver Island air samples collected at the same time of the year (Table 2). *C. gattii* concentration in soil from northern Washington and 2 of the Gulf Islands was ≈5× lower than in soil from Vancouver Island. Only in a limited area of 1 particular

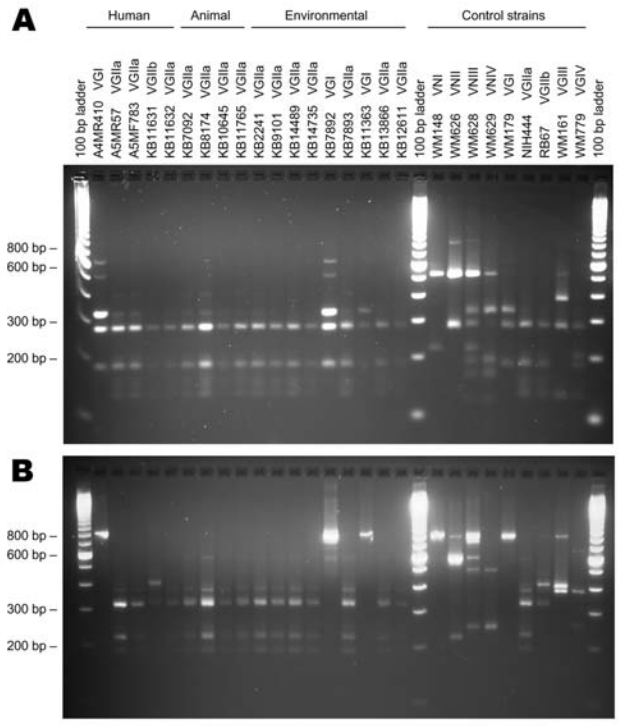

Figure 4. URA5-restriction fragment length polymorphism (RFLP) profiles for selected human, animal, and environmental *Cryptococcus gattii* isolates. A) URA5-RFLP to determine the molecular type using *Hha* I and *Sau96* I endonucleases (*14*). B) URA5-RFLP to confirm molecular type and determine VGII subtype, using *Hha* I, *Dde* I, and *BsrG* I endonucleases.

Gulf Island in grid 092B/14 was *C. gattii* concentration in soil higher (3.3-fold) than in soil from Vancouver Island.

While direct comparisons with infected persons living on or traveling to Vancouver Island are not possible because of the small number of off-island cases, humans affected by *C. gattii* in off-island environments may have a higher rate of serious underlying health conditions. Among cases in BC and Oregon, persons were affected by renal failure, chronic hepatitis C infection, and cancer (i.e., chronic lymphocytic leukemia, breast cancer). In an age-matched case-control study, persons from Vancouver Island with *C. gattii* infection were not significantly more likely than noninfected island residents to have had cancer (M. Fyfe, unpub. data) or liver disease (L. MacDougall, unpub. data). Persons with compromised immune systems may be more susceptible to infection with *C. gattii* at the lower concentrations observed in most off-island environments.

Even with ongoing surveillance in BC, the last reported case of symptom onset in a person with *C. gattii* infection who had not traveled to Vancouver Island was in December 2004. As of May 2006, no further cases had been detected, a finding at odds with the notion of permanent colonization. The onset of infections in the lower mainland of BC (September–December 2004) coincides with positive air samples on the mainland some months earlier (July 2004), given known variations in individual incubation periods (Figure 3) (*5*). Although animal cases did continue to occur during 2005–2006, environmental sampling attempts throughout 2005 did not detect the organism in the lower mainland. This result indicates either that permanent colonization did not occur in the sampled areas or that *C. gattii* was present below the limits of detection.

As on Vancouver Island, *C. gattii* in off-island areas was first detected in companion animals. Animal cases began to be regularly identified in March 2004, ≈6 months before human illness was reported in this area. As on Vancouver Island and in Australia, cats were affected more often than other companion animals (*20*,*21*). However, illness occurred in a ferret and llama, rare animals also infected early in the emergence on Vancouver Island, which may suggest that these species are particularly sensitive to infection. Despite substantial underreporting of animal cases, data from Vancouver Island suggest that animal cases exceeded human cases by almost 75%, highlighting their value as a sentinel indicator of disease (*20*).

Clinical and environmental isolates from the BC mainland, Gulf Islands, and northern Washington tested by MLST were identical to representative isolates from Vancouver Island at the 6 loci investigated (Table 4) (*9*,*16*). However, although isolates from the *C. gattii* human case-patients living in Oregon were typed as VGIIa and VGIIb, MLST analyses indicated that these isolates were genetically distinct from BC and Washington clinical and environmental isolates. A BLAST comparison (www.ncbi.nlm.gov/BLAST) to sequences from previous studies and those represented in the National Center for Biotechnology Information database identified Oregon strains as genotypically unique (*6*,*16*). We have not identified an environmental source of *C. gattii* within Oregon or any isolate possessing the same MLST profile as the Oregon clinical strains. Oregon strains could represent an independent population; alternatively, they may have evolved from the VGIIa or VGIIb strains previously described in BC or from VGIIa strains from California (*6*,*9*,*16*), either through random genetic drift or through sexual recombination. Recent studies suggest that same-sex mating can occur among cryptococcal isolates and that the VGIIa genotype may have arisen from same-sex mating between a strain of the VGIIb genotype and another unknown strain (*16*,*22*).

Conclusion

C. gattii infections have been shown in human and animal residents of the BC lower mainland and in Washington and Oregon in the United States, despite no

Table 4. Multilocus sequence typing (MLST) profiles of representative VGII strains from Vancouver Island compared with *Cryptococcus gattii* isolates from clinical and environmental sources in other locations*

Culture no.	Origin	Source	RFLP genotype	MLST profiles					
				URA5	LAC	FTR1	CAP1	PLB1	IGS
A1M R265	VI	Human	VGIIa	5†	3†	4†	2†	1†	1†
A1M R272	VI	Human	VGIIb	7‡	3	4	3‡	2‡	2‡
A5M R57	LM	Human	VGIIa	5	3	4	2	1	1
A5M F738	LM	Human	VGIIa	5	3	4	2	1	1
KB7092	LM	Animal	VGIIa	5	3	4	2	1	1
KB11765	LM	Animal	VGIIa	5	3	4	2	1	1
KB2045	LM	Air	VGIIa	5	3	4	2	1	1
KB13866	GI	Soil	VGIIa	5	3	4	2	1	1
KB11377	GI	Soil	VGIIa	5	3	4	2	1	1
KB14489	WA	Fence post	VGIIa	5	3	4	2	1	1
KB14735	WA	Soil	VGIIa	5	3	4	2	1	1
KB11632	OR	Human	VGIIa	12§	3	4	2	1	1
A6M R38	OR	Human	VGIIb	11¶	3	9¶	4#	1	3¶

*RFLP, restriction fragment length polymorphism; VI, Vancouver Island; LM, British Columbia lower mainland; GI, Gulf Islands; WA, northern Washington, USA; OR, Oregon, USA.
†Representative sequence accession nos.: URA5 = AY973136, LAC = AY973094, FTR1 = AY972024, CAP1 = AY971991, PLB1 = DQ777861, IGS = DQ777859.
‡ Representative sequence accession nos.: URA5 = AY973141, CAP1 = AY971981, PLB1 = DQ777862, IGS = DQ777860.
§Accession no. for unique sequences: URA5 = DQ777864.
¶Accession no. for unique sequences: URA5 = DQ777863, FTR1 DQ777857, IGS DQ777858.
#Representative sequence accession no.: CAP1 = AY971973.

contact with Vancouver Island or other known disease-endemic areas. These findings may represent an expansion of recognized areas where the disease is endemic.

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Cryptococcus gattii Dispersal Mechanisms, British Columbia, Canada

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Recent *Cryptococcus gattii* infections in humans and animals without travel history to Vancouver Island, as well as environmental isolations of the organism in other areas of the Pacific Northwest, led to an investigation of potential dispersal mechanisms. Longitudinal analysis of *C. gattii* presence in trees and soil showed patterns of permanent, intermittent, and transient colonization, reflecting C. gattii population dynamics once the pathogen is introduced to a new site. Systematic sampling showed *C. gattii* was associated with high-traffic locations. In addition, *C. gattii* was isolated from the wheel wells of vehicles on Vancouver Island and the mainland and on footwear, consistent with anthropogenic dispersal of the organism. Increased levels of airborne *C. gattii* were detected during forestry and municipal activities such as wood chipping, the byproducts of which are frequently used in park landscaping. *C. gattii* dispersal by these mechanisms may be a useful model for other emerging pathogens.

The basidiomycete fungal pathogen *Cryptococcus gattii* can infect the pulmonary and central nervous systems of humans and animals and was until recently regarded as a predominantly tropical organism (*1*,*2*). *C. gattii* began to emerge as a primary pathogen on Vancouver Island, British Columbia (BC), in 1999 (*3*). Most BC *C. gattii* cases were among humans or animals that had contact with the Coastal Douglas Fir and Coastal Western Hemlock xeric maritime biogeoclimatic zones of Vancouver Island (*3*–*5*). However, a number of infections in humans and animals with no travel to *C. gattii*–endemic areas were recently confirmed on the BC mainland and in Washington and Oregon in the United States (*6*), indicating dispersal within the Pacific Northwest. Strains of the *C. gattii* VGIIa genotype were isolated on 3 Gulf Islands that are clustered with the San Juan Islands between Vancouver Island and the mainland, as well from air samples on the BC lower mainland, and from soil and a fencepost in northern Washington (*6*). These findings indicated that, in contrast to a previous report (*7*), the Strait of Georgia/Juan de Fuca does not form a geographic barrier to *C. gattii* dispersal and that mechanisms for the dispersal of *C. gattii* exist in the Pacific Northwest. A recent gene genealogy study found evidence for global dispersal of *C. gattii* (*8*).

A large-scale study of the environmental distribution of *C. gattii* in the Pacific Northwest showed that focal areas were characterized by comparatively high concentrations (S.E. Kidd et al., in press). With the exception of 1 Gulf Island location with extremely high *C. gattii* concentrations in soil, these "hotspot" areas were all on Vancouver Island, which suggests that this is the primary area of *C. gattii* colonization. Approximately 10% of trees were positive for *C. gattii*, including >10 species. The highest airborne *C. gattii* concentrations were detected during the Northern Hemisphere summer but with propagules sufficiently small to cause infection present throughout the year. *C. gattii* was detected in fresh water and seawater in several locations.

While the association between *C. gattii* and exported tree species, particularly eucalypts, has been speculatively linked to its dispersal (*6*,*9*–*11*), no evidence for this has been found in BC. We investigated potential mechanisms for *C. gattii* dispersal and transmission within the Pacific Northwest region, given recent indications of an expanding distribution. We addressed the potential for *C. gattii* mobility in the environment through distribution of tree byproducts, aerosolization, water flow, and anthropogenic

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factors. An improved understanding of the mechanisms of dispersal and the risk of exposure to *C. gattii* could facilitate a model to effectively manage the emergence of cryptococcal and other infectious diseases in previously non–disease-endemic areas.

Materials and Methods

Environmental Sampling Strategies

Trees, soil, debris, wood chips, water, and air samples were collected according to previously described techniques, with limits of detection as previously described (S.E. Kidd et al., in press). Sampling was conducted in the environments surrounding the residences of those with reported infections, including homes, habitats, and nearby parks and wooded areas.

A 35-km traffic corridor connecting 2 highly visited provincial parks was sampled at ≈500-m intervals (publicly accessible areas only). This sampling was performed on 6 nonconsecutive days in October 2004 and included 92 sites, 64 located at the roadside (designated "road sites") and 28 located ≈100 m from the road (designated "forest sites"). For this series of samples, 169 trees of 8 different species were swabbed, and soil samples were collected from the rhizospheres of 77 of these trees. Global positioning system coordinates and tags were used to identify trees. Sites were designated positive by the presence of at least 1 tree or soil sample positive for *C. gattii.* Positive sites were resampled in June and December of 2005, including the original positive tree(s) and adjacent trees.

Many sites were sampled multiple times within 2–3 years to investigate the longitudinal colonization patterns of *C. gattii* in the environment. Except in these analyses, all data presented consider only the first swab, soil, and air samples collected at each sampling point to minimize statistical artifacts.

To investigate the effect of forestry activities on *C. gattii* aerosolization, air samples were collected during 2 independent tree removal efforts in a *C. gattii*–endemic area of Vancouver Island. A red alder (*Alnus rubra*) and a Douglas fir (*Pseudotsuga menziesii* var. *menziesii*) tree were removed by arborists on the same day in August 2002. The trees were tested for *C. gattii* colonization by swab and air sampling immediately before felling; in addition, air samples were collected at different tree heights during felling.

In collaboration with a municipal garden waste removal service, swabs of garden waste and nearby trees were collected from properties on 17 residential streets in October 2003. Air samples were collected around the garden waste and at the outlet of the wood chipper. Samples of wood chips were also collected from the wood chipper.

The wheel wells of vehicles used for Vancouver Island sampling were routinely swabbed; 63 swabs have been taken since July 2003. In addition, wheel wells of privately owned mainland- and Vancouver Island–based vehicles were swabbed to evaluate *C. gattii* carriage on vehicles not involved in the sampling effort. Two hundred vehicles in a mainland university campus parking lot and 200 vehicles in a Vancouver Island university campus parking lot were randomly selected for testing.

Eighty swabs of footwear worn by personnel participating in *C. gattii* sampling were taken at various Vancouver Island sampling sites during July 2003–June 2004. In addition, 9 swabs were taken from surfaces that came in contact with *C. gattii*–positive footwear (i.e., carpet, steel flooring, and a plastic bag). To investigate the potential for survival of *C. gattii* on footwear, shoes worn in a *C. gattii*–endemic area of Vancouver Island were stored for 333 days, with swabs taken periodically. The shoes were worn in nonendemic areas for short periods (≈4 h) on days 144, 153, and 154.

Identification and Genotyping of *C. gattii* Isolates

Cryptococci were initially identified by using Staib agar (*12*). Isolates were subcultured on malt extract agar and confirmed as *C. gattii* by using canavanine-glycine-bromothymol blue media (*13*), serotyping, or both (Iatron Laboratories, Tokyo, Japan). Sample positivity was scored both binarily and by the detected *C. gattii* concentration. Swab concentration was estimated by the presence of colonies on progressive streak lines, on a scale of 0 to 4+. The molecular types of selected isolates were identified by using previously described restriction fragment length polymorphism (RFLP) methods (*6*,*14*).

Data Analyses

Environmental sampling data was compiled by using Microsoft (Redmond, WA, USA) Access 2002, and statistical analyses were performed with SPSS 14 (SPSS Inc., Chicago, IL, USA). Geographic data were assembled, and sampling points were mapped on 1:50,000 scale National Topographic System of Canada (NTS) grids (S.E. Kidd et al., in press).

Results and Discussion

Patterns of *C. gattii* Colonization

We initially investigated longitudinal patterns of *C. gattii* tree and soil colonization. Figure 1 illustrates these colonization patterns. Consistently positive swab and soil results were observed for some trees and their rhizospheres over 2–3 years, and these were designated "permanently colonized." For other trees, an initial positive swab result was followed by a series of negative samples, which indicated a transient presence of *C. gattii* in these locations.

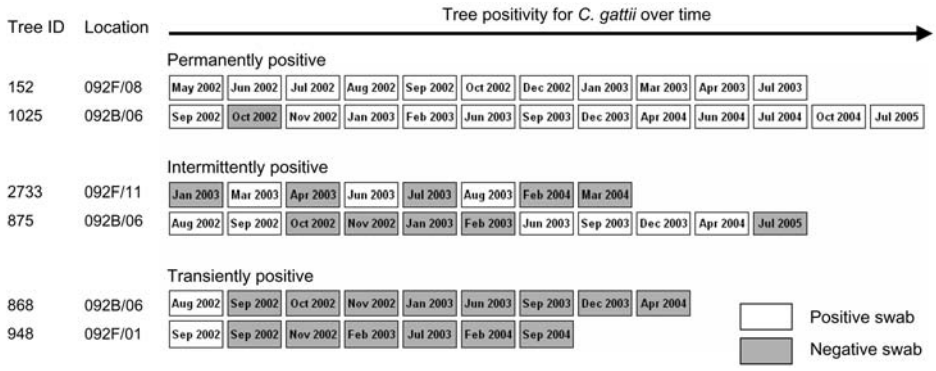

Figure 1. Example of longitudinal swab sampling profiles from trees designated permanently, intermittently, or transiently colonized with *Cryptococcus gattii*. Samples were collected during a 3-year period.

Intermittently positive swab and soil results were also observed, in which the series of samples effectively oscillated between positive and negative for *C. gattii* over time with no discernable seasonal pattern. This intermittent positivity was probably due to fluctuations in the cryptococcal population over time, above and below limits of detection. This situation might arise following the introduction of *C. gattii* to a new location or substrate.

We hypothesize that permanent colonization is established once the cryptococcal population reaches a critical mass. This concept forms the basis of a model for *C. gattii* introduction to new areas of the Pacific Northwest, in which the fungus must adapt to new microclimates and compete with local microbiota. Areas of recent *C. gattii* dispersal may go through a period of intermittent positivity before either failing to become colonized (transience) or becoming permanently colonized. Determinants of colonization resulting in high *C. gattii* concentrations include low soil moisture and organic carbon content (S.E. Kidd et al., in press).

Human-mediated *C. gattii* Dispersal

Using systematic sampling strategies, we acquired evidence of anthropogenic distribution of *C. gattii* in BC. We assessed *C. gattii* positivity at sites along a largely recreational traffic corridor that traverses both the Coastal Douglas Fir and Coastal Western Hemlock xeric maritime biogeoclimatic zones. Of the 169 trees sampled by swabbing on this route, 12 (7.1%) were positive for *C. gattii*, representing 10 sampling sites. Two of 77 soil samples (2.6%) from different sites were positive at low concentrations (25–50 CFU/g). No significant difference was observed between the *C. gattii* positivity of road sites and forest sites. Interestingly, the positive sampling sites were clustered at small towns, services, or attractions such as provincial parks (Figure 2). No sampling sites tested

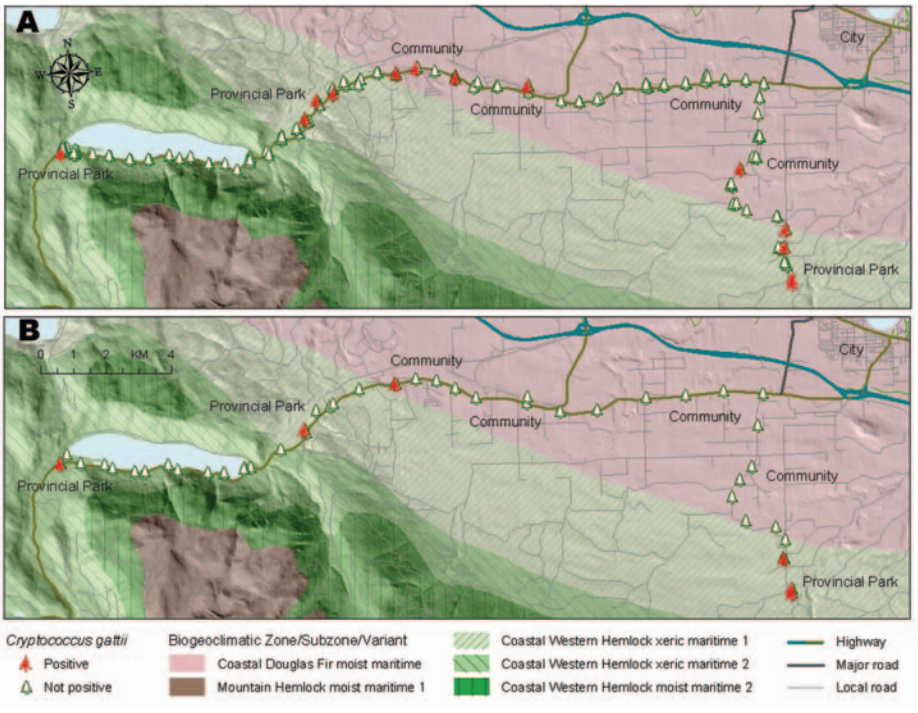

Figure 2. Distribution of positive and negative environmental samples for a systematic sampling along a 35-km traffic corridor traversing National Topographic System of Canada grids 092F/06 and 092F/07, highlighting transience of *Cryptococcus gattii* isolations. A) Sites of initial samples, collected in October 2004. B) Positive sites that were resampled in June 2005.

positive in areas of the sampling corridor where there was no apparent reason or safe location for a vehicle to pull off the road. These results support a model of *C. gattii* dispersal facilitated in part through human interaction with the environment.

In a resampling of trees and soil at these positive sites 8 months later, *C. gattii* was detected at only 6 of 12 previously positive sites (Figure 2). Specifically, only 2 of the 12 previously positive trees yielded positive swab results on the second sampling. Only 1 of the previously positive sampling sites remained positive at a third sampling in December 2005. This site, considered to be permanently positive, is located at the entrance to a highly visited provincial park (NTS grid 092F/08) where *C. gattii* is now regarded as endemic. RFLP genotyping indicated that all isolates from this sampling study belonged to the predominant genotype, VGIIa. Most trees and sampling sites in which *C. gattii* was initially detected appear to have been transiently or intermittently positive at that time, which is consistent with a model of recent dispersal, perhaps as a result of human activity.

Water sampling at a different provincial park (NTS grid 092F/07) also yielded evidence in support of anthropogenic *C. gattii* dispersal. The park contains a lake (≈550 km^2) with a boat launch, hiking trails, and facilities for camping and picnics; 170,900 persons visited the park in 2005 (D. Forman, pers. comm.). Forty water samples were collected at 4 park sites during 11 separate sampling trips in a 32-month period, and at least 1 positive sample was obtained on each sampling trip. Table 1 provides a summary of the sampling sites at this lake and the *C. gattii* concentration detected at each. The boat launch site was associated with the highest rate of sample positivity and *C. gattii* concentration, followed by the picnic site and a historic artifact site, all of which are high-traffic visitor areas. Within the campground, water samples were collected from a creek feeding into the lake near the picnic site. These positive creek samples probably account for some of the *C. gattii* isolated from lake samples at the picnic site, although the observed *C. gattii* concentrations from creek samples were far lower than that of the lake samples. By contrast, 6 water samples taken in areas of the lake with limited public access were all negative for *C. gattii*. Many swab and soil samples collected from the public sites were positive for *C. gattii* (data not shown). While *C. gattii* in the water from these locations may be in part seeded by contaminated soil or tree debris, we suggest that human activities in and around the lake contributed to the dispersal of *C. gattii* to these recreational areas.

Sampling in other areas of BC detected *C. gattii* in several bodies of fresh water and seawater around Vancouver Island, and viability assays indicate the organism's potential to survive for at least 1 year in fresh water and seawater (S.E. Kidd et al., in press). These data provide some insight into the mechanism of transmission of cryptococcal infection reported for a considerable number of wild marine mammals in the Strait of Georgia (*4*,*15*).

The wheel wells of vehicles used for sampling were routinely swabbed to further investigate the role of humans in dispersal. *C. gattii* was detected in 22 (35%) of 63 samples, including samples taken several weeks after return of the vehicle to a non–*C. gattii*–endemic area, after the vehicle had been professionally washed. In addition, *C. gattii* was detected on 10 (5%) of 200 privately owned vehicles on Vancouver Island and 1 (0.5 %) of 200 on the BC mainland. The isolates obtained from the Vancouver Island–based vehicles represented molecular types VGIIa, VGIIb, and VGI (including coisolation of VGIIa and VGIIb from 1 vehicle), while the isolate from the mainland-based vehicle represented VGIIa. While we have not assessed the *C. gattii* positivity of cars that are actively traveling between Vancouver Island and the mainland, vehicles could certainly be involved in the mechanical dispersal of *C. gattii* propagules. Approximately 8 million private and commercial vehicles are transported between Vancouver Island and the BC mainland each year (*16*), and given the potential for extensive travel beyond these areas, dispersal of *C. gattii* in the Pacific Northwest likely can be attributed at least in part to the use of vehicles.

C. gattii was detected in 43 (54%) of 80 swab samples from the footwear of persons participating in sampling. In addition, 5 (56%) of 9 swabs of surfaces contacted by positive footwear were positive for *C. gattii*, which indicates that the fungus can be transferred to contacting surfaces

Table 1. *Cryptococcus gattii* positivity and concentration among water samples collected from different lake sites at a highly visited provincial park located within the NTS grid 092F/07*

Sampling site	Total samples	Positive samples (%)	*C. gattii* GM concentration (CFU/100 mL)†	GSD
Boat launch (lake)	11	10 (91)	11.6	10.6
Picnic site (lake)	9	6 (67)	4.9	2.8
Historic site (lake)	10	7 (70)	5.0	24.3
Campground (creek)	10	4 (40)	0.8	2.8
Other sites, limited public access (lake)	6	0	–	–

*NTS, National Topographic System of Canada; GM, geometric mean for positive samples; GSD, geometric standard deviation for positive samples.
†Limit of detection of method: 0.2 CFU/100 mL (1 CFU/500 mL).

and may be redistributed to some extent by this mechanism. Similarly, passive transport of *C. gattii* by wild and domestic animals could be involved in dispersal.

The viability of *C. gattii* carried on footwear was investigated over time. Swabs from shoes worn for environmental sampling were positive at day 0 and were consistently scored as 2+ when unworn (up to day 144). The active wearing of the shoes reduced viable *C. gattii* levels; *C. gattii* was detected following activity on day 144 but not after activity on days 153 and 154. However, viable levels rebounded slightly by day 183 (1+) and remained detectable on day 333 (1+). Genotyping of isolates from this footwear showed isolates belonging to the VGIIa, VGIIb, and VGI subtypes, reflecting the diversity observed at the sampling site where the shoes became contaminated on day 0 (data not shown). While different footwear materials and activity patterns are likely to influence viability and dispersal of *C. gattii*, these observations suggest that footwear could serve as mechanical vectors for *C. gattii*.

C. gattii Dispersal through Forestry Activity

The concentration of airborne *C. gattii* was investigated during the scheduled removal of 2 trees in an area of Vancouver Island where *C. gattii* had been found. Both trees tested positive for *C. gattii* by swab and by adjacent air sampling done immediately before they were felled. Table 2 shows the detected *C. gattii* concentrations in air samples taken during tree cutting, limb removal, and chipping activities. All air samples, collected at varying heights, tested positive during the felling of both trees. Airborne concentrations increased during felling of the red alder, but no substantial change was observed for the Douglas fir. However, for both trees, air samples taken during branch chipping indicated much greater (10- to 140-fold) airborne *C. gattii* concentrations than were observed during quiescence. Aerosolization of *C. gattii* through such activities is likely to increase the risk of exposure and the dispersal of cryptococci through wind.

A log and a sample of wood chips from 1 of the removed trees were retained in the laboratory for 2 years, stored in sealed plastic bags at room temperature. Air samples taken close to the log after 1 and 2 years detected 25 CFU/m^3 and <5 CFU/m^3 *C. gattii*, respectively. Similarly, air samples taken at the opening of the wood-chips storage bag detected 2,256 and 1,494 CFU/m^3 after 1 and 2 years, respectively, indicating long-term aerosolization of propagules from these tree byproducts. *C. gattii* was also isolated from 2 samples of wood chips (2,143 and 145 CFU/g) collected from within a wood chipping machine during a municipal cleanup of garden waste. Wood debris sampled directly from the chipper blade yielded 18 CFU/g of *C. gattii*, and an air sample taken at the outlet of the chipper yielded 19 CFU/m^3 of *C. gattii*. Woodchips obtained from this service are used primarily to cover trails in the local parks (P. Crawshaw, 2003, pers. comm.). These data indicate that forestry activities and the distribution of tree byproducts may facilitate the mobility of *C. gattii* through both aerosolization and mechanical dispersal.

Residing within 10 km of sites of commercial soil disturbance or vegetation clearing has been reported as the most significant risk factor for *C. gattii* infection in domestic cats and dogs in BC (*5*). While we have not yet specifically tested the effect of soil disturbance on the aerosolization of *C. gattii*, we have observed that the highest concentration of *C. gattii* occurs within the top 15 cm of soil (S.E. Kidd et al., in press) and could potentially be aerosolized through both large- and small-scale soil disturbances such as deforestation, landscaping and gardening, vehicles traveling on dirt roads, or rain splash (*17*).

C. gattii Dispersal as a Model for Emerging Infectious Diseases

The emergence of *C. gattii* infection among humans and animals with no travel history to endemic areas raised the possibility of dispersal within the Pacific Northwest (*6*) and the observed colonization of *C. gattii* on wood products such as wood chips and mulch, in bodies of fresh

Table 2. Airborne *Cryptococcus gattii* concentration before and during contracted tree-cutting activities

Tree-cutting activity	Sampling method	*C. gattii* concentration in air (CFU/m^3)	
		Red alder	Douglas fir
Quiescent	Swab	Positive	Positive
	Air; Andersen 6-stage*	381	2,073
Limb removal	Air; Andersen 6-stage	5,707	940
	Air; Andersen 6-stage	3,622	1,279
Felling	Air; RCS-Plus,† 12–15 m above ground	906	294
	Air; RCS-Plus, 12–15 m above ground	881	213
Cutting limbs	Air; RCS-Plus, 6 m above ground	750	1,719
	Air; RCS-Plus, 6 m above ground	–	2,968
Cutting tree trunk	Air; RCS-Plus, 0–3 m above ground	21,250	225
Wood chipping	Air; RCS-Plus	53,125	21,250

*Limit of detection of method: 6 CFU/m^3.
†RCS, Reuter centrifugal sampler; limit of detection of method: 5 CFU/m^3.

water and seawater, in air, and in soil, suggests there could be several mechanisms for this dispersal (S.E. Kidd et al., in press). The unique opportunity for investigation of *C. gattii* as an emerging pathogen in BC has facilitated insight into the ecology and distribution of this pathogen. We believe the dispersal mechanisms of *C. gattii* could be applied as a model for other organisms.

The mechanisms of *C. gattii* dispersal discussed here are similar to those described in a number of reports for other mammalian pathogens. For example, *Coccidioides immitis* is similar to *C. gattii* in that it primarily colonizes soil, and disease is acquired by inhaling aerosolized spores. Coccidioidomycosis outbreaks have occurred as a result of soil disturbances as well as windborne dispersal. Such outbreaks were documented after a California earthquake (*18*), after separate point-source exposures among archaeology students in northern California and Utah (*19*,*20*), and after a windstorm in Kern County, California, that led to many coccidioidomycosis cases in non–disease-endemic areas of the San Joaquin Valley in California (*21*).

Blastomyces dermatitidis is the cause of blastomycosis outbreaks in humans and animals in Wisconsin (*22*,*23*), and dispersal occurs by way of rivers (*24–26*). This dispersal is similar to the isolation of *C. gattii* from bodies of water in BC. *B. dermatitidis* infection has also been reported in 2 captive California sea lions in the adjacent US states of Wisconsin and Illinois *(27*), although no related environmental sampling was discussed in these cases.

We have compiled evidence that suggests human-mediated dispersal of *C. gattii* may be important, including the detection of multiple *C. gattii* strains in the wheel wells of vehicles and on footwear. Footwear and farm vehicles were found to be involved in mechanical transmission of porcine reproductive and respiratory syndrome virus in Minnesota (*28*). Similarly, contaminated footwear and vehicles have been implicated in the spread of foot and mouth disease, resulting in the establishment of strict disinfection protocols in certain parts of the world (*29*,*30*). In addition, dispersal of the oomycete plant pathogen *Phytophthora ramorum*, causing sudden oak death in North America and Europe, is mediated by human activity as well as natural factors (*31–33*).

A specific route of *C. gattii* introduction to the Pacific Northwest has not been established, although 1 hypothesis implicates importation of contaminated trees (*9*,*10*). The data presented here indicate that *C. gattii* could also have been introduced by mechanical vectors such as vehicles or footwear, or by wooden pallets or crates (*34*) that are not routinely inspected for microbial contamination upon entry into Canada. Bird and animal migration may be involved in *C. gattii* dispersal through passive transport as well. Certainly, a large number of migratory birds pass through the disease-endemic area on Vancouver Island.

Given numerous possibilities for dispersal of *C. gattii*, until more study is done on conditions favoring or inhibiting de novo colonization, attempts to restrict further dispersal of the organism would be impractical and unlikely to succeed. However, public health, medical, and veterinary personnel, as well as the public in the Pacific Northwest and elsewhere, must be made aware of *C. gattii* and symptoms of infection so the disease can be identified correctly and managed effectively.

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Similar Biochemical Signatures and Prion Protein Genotypes in Atypical Scrapie and Nor98 Cases, France and Norway

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Isolates of atypical scrapie recently identified in sheep and goats in France were compared with Nor98 isolates reported in Norway. Western blot methods for characterization of the protease-resistant prion protein showed that all these isolates shared a unique biochemical signature: 5 groups of bands, including a characteristic band of apparent low molecular weight (11 kDa). This pattern could originate from the presence of 3 different protease cleavage products, including the 11 kDa most likely cleaved at both N- and C-sides of the protein. Genetic data, which strongly suggested the higher susceptibility of AHQ and $AF_{141}RQ$ animals in French cases, resembled earlier data from Nor98 scrapie.

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders that occur in sheep and goats (scrapie), cattle (bovine spongiform encephalopathy [BSE]), or humans (Creutzfeldt-Jakob disease). The biochemical marker of the disease is currently considered to be the accumulation of an abnormal isoform (PrPres) of the normal cellular prion protein (PrPc). PrPres can be identified by its partial resistance to proteases and insolubility in detergents. Classically, after pK treatment and Western blot (WB), PrPres exhibits a typical 3-band pattern comprising 18–30 kDa, whereas PrPc is totally digested (*1*,*2*).

Ovine susceptibility to scrapie is largely controlled by polymorphisms at the PrP gene (*prnp)*. The major polymorphisms associated with susceptibility or resistance are located at codons 136 (A or V), 154 (R or H), and 171 (R, Q, or H) (*3*,*4*). $V^{136}R^{154}Q^{171}$/VRQ, ARQ/VRQ, and ARQ/ARQ PrP animals are considered the most susceptible to scrapie, whereas homozygous or heterozygous AHQ and heterozygous ARR animals show only marginal susceptibility (*5*). ARR/ARR sheep are considered to be the more resistant (*4*,*6*), but after oral challenge with BSE agent they can accumulate PrPres in the spleen (*7*).

In 1998, a novel and unusual TSE type (called Nor98) was identified in sheep in Norway (*8*). A large proportion of animals were carriers of AHQ and $AF_{141}RQ$ alleles (*9*). The PrP WB signature in these cases differed from the known scrapie profile; the classic 3-band WB pattern was replaced by a multiband pattern with a prominent band of low molecular mass (≈12 kDa).

Since 2002, an active surveillance program for TSE has been implemented in small ruminants in European Union (EU) countries. As a result of this program, unusual TSE isolates were rapidly identified in sheep and goats in France, Germany (*10*,*11*), and Great Britain (*12*). These atypical TSE isolates had the following characteristics: 1) they came from sheep carrying PrP alleles reportedly associated with resistance to TSEs; 2) their rapid diagnostic test results based on PrPres detection showed discrepancies; and 3) they could not be readily confirmed by recommended Office International des Epizooties diagnostic methods (*10*). In this study we investigated a panel of 54 French atypical isolates from sheep (n = 51) and goats (n = 3) and compared their PrP genotypes and advanced PrPres biochemical signatures (WB electrophoretic mobility, epitope mapping, and PrPres deglycosylation pattern) with those of Nor98 cases.

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Materials and Methods

Small Ruminant Isolates

The biologic samples (Table) consisted of 54 brain stems from 51 sheep from France and 3 goats previously classified as having atypical scrapie and collected during the active surveillance program between 2002 and 2004. These samples were compared with Nor98 samples from 4 Norwegian sheep.

The *prnp* polymorphisms from these atypical cases were compared with a panel from animals with classic scrapie (74 clinically affected sheep obtained from 60 flocks between 2000 and 2002 and 60 scrapie-positive goats obtained from 13 flocks between 2002 and 2004. The animals with classic scrapie were not matched for age, breed, or flock structure with animals with atypical scrapie.

prnp ORF Sequencing

In each case, DNA was directly recovered from brain stem (30 mg) by using a commercial DNA extraction kit (Qiaprep DNeasy Minikit (QIAGEN, Courtaboeuf, France) according to manufacturer's recommendations. The complete open reading frame (ORF) sequence of the *prnp* gene was determined by sequencing both strands of 2 overlapping PCR fragments covering the complete ovine *prnp* ORF (primer 1F: GTGGGCATTTGATGCTGACAC, primer 1R: TGGTTGGGGTAACGGTACATG, Tm1 59°C, primer 2F: TCAGCCCCATGGTGGTGGCT, primer 2R: CTGCAGGTAGACACTCCCTCC, Tm2 61°C). The same primers were used for the goat samples.

The PCR products were amplified for 35 cycles (extension time 45 s) and allowed to migrate on 1% agarose gel. They were then purified, and both strands were sequenced. The appropriate software (SemanII, DNAstar, Monluçon, France) was then used to reconstitute the ORF sequence and align it with reference sequences from both ovine and caprine species.

PrPres Purification and Western Blotting

Samples were examined by TeSeE WB (Bio-Rad, Marnes la Coquette, France), according to manufacturer's recommendations. Briefly, 20% brain homogenate was incubated with pK and detergent solution for 10 min at 37°C before buffer B was added. Samples were then centrifuged at 15,000× *g* for 7 min and the pellet solubilized by incubation at 100°C for 5 min in 100 µL Laemmli solution completed (Bio-Rad) with 5% (v/v) β-mercaptoethanol and 2% (w/v) sodium dodecyl sulfate (SDS). Samples were centrifuged at 15,000× *g* for 15 min. The supernatants were then heated at 100°C for 5 min and subjected to electrophoresis. The undiluted sample (equivalent to 15 mg of tissue) was loaded onto homemade acrylamide SDS-polyacrylamide gels.

Gels (15% resolving gel and 4% stacking gel) were subjected to electrophoresis for 60 min at 200 V. The proteins were transferred onto a polyvinylidene difluoride membrane at 115 V for 60 min. The membrane was soaked successively with phosphate-buffered saline (PBS), ethanol, and distilled water; saturated with blocking solution for 30 min; and then incubated for 30 min at room temperature with Sha 31 (4 µg/mL in PBS-Tween [PBST]) against the YEDRYYRE (148–155) ovine PrP sequence (*13*). The membrane was then washed with PBST and incubated for 20 min with goat anti-mouse immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase diluted 1:10 in PBST. It was the subjected to the

Table. Atypical, Nor98, and classic scrapie isolates from sheep and goats and distribution of genotypes*

Genotype		Sheep isolates			Goat isolates	
		Atypical, n = 51 (no. of fully characterized isolates)	Nor98, n = 4	Classic, n = 74	Atypical, n = 3	Classic, n = 60
$AF_{141}RQ$/	ARR	16 (3)	1 (0)	–	–	–
	ARQ	9 (3)	–	5	–	–
	$AF_{141}RQ$	7 (3)	1 (0)	–	–	–
	VRQ	3 (0)	–	4	–	–
AHQ/	ARR	2 (1)	–	–	–	–
	AHQ	2 (0)	2 (1)	–	1 (1)	–
	ARQ	1 (0)	–	–	1 (1)	–
	ARH	1 (1)	–	–	–	–
	VRQ	1 (0)	–	–	–	–
ARR/	ARR	6 (3)	–	–	–	–
ARQ/	ARR	2 (2)	–	–	–	–
	ARQ	–	–	29	1 (0)	60
	ARH	–	–	13	–	–
ARH/	ARH	1 (1)	–	3	–	–
VRQ/	ARQ	–	–	10	–	–
	VRQ	–	–	9	–	–
	ARH	–	–	1	–	–

*Amino acids encoded at positions 136, 141, 154, and 171 are indicated.

enhanced chemiluminescence WB detection (Amersham or Supersignal, Pierce, Orsay, France) and visualized by using the Versa Doc image analysis system (Bio-Rad).

MW Determination

A panel of 20 of these samples from 17 French sheep with atypical cases, 2 French goats with atypical cases, and a Norwegian sheep with Nor98 (Table) were selected for detailed molecular characterization. The apparent molecular weights (MWs) were determined with a protein standard (B2787; Sigma, Saint Louis, MO, USA). Each band was measured (apparent MWs and proportions) by using Quantity One software (Bio-Rad).

Epitope Mapping

Different monoclonal antibodies (MAbs) were used for detection of PrPres fragments: Sha 31 (4 μg/mL in PBST), P4 (1/2,500 in PBST) (R-Biopharm, Saint-Didier Au Mont d'Or, France), 4F2 (1/2,500 in PBST), and 99/97.6.1 (1/2,500 in PBST). They recognized the following respective ovine sequences: YEDRYYRE (148–155) (*13*), WGQGGSH (93–99) (*14*),QPHGGGW (62–93), and 99/97.6.1 YQRE (221–224) (J. Langeveld, unpub. Pepscan data). The membranes were washed and then incubated with peroxidase-labeled conjugates against mouse Ig (1/2,500 in PBST) (Ozyme, Saint Quentin/Yvelines, France).

Deglycosylation Experiments

Deglycosylation experiments were performed on 6 French and 1 Nor98 isolates with PNGaseF, following the Ozyme manufacturer's instructions (P0704S) and TeSeE WB protocol for sample purification. Briefly, after PrPres purification, the pellet was solubilized by incubating at 100°C for 10 min in glycoprotein denaturing buffer 1× instead of Laemmli (Bio-Rad) solution. Samples were treated with PNGase F for 1 h at 37°C (reaction buffer 1×, 10% NP-40, PNGase F); buffer B was then added. Samples were centrifuged at 15,000× *g* for 7 min, and the pellets were solubilized in 100 μL completed Laemmli solution by incubation at 100°C for 5 min. Samples were then centrifuged at 15,000× *g* for 15 min, and the supernatants were heated at 100°C for 5 min before electrophoresis.

Results

All the atypical cases detected in France by the surveillance program were initially identified by an ELISA rapid diagnosis test (TeSeE Bio-Rad). The brain stem samples in our atypical scrapie panel (n = 54) invariably gave negative results with modified SAF (Scrapie-associated fibrils) Immunoblot (*10*,*15*). However, the 51 sheep and 3 goat isolates analyzed gave positive results with the highly sensitive WB method (TeSeE Bio-Rad), which used the classic pK concentration for sample digestion and Sha31 MAb for PrPres detection.

PrP Genetics of Classic and Atypical Cases

The *prnp* genotypes at codons 136, 141, 154, and 171 are shown in the Table. Most (87.8%) samples from sheep with classic scrapie were observed in genotypes with combinations of the ARQ, ARH, and VRQ alleles. A small proportion (12%) of classic scrapie cases were observed in $AF_{141}RQ$ carriers, but no classic scrapie was found in animals carrying the AHQ or ARR allele.

A large proportion (82.3%) of animals in the atypical sheep scrapie group carried the $AF_{141}RQ$ (n = 35, 68.6%) or AHQ (n = 7, 13.7%) allele. An unusually high proportion of atypical cases were ARR heterozygous (n = 20, 39.2%), but in 16 cases the ARR allele was associated with either the $AF_{141}RQ$ or AHQ allele. Six of the atypical cases were ARR/ARR homozygous (11.8%).

Only 3 of the atypical cases had genotypes combining the ARQ, ARH, and VRQ alleles association, which was strikingly different from the group of classic sheep scrapie cases. Similarly, only 4 animals (7.8%) carried the highly susceptible VRQ allele, and in all cases this was associated with the $AF_{141}RQ$ allele. Two of the 3 goats with atypical cases carried the AHQ allele (1 homozygous and 1 heterozygous), whereas none of the 60 sheep with cases of classic scrapie carried the AHQ allele (Table). Taken together, these data strongly suggest that AHQ and $AF_{141}RQ$ animals are more susceptibile to atypical scrapie than to classic scrapie.

PrPres WB Pattern of Atypical Scrapie and Nor98 Isolates

All the atypical isolates showed a complex multiband pattern that differed dramatically from the 3-band pattern observed in classic scrapie (Figure 1A, B). Five major bands (designated I to V according to increasing electromobility) could be distinguished in all atypical cases, irrespective of genotype or species (sheep or goat); in all 54 cases, a V band was clearly observed around 11 kDa.

Twenty isolates, from 17 sheep with various genotypes, 2 goats, and 1 Nor98 sheep (Table), were subjected to repeated electrophoresis to measure the bands' apparent form. According to our measurements, the patterns from the 19 French atypical cases were very similar to each other and indistinguishable from those from the Norwegian Nor98 isolate (Figure 1C).

However, a slight variability could be observed in the apparent MWs between cases. Variations of the WB method were further examined in repeated runs of PrPres isolated from a classic scrapie isolate to assess their possible significance. The analysis of 15 different runs of such

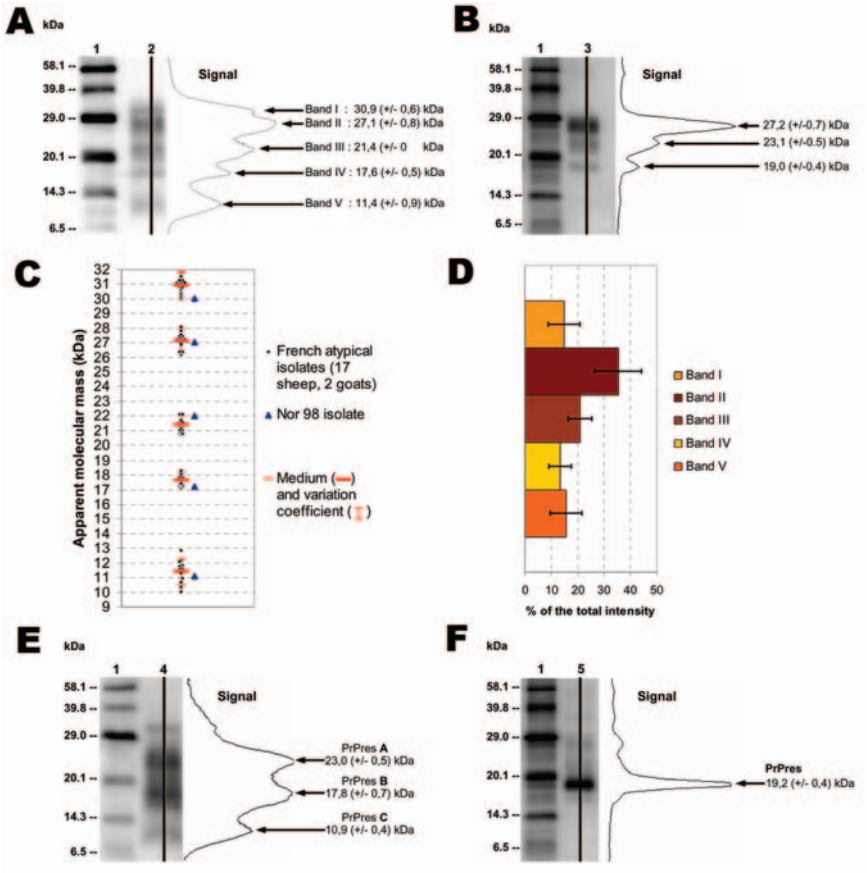


Figure 1. Atypical scrapie and Nor98 isolates PrPres Western blot pattern. Western blot (WB) profile in atypical (A, lane 2) and classic (B, lane 3) scrapie isolates with curves of chemiluminescence measured along the lane and corresponding apparent MWs (MWs), assessed by Bio-Rad Quantity One software analysis after signal capture using Versa Doc5000. Molecular weight (MW) standard (lanes 1). WB profiles of French atypical isolates in sheep (n = 17) and goats (n = 2) were compared with those of a Nor98 isolate. Apparent molecular masses (C) and proportions (D) of bands I to V were assessed from 3 independent runs for each sample by Bio-Rad Quantity One software analysis after signal capture using Versa Doc5000. Apparent MWs are measures for each of the atypical scrapie isolates, and proportions of bands are the means and standard deviations in the 19 atypical scrapie isolates. WB profiles of PrPres after PNGase deglycosylation with curves of chemiluminescence in atypical (E, lane 4) and classic (F, lane 5) scrapie isolates. Apparent MWs were estimated by comparison with a MW standard (lane 1) from 10 independent runs.

a sample showed a variation coefficient (standard deviation divided by mean) of 2.1% (±0.1%) for the 3 bands. The observed variations in the individual measures for atypical scrapie samples were 2.0% (±1.0%) for bands I to IV and 4.5% (±3.0%) for band V. These findings strongly suggest that the observed variations between the different samples were probably due to the method rather than to significant differences between samples.

The proportion of total PrPres WB signal represented by each band in the same panel of 19 French atypical cases was measured (Figure 1D**)**. Band II was significantly more intense (mean 35%) than band III (mean 20%) or bands I, IV, and V (means 10%–15%). Two individual peaks could be identified in bands II and III in runs and lanes with the highest resolution. These 2 peaks were located at 28.5 (±0.6) and 26.6 (±0.55) kDa in band II and at 22.5 (±0.8) and 20.9 (±0.4) kDa in band III (Figure 2, small arrows).

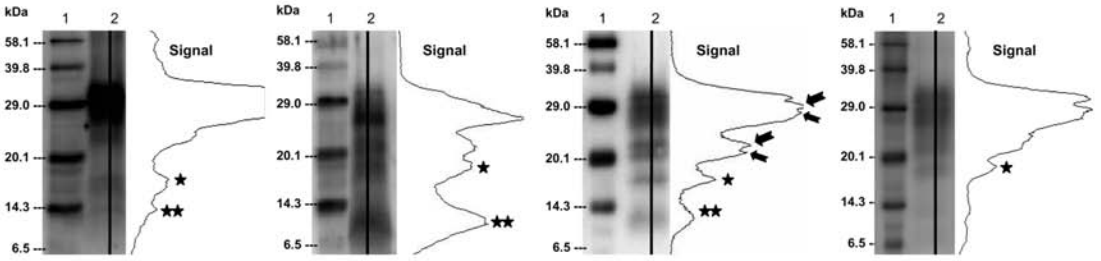
PrPres Deglycosylation and Epitope Mapping

Deglycosylations experiments with PNGase before WB analysis were then conducted to investigate the origin of this complex banding pattern. Because of the limited amount of field-collected material (brain stem only) and their low PrPres levels, only 6 atypical sheep isolates and 1 Nor98 isolate could be investigated. A similar pattern of 3 bands at 23.0 (±0.5), 17.8 (±0.7), and 10.9 (±0.4) kDa (referred to as A, B, and C forms, respectively) (Figure 1E) was observed in all 7 samples. PNGase treatment of classic scrapie cases resulted in a single band at 19.2 (±0.4) kDa (Figure 1F), which is consistent with already published data.

The biochemical pattern was characterized by epitope mapping of PrPres using 4F2 (62–93), P4 (93–99), Sha 31 (148–155), and 99/97.6.1 (221–224) (Figure 2). Bands I to III were strongly recognized by all 4 MAbs in our panel, which indicated that the 3 bands at least contained the 85– to 155–amino acid sequence. Band IV was clearly recognized by P4 and Sha31 antibodies and faintly by 4F2 and 99/97.6.1. Band V was not recognized by the more C-terminal 99/97.6.1 antibody, which suggests that this band corresponds to a C-terminal cleaved PrPres fragment but was labeled by the 3 other MAbs, although more weakly by the 4F2 antibody.

Discussion

In this study, we characterized a series of TSE isolates from French sheep and goats originally classified as having atypical scrapie cases (*10*) on the basis of discrepancies between rapid diagnostic tests and confirmatory methods used to detect PrPres in brain stem samples. Similar dis-



Biochemical pattern	4F2	P4	Sha31	99/97.6.1
Band I	+	+	+	+
Band II	+	+	+	+
Band III	+	+	+	+
Band IV [★]	+/-	+	+	+/-
Band V [★★]	+/-	+	+	-

Figure 2. Western blot profiles of PrPres in an atypical scrapie isolate (lane 2) detected by using N-terminal (4F2, P4), central (Sha31), or C-terminal (99/97.6.1) monoclonal antibodies. Molecular weight (MW) standard (lane 1). Immunoreactivities obtained with each antibody on 10 different atypical scrapie isolates are indicated (+, strong, ±, low, -, absent).

crepancies had been observed in the early description of so-called Nor98 scrapie isolates (*8*). The long-debated hypothesis that atypical cases and Nor98 cases could be only artifacts and not true TSE was recently ruled out by the successful transmission of 10 of these French atypical isolates and 3 Nor98 isolates to transgenic mice overexpressing the ovine PrP (V_{136} R_{154} Q_{171} allele) (*16*). Similarly, PrPres could be detected by using the TeSeE Bio-Rad WB method for all the French atypical and Nor98 cases studied here.

Original PrPres WB Signature

We found that PrPres showed a unique biochemical signature in all cases (54 French atypical and 4 Nor98 isolates) in comparison to classic scrapie, with 1) a multiband pattern with a characteristic band of low MW (11 kDa) and 2) 3 distinct deglycosylated PrPres forms of 23, 18, and 11 kDa (A, B, and C fragments, respectively). Given the theoretical MW of ≈22.8 kDa of the mature ovine PrP protein, the results obtained with all 4 antibodies in our panel, and the C-terminal 99/97.6.1 epitope undetected from only band V, the following hypothetical sequences of the A, B, and C fragments can be inferred (Figure 3A ;[*17*]). The PrPres fragment A (23 kDa) might correspond to a native (uncleaved or marginally cleaved by pK treatment) PrP fragment. The PrPres fragment B could be N terminally cleaved (nearby 4F2 epitope), as in classic scrapie, although cleavage of the C-terminal end cannot be fully excluded. While this scenario is already suggested by the faint labeling with 99/97.6.1 antibody, it would also be consistent with the observation that, despite the presence of the P4 epitope, PrPres B apparently has a lower mass than PrPres in ovine BSE (*18*). The C fragment (11 kDa) could correspond to an N (nearby 4F2 epitope) and C terminally cleaved PrPres protein.

If one assumes that the PrPres glycosylation process could be similar in classic (+3.8 or +7.9 for monoglycosylation and biglycosylation, respectively) and atypical scrapie cases, the theoretical MWs of the unglycosylated, monoglycosylated, and biglycosylated forms that could be derived from A, B, and C fragments can be reconstituted and compared with the banding pattern observed with different antibodies, such as Sha31 MAb (Figure 3B). Glycosylations of the 23-kDa A form would result in bands at 26.8 kDa and 30.9 kDa and those of the 18-kDa B form in bands at 21.6 and 25.7 kDa. These forms are consistent with bands I to IV, as well as with the presence of 2 distinct PrP forms detectable in both band II and band III in WB with the highest resolution (Figure 3B). The absence of detectable bands at 15 kDa and 19 kDa with any of the antibodies tested could suggest that the C form would only be present in the unglycosylated form. This lack of glycosylation would be consistent with a C-terminal cleavage of this PrP form upstream from the N-glycosylation sites (amino acids 184 and 200). Taken together, these hypotheses could explain the unique WB pattern identified in all the French atypical and Nor98 isolates studied here (Figure 3B). However, other hypotheses, such as the existence of random pK-digested fragments resulting in 3 major PrPres with variable sequences after deglycosylation, cannot be fully excluded.

These hypotheses need to be considered in the light of results recently published by Klingeborn et al. (*19*). After purification of PrPres, including a pK treatment at 100 μg/mL for 1 h at 37°C, and concentration by precipitation with trichloroacetic acid, these authors detected 2 PrPres

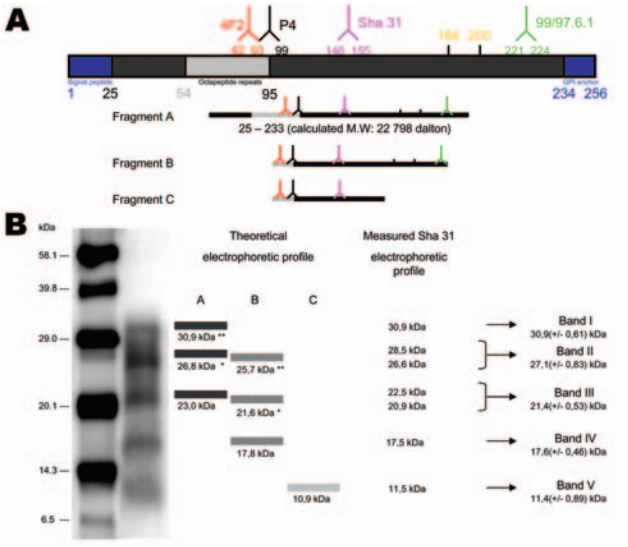


Figure 3. A) Schematic representation of ovine PrPc with location of epitopes recognized by the monoclonal antibodies used during the study and approaching sizes of PrPres fragments in atypical scrapie and Nor98 isolates. Theoretical apparent molecular weights (MWs) of PrP fragments were calculated, by using those of each amino acid included in the known ARQ sheep PrP sequence, according to Sambrook and Russell (*17*). B) Interpretation of PrPres Western blot (WB) profiles in atypical scrapie and Nor98 isolates. Theoretical WB profile shows the expected apparent molecular masses of glycosylated PrP forms estimated by addition of 3.8 (*, monoglycosylated) or 7.9 (**, diglycosylated) kDa to the apparent molecular masses of A and B PrPres forms observed after PNGaseF deglycosylation. Values of 3.8 and 7.9 kDa were estimated from comparisons of glycosylated and unglycosylated forms in a classic scrapie isolate. The Sha31 WB profile included the mean apparent MWs assessed from highest resolution WB analysis (n = 32) and showed 2 separate peaks of maximal intensity in pictograms of signal intensities of bands II and III (19 sheep scrapie isolates).

products at 7 kDa (Nor98-PrP7) and 14 kDa (PrP-CTF14) in Nor98 case isolates from Swedish sheep. Importantly, 1 of these Swedish Nor98 cases was investigated in a laboratory taking part in this study (that of S.L. Benestad) and showed the same pattern with the band of low MW at 11 kDa with TeSeE Bio-Rad WB. Differences in the apparent molecular masses between the isolates from the 2 studies could result, at least in part, from the different methods used for PrPres purification and concentration, when one considers the high pK sensitivity of PrPres in atypical scrapie (*10*,*12*). The choice of antibodies could also contribute to the observed differences. For instance, in our study, band V consistently showed an apparently lower molecular mass (9–10 kDa), with P4 antibody (antibody used in the Klingeborn et al. study). Therefore, fragments C (11 kDa) and B (18 kDa) could correspond to Nor98-PrP7 and PrP-CTF14, respectively, in the harsher pK conditions used by Klingeborn et al. PrPres fragment A (23 kDa) was not observed by Klingeborn et al., which suggests that this PrPres form might be completely digested or transformed into fragments of Nor98-PrP7, PrP-CTF14, or both. Despite minor differences, the results of the 2 studies in regard to the particular molecular features of atypical scrapie/Nor98 isolates are consistent.

The presence of a PrPres fragment with an apparently low molecular mass, which is a salient feature of atypical and Nor98 cases, has already been reported in several human diseases. N terminally truncated PrPres migrating to either 12 kDa or 13 kDa have been reported in some sporadic Creutzfeldt-Jakob disease cases (*20*). The identification of a low-MW fragment cleaved at both C- and N-terminal ends of the prion protein has even been described as the hallmark of Gerstmann-Sträussler-Scheinker syndrome in humans (*2*,*21*–*24*). However, even if these atypical isolates appear to have similarities with those from rare human prion diseases, atypical cases of scrapie are not rare in sheep and goats and, in some countries, are more frequent than the classic disease.

Biodiversity in Atypical Cases

The isolates from the 54 atypical cases we investigated here, which included a large panel of different PrP genotypes and 2 species (sheep and goat), possessed a unique biochemical signature, indistinguishable from that of the Nor98 isolates. All of the isolates from the French atypical and Nor98 cases that were transmitted to Tg338 ovine transgenic mice also shared the same biologic signature, with comparable incubation periods, clinical signs, lesion profiles, and PrPres deposits patterns in the central nervous system (*16*). Moreover, the PrPres biochemical signature in the inoculated Tg338 was strikingly comparable to that observed in the present study. Taken together, these data strongly suggest that the prions involved in atypical (sheep and goat) and Nor98 cases are in fact a unique TSE agent. However, because our cases were obtained from only 2 countries, whereas atypical cases have been identified throughout Europe (*12*,*25*–*29*), further studies are required before definitive conclusions can be drawn. Nevertheless, the lack of diversity in our panel, combined with the identity with Nor98 cases, suggests that the biodiversity of the TSE agents of atypical scrapie is not large.

Allelic Tropism in Atypical Cases

Although polymorphisms associated with classic scrapie have been widely documented (*4*,*6*), both the atypical and Nor98 cases (*9*) seemed to deviate from the established concepts of classic scrapie. In our panel, an obviously high susceptibility seemed to be associated with the AHQ and $AF_{141}RQ$ alleles, whereas the VRQ allele was poorly represented. Similar observations were reported for Nor98 cases (*8*,*9*).

However, a proper analysis of the risk associated with each genotype in atypical cases will require much more data than those presented here. Indeed, all our atypical case data were collected through active surveillance network, by using a particular rapid diagnosis test. These affected animals would need to be matched for breed, age, and population (detection within the same active surveillance program with similar tests) to permit an appropriate risk factor analysis and comparison of susceptibility with animals with classic scrapie. Moreover, the distribution of F_{141} within each breed is currently unknown. This work is ongoing in France, and results should be presented soon.

The involvement of ARR/ARR genotype animals not only in our study (6 cases) but also in several EU countries (*11*,*30*) is also of some concern. Based on the observed resistance to TSE in homozygous ARR animals, a breeding program for resistance to scrapie and BSE has been implemented in several EU countries to control human exposure to TSE risk. This unusual susceptibility of small ruminants believed to be genetically resistant to TSE could lead to a reevaluation of such a policy. In this context, determining the distribution of infectivity in different tissues of affected animals and whether or not atypical scrapie is naturally transmissible between animals within affected flocks would also be helpful.

Conclusion

Our data provide new information about the recently described atypical cases of TSE. These cases appear to be associated with a novel PrPres biochemical pattern; they shared similarities with some rare prion diseases in humans and were clearly distinct from classic scrapie or BSE. This potential similarity in PrPres formation mechanisms with some other rare prion diseases in humans is intriguing. However, the unusual properties of these atypical cases illustrate our decades of underestimating the biodiversity of TSEs in small ruminants and the consequences. This finding should lead to a general reexamination of our conceptual approach in the control of TSEs in small ruminants.

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Mr Arsac is a doctoral student researching the diagnosis and characterization of prion diseases in ruminants. His research interests include molecular characterization of prion diseases from small ruminants in their natural host and in experimental mouse models.

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Primary *Pneumocystis* Infection in Infants Hospitalized with Acute Respiratory Tract Infection

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Acquisition of *Pneumocystis jirovecii* infection early in life has been confirmed by serologic studies. However, no evidence of clinical illness correlated with the primary infection has been found in immunocompetent children. We analyzed 458 nasopharyngeal aspirates from 422 patients hospitalized with 431 episodes of acute respiratory tract infection (RTI) by using a real-time PCR assay. In 68 episodes in 67 infants, *P. jirovecii* was identified. The odds ratio (95% confidence interval) of a positive signal compared with the first quartile of age (7–49 days) was 47.4 (11.0–203), 8.7 (1.9–39.7), and 0.6 (0.1–6.7) for infants in the second (50–112 days), third (113–265 days), and fourth (268–4,430 days) age quartiles, respectively. Infants with an episode of upper RTI (URTI) were 2.0 (1.05–3.82) times more likely to harbor *P. jirovecii* than infants with a lower RTI. *P. jirovecii* may manifest itself as a self-limiting URTI in infants, predominantly those 1.5–4 months of age.

The opportunistic fungus *Pneumocystis jirovecii* (formerly *Pneumocystis carinii* f.sp. *hominis* [*1*]) may cause severe pneumonia (PCP) in patients with AIDS and other immunodeficiencies. The epidemiology of *P. jirovecii* infection is still not well understood, however. Serologic studies have shown that children are exposed to *P. jirovecii* early in life (*2–5*).

To our knowledge, no previous evidence exists of a correlation between clinical illness and primary infection in the competent host (*6*). Recently, *P. jirovecii* has been found in respiratory secretions from infants with respiratory tract infection (RTI) as well as in autopsy lung tissue from infants who died of sudden infant death syndrome (*7–10*).

The role of a human reservoir for the pathogen, consisting of HIV-positive or HIV-negative adults, has recently been debated (*11*). Also, immunocompetent children may contribute to the circulation of the organism. In addition, detecting abundant infection in infants could reflect widespread exposure from an environmental reservoir.

We conducted a blinded, retrospective study to determine the prevalence of *P. jirovecii* harbored in the respiratory tracts of Danish children with acute RTI, and whether clinical and laboratory characteristics separate those with and without *P. jirovecii* infection. The detection method employed was a single-round, closed-tube, real-time PCR assay. The study was approved by the Ethical Committee of Copenhagen (KF 01–028/03).

Methods

Patient Population and Samples

All available routine nasopharyngeal aspirates (NPAs) obtained for respiratory syncytial virus (RSV) analysis during 1999–2002 from children hospitalized at the Departments of Pediatrics, Hvidovre University Hospital and Amager Hospital, Copenhagen, Denmark, were included in the study. Thus, included samples and subjects were NPAs from children in whom the treating physician suspected or wished to rule out an RSV infection. Therefore, most children were <24 months of age. Samples collected within 3 weeks from the same person were regarded as being from the same episode of respiratory disease.

Clinical Data Collection

Clinical data were obtained by reviewing medical records of the patients using uniform data abstraction forms. The reviewer, a pediatrician, was blinded to PCR data. A diagnosis of lower RTI (LRTI), upper RTI (URTI), or "other" was made on the basis of recorded clinical findings (*12*). In brief, a diagnosis of URTI was made if the

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infant had one or more of the following clinical signs without evidence of LRTI: cough, nasal discharge, a red bulging tympanic membrane, and pharyngotonsillar erythema or exudate. A diagnosis of LRTI was made if the infant also had abnormal sounds on lung auscultation and chest indrawing or tachypnea. Infants with an RTI who were hospitalized primarily for other reasons received the diagnosis "other."

PCR Analysis

All samples were extracted and assayed in the Clinical Microbiology Laboratory at Hvidovre University Hospital. Universal PCR laboratory procedures were used, such as physical separation of the steps involved in PCR and unidirectional workflow; specimens were processed carefully with observance of universal PCR laboratory precautions. In addition, a single-round, nonnested, closed-tube PCR assay, with no manipulations of amplicons required, inherently reduces the risks of carryover contamination. To further reduce this risk, uracil-N-glycosylase and deoxyuridine triphosphate were used to prevent amplicon carryover (*13*). PCR analysis was carried out with researchers blinded to all clinical data. The code was only broken at the time PCR analysis and clinical data collection were completed.

DNA Extraction

DNA was extracted from patient and control specimens with the automated MagnaPure (Roche Diagnostics GmbH, Mannheim, Germany) system, using the MagNA Pure LC DNA Kit III (bacteria, fungi) (Roche), according to the manufacturer's recommendations. A sample volume of 100 μL was used for extraction, and a preincubation step was carried out by adding lysis buffer and protein K mixture to the sample, which was then incubated for 15 min at 65°C, followed by 10 min at 95°C. Extracted material was stored at –80°C.

PCR Controls

P. jirovecii–positive and –negative respiratory samples, as determined by results of previous microscopy and PCR, were included with each DNA extraction and in each PCR run as external controls. An internal control amplifiable by the *P. jirovecii* primers was included to detect PCR inhibitors in the patient specimens (*14*).

The following standards were set. For *Pneumocystis*, 10-fold serial dilutions (10^{-2}–10^{5} copies/μL) of a plasmid containing a *P. jirovecii* major surface glycoprotein (MSG) gene insert were prepared (*14*). Standard curves for quantification of positive patient samples were generated by assaying the serial dilution in triplicate. For betaglobin, 10-fold serial dilutions (1.5×10^{-4}–1.5×10^{1} ng/μL) of human genomic DNA provided with the Control Kit DNA (Roche) were used to generate standard curves, according to the manufacturer's instructions.

DNA amplification and detection were carried out as follows. For *Pneumocystis*, we used a quantitative touchdown PCR method that targeted the multicopy MSG gene of *P. jirovecii* (*14*). In brief, primers JKK14/15 and JKK17 amplify a 250-bp segment of the multicopy MSG gene family. The MSG primers also amplify a 295-bp fragment of the artificially constructed internal control. Detection was carried out by using 2 separate sets of fluorescence resonance energy transfer (FRET) probes, which detected the MSG (PCMSGFRET1U and PCMSGFRET1D) and internal control target (PCMIM1U and PCMIM1D), respectively. The probes were labeled with Red640 and Red705, respectively, for simultaneous amplification and detection to take place in the same reaction tube. PCR conditions were as previously described (*14*). First, all samples were assayed with the internal control included. *P. jirovecii*–positive samples were then assayed for quantification without an internal control, including 2 standards (10^{3} copies/μL) in the experiment, and the generated external standard curve was imported for quantification.

For betaglobin, a commercial kit, Control Kit DNA (Roche), was used to estimate the amount of human DNA present in the samples. PCR conditions were as recommended by the company. Two standards (1.5×10^{-1} ng/μL) were included in each experiment, and the generated external standard curve was imported for quantification. All *P. jirovecii*–positive samples and a randomly selected subgroup of *P. jirovecii*–negative samples (all negative samples from patients born on the first through third days of the month) were assayed; 5 μL of patient specimen or the standard dilution was added per tube.

Interpretation

If a PCR-positive sample was negative by the second analysis, the sample was reextracted and reassayed in 2 tubes. If at least 2 of 4 tubes were positive, the sample was recorded as positive for *P. jirovecii*.

A negative MSG result had to have a positive result for the internal control to be considered valid, to ensure absence of inhibitors in the specimen. If PCR inhibitors were detected, the sample was to be diluted 1:5.

Data Analysis

All acquired fluorescence data were analyzed with LightCycler software (Roche). Clinical data were recorded with EpiData 2.1a (EpiData Association, Odense, Denmark). Statistics were calculated by using the SAS System, version 9.1 for Windows (SAS Institute Inc., Cary, NC, USA).

Wilcoxon 2-sample test or Kruskal-Wallis test was used to compare quantitative data when appropriate. Fisher

exact test was used to compare groups. A 2-sided p value of <0.05 was considered significant. Values presented are medians with ranges or interquartile ranges (IQR). Logistic regression was used for univariate and multivariate analyses.

Results

Patients and Episodes

Four hundred sixty-one NPAs from 423 patients with 432 episodes were available for analysis. One HIV-infected child with PCP was excluded from analysis. The remaining infants were presumed to be uninfected with HIV of the basis of review of their medical charts. Two hundred ninety-six (70%) patients (with 303 episodes [70%]) were hospitalized at Hvidovre University Hospital and the rest at Amager Hospital. Sixty-four percent of the episodes received a diagnosis of LRTI, 28% a diagnosis of URTI, and 8% "other." The median age was 112 days (IQR 49–265), and 52.7% of the NPAs were from male patients.

PCR Results

No samples exhibited inhibition. All controls were appropriate. Sixty-seven (16%) of the 422 patients had positive test results for *P. jirovecii* in 68 (16%) of 431 episodes. More than 1 NPA was collected in 21 episodes, of which 4 (19%) were *P. jirovecii* positive, and PCR results were concordant in 96% (46/48 samples) of the NPAs. NPAs from 8 pairs of siblings were collected, and all pairs were concordant (1/8 pairs positive).

Basic demographic data for the *P. jirovecii*–positive and –negative groups are presented in Table 1, and age distribution in quartiles is presented in Table 2. Significant differences were found in age, days admitted to hospital, and occurrence of reported fever. However, no significant difference was found in temperature at admission. No difference in positivity rate was seen between the 2 hospitals. Univariate and multivariate analyses are presented in Table 3. By univariate analysis, URTI versus LRTI, age quartiles 2 and 3 versus 1, and reported fever were associated with the presence of *P. jirovecii*, but days admitted to the hospital was not. Age quartiles 2 and 3 versus quartile 1 and URTI versus LRTI were independently associated with *P. jirovecii* positivity by multivariate analysis. The distribution of number of episodes by clinical diagnosis and age is illustrated by online Appendix Figure A (available from www.cdc.gov/ncidod/EID/13/1/66-appG.htm#A) and the frequency of *P. jirovecii*–positive episodes by online Appendix Figure B (available from www.cdc.gov/ncidod/EID/13/1/66-appG.htm#B).

Quantitative Analysis of PCR-positive Results

If >1 NPAs were collected during the same episode, average numbers of copies were calculated for that episode. The *P. jirovecii*–positive episodes had a median of 9 copies/tube (IQR 2.8–25).

Of the 387 *P. jirovecii*–negative NPAs, 49 (12.7%) were randomly selected for betaglobin analysis. The *P. jirovecii*–positive and –negative samples had a median of 129,400 (IQR 49,540–298,800) versus 95,410 (IQR 27,610–228,800) pg/tube, with no significant difference (p = 0.09).

Due to the natural variation of the specimens, *P. jirovecii* copy numbers were corrected for amount of human DNA in the sample (copies MSG per ng betaglobin). The PCR-positive episodes had a median of 0.069 (IQR 0.021–0.315) copies/ng betaglobin per tube.

The quantitative data were normally distributed when logarithm transformed (Figure). Quantitative data for age and clinical diagnosis subgroups are presented in Table 4. No significant differences were found among groups.

Discussion

In this study, *P. jirovecii* was detected in NPAs from 16% of infants hospitalized with acute RTI. A marked difference occurred in the age distribution, as the prevalence was 48% in infants ages 50 to 112 days (second quartile), 13% in infants ages 113 to 265 days (third quartile), and negligible in the youngest and oldest infants (Table 2, online Appendix Figure). Similarly, ORs of 47 and 8.7 were found for the second and third quartiles, respectively, when compared with that of the youngest group for being *P. jirovecii* positive by multivariate analysis (Table 3). These data indicate that infants were exposed very early to

Table 1. Basic demographic data for the *Pneumocystis jirovecii*–positive and *P. jirovecii*–negative groups

Demographic factor	No. *P. jirovecii* positive†	No. *P. jirovecii* negative*	p value
RSV† positive	30/68 (44)	165/363 (45)	0.93
Sex (male)	36/67 (54)	188/358 (53)	0.89
Coexisting conditions	8/67 (12)	65/358 (18)	0.29
Reported fever	35/64 (55)	247/351 (70)	0.02
Admission temperature (°C)	37.6 (37.0–37.9)	37.7 (37.1–38.5)	0.11
Age, d	90 (73–112)	140 (44–292)	0.04
Hospital days	2 (0–5)	3 (1–6)	0.04
Hospital (Hvidovre)	50/68 (74)	253/363 (70)	0.52

*Denominators are total number of episodes in each group. Values are numbers (%) or median (interquartile range).
†RSV, respiratory syncytial virus.

Table 2. Quartiles of age in days with rate of RSV and *Pneumocystis jiroveci* positivity*

Age quartile	n	Age, d†	RSV positive, %	*P. jirovecii* positive, %
1	108	7–49 (32.5, [22.5–42])	48	2
2	105	50–112 (78, [63–96])	51	48
3	107	113–265 (173, [139–214])	44	13
4	105	268–4,430 (415, [319–542])	38	1

*RSV, respiratory syncytial virus.
†Median in parentheses with interquartile range in brackets.

P. jirovecii, and this raises the question of whether this diagnosis should be considered in infants ages 1.5–4 months who exhibit symptoms of an acute RTI.

The relative absence of *P. jirovecii* among the youngest infants (ages ≤50 days) could indicate either differences in exposure or in immunity, or reflect the incubation time of the infection. One could hypothesize that the increased rate of *P. jirovecii* positivity was coincidental with the infants' introduction to a daycare facility/institution. However, the infants were cared for at home and not at an institution in 64 (96%) of the 67 *P. jirovecii*–positive episodes.

Another possible explanation is differences in immunity, which could be mediated by maternal antibodies in the youngest infants. Animal studies have shown that maternal antibodies are protective in infants (*15–17*). Likewise, *P. jirovecii* was seldom found in the oldest infants (>265 days of age), which may have been due to acquired immunity. Previous studies suggest that the clearance of organisms is complete; no detectable organisms were found by microscopy or PCR in postmortem lung specimens from immunocompetent adult patients (*18*). However, primary infection could possibly be acquired later in life and produce a milder illness (one that does not require hospitalization) in older children, and therefore these cases are not included in the current study.

Also, the absence of *P. jirovecii* among the youngest infants (ages ≤50 days) may have been a result of the incubation period of the infection, assuming that organism burden during the incubation period was below level of detection of the assay. Animal studies have indicated that the peak organism load in healthy mice occurs 5–6 weeks after exposure (*19*,*20*). Thus, the infants could have been exposed shortly after birth in order for symptoms to develop in infants at the ages found here. In fact, animal studies have found early exposure of newborn infants by a maternal source, and asymptomatic carriage by pregnant women has been reported recently (*21–23*). In a reported case of probable mother-to-infant transmission, the mother became symptomatic at 3 days postpartum and the infant at 29 days of age (*24*). The shorter incubation period in this case may reflect a higher level of infectious inoculum in this infant.

The age distribution was in concordance with the trend reported in a recent study on autopsied lung specimens from 112 infants (*25*). Similarly, serologic studies have indicated that most children seroconvert early in life (*2–5*,*8*,*26*). Among children with perinatally acquired HIV, the incidence of PCP was highest from 3 to 6 months of age (*27*,*28*). That is, these infants were slightly older when PCP was diagnosed. Assuming that they were exposed to *P. jirovecii* at the same time as healthy infants, the difference could be because a longer incubation time is needed for clinical PCP to develop in susceptible immunocompromised persons. Previous studies have reported an overall *P. jirovecii* prevalence of 25% (*8*), and 32% (*9*) in infants with acute RTI. When episodes were considered, however, the prevalence in the latter study was 17%, which was in concordance with the findings in our current study. The former study comprised 178 infants but did not include clinical data, and the latter study comprised a smaller population (74 infants with 178 episodes). Geographic variation or methodologic differences may account for the slight difference in reported prevalence. Our study used a single-round, closed-tube, PCR format for detection, which has a high sensitivity and a greatly reduced risk for carryover contamination (*14*). The difference in amount of human DNA detected in *P. jirovecii*–positive and –negative samples did not reach the 5% level of significance. If, in fact, a difference exists, this could be because sample

Table 3. Univariate and multivariate OR (95% CI) for *Pneumocystis jirovecii* positivity (logistic regression analysis)*

	OR univariate (95% CI)	OR multivariate (95% CI)†
LRTI	1	1
URTI (vs LRTI)	2.74 (1.58–4.73)	2.00 (1.05–3.82)
Other (vs LRTI)	0.70 (0.20–2.41)	1.06 (0.27–4.20)
Age Q1	1	1
Age Q2 vs Q1	48.2 (11.3–205)	47.4 (11.0–203)
Age Q3 vs Q1	7.98 (1.77–36.0)	8.74 (1.92–39.7)
Age Q4 vs Q1	0.51 (0.05–5.71)	0.60 (0.05–6.71)
Sex (M vs F)	1.05 (0.62–1.77)	
Coexisting conditions	0.61 (0.28–1.34)	
Reported fever	0.51 (0.30–0.88)	
Fever‡	0.86 (0.50–1.49)	
RSV	0.94 (0.56–1.58)	
Hospital days	0.94 (0.88–1.01)	

*OR, odds ratio; CI, confidence interval; LRTI, lower respiratory tract infection; URTI, upper respiratory tract infection; M, male; F, female; RSV, respiratory syncytial virus.
†Including more variables in the model did not increase the fitness; including RSV showed it was not a confounder.
‡Defined by temperature at admission >37.5°C.

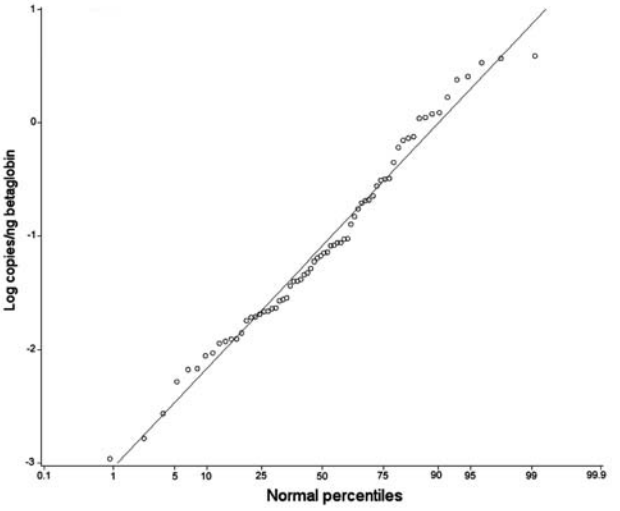

Figure. Plot of logarithmic transformed values of copies/ng betaglobin detected in PCR-positive tubes (○). Solid line denotes normal distribution.

quality varied, which means that we may have underestimated the true prevalence of *P. jirovecii* carriage. The difference could also have occurred because the presence of *P. jirovecii* increases the amount of, for example, inflammatory cells in the nasopharyngeal secretions (*29*), thereby increasing the amount of human DNA sampled. The current study confirms the previous reports that *P. jirovecii* can be detected in respiratory tract specimens from otherwise healthy infants with an acute RTI.

Pneumocystis is likely transmitted through the respiratory route (*30*). The reservoir for *P. jirovecii* is unknown but could include other persons or environmental sources, whereas animal reservoirs are unlikely because of the host specificity (*6*,*31*). Animal studies have shown that colonized mice may transmit the organism to immunosuppressed mice (*32*). Therefore, healthy children with a primary *P. jirovecii* infection may play a role in the circulation of the organism as previously suggested (*25*), although recent genotyping studies have yielded conflicting results (*10*,*33*).

P. jirovecii–positive episodes could represent either colonization or clinical overt disease. We have previously shown the assay used here provides reproducible quantitative results, and that a similar real-time quantitative PCR assay correlates well with the number of whole organisms in the sample (*14*,*34*). The fact that the quantitative data were normally distributed after logarithmic transformation (Figure), and that no differences in copy numbers were detected among groups (Table 4), may indicate that the *P. jirovecii*–positive episodes represent 1 biologic phenomenon.

To our knowledge, no previous evidence has shown a connection between clinical illness or specific symptoms and primary infection in immunocompetent children. It has been presumed to be an asymptomatic or mild, nonspecific disease (*6*,*22*). In the study by Vargas et al., no differences in clinical diagnosis were observed (*8*). In the current study, we found that infants with an episode of URTI were 2.0× more likely to be carrying *P. jirovecii* than infants with LRTI, when findings were adjusted for age (online Appendix Figure B, Table 3). This finding is somewhat surprising because the organism causes LRTI in immunocompromised subjects. It is unlikely that the finding is due to differences in sample quality, because the amount of betaglobin detected in samples from patients with URTI and LRTI was similar (data not shown), and no difference in adjusted *Pneumocystis* DNA was detected (Table 4). Parents reported that the child had a history of fever less often in *P. jirovecii*–positive episodes by univariate analysis, but no differences were found in the presence of fever as assessed at admission (Tables 1 and 3). Also, *P. jirovecii*–positive infants tended to be hospitalized for a marginally shorter duration (Tables 1 and 3).

The limitations of this study are primarily the lack of a healthy control group without respiratory symptoms, and lack of serologic data from the patients. Also, a comprehensive analysis of the specimens was not done for known respiratory pathogens other than RSV and *P. jirovecii*. Further investigation is therefore needed to confirm these findings before recommendations can be made for routine

Table 4. Number of copies per ng of betaglobin detected per tube (median, IQR) in PCR-positive specimens in the 4 age groups and 3 diagnosis groups*†

	n	Median copies/ng betaglobin/tube (IQR)	p value
Age quartile			
1	2	0.854 (0.023–1.685)	0.37
2	50	0.063 (0.021–0.311)	
3	14	0.085 (0.022–0.318)	
4	1	0.001	
Clinical diagnosis			
LRTI	32	0.033 (0.016–0.269)	0.16
URTI	32	0.082 (0.033–0.526)	
Other	3	0.094 (0.087–1.098)	

*IQR, interquartile range; LRTI, lower respiratory tract infection; URTI, upper respiratory tract infection.
†No significant differences were detected by Kruskal-Wallis tests.

diagnostic testing for *Pneumocystis* in defined populations of infants, because it remains possible that *Pneumocystis* carriage in this population could represent a bystander phenomenon. Similarly, one should be cautious in inferring these results to infants without RTI or to those with an RTI that does not require hospitalization.

In this study, we found an overall prevalence of *P. jirovecii* in the respiratory tracts of 16% of infants hospitalized with an episode of acute RTI. Infants ages 50–112 days harbored *P. jirovecii* in 48% of the episodes. Our data suggest that primary *P. jirovecii* infection acquired early in life may present itself as a self-limiting URTI.

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Panmicrobial Oligonucleotide Array for Diagnosis of Infectious Diseases

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To facilitate rapid, unbiased, differential diagnosis of infectious diseases, we designed GreeneChipPm, a panmicrobial microarray comprising 29,455 sixty-mer oligonucleotide probes for vertebrate viruses, bacteria, fungi, and parasites. Methods for nucleic acid preparation, random primed PCR amplification, and labeling were optimized to allow the sensitivity required for application with nucleic acid extracted from clinical materials and cultured isolates. Analysis of nasopharyngeal aspirates, blood, urine, and tissue from persons with various infectious diseases confirmed the presence of viruses and bacteria identified by other methods, and implicated *Plasmodium falciparum* in an unexplained fatal case of hemorrhagic feverlike disease during the Marburg hemorrhagic fever outbreak in Angola in 2004–2005.

Rapid differential diagnosis of infectious diseases is increasingly important as novel pathogens emerge in new contexts and treatment strategies are beginning to be tailored to specific infectious agents. Because clinical syndromes are rarely specific for single pathogens, unbiased multiplex assays are essential. Methods for direct molecular detection of microbial pathogens in clinical specimens are rapid, sensitive, and may succeed when fastidious requirements for agent replication or the need for high-level biocontainment confound cultivation.

We have adopted a staged strategy for molecular pathogen surveillance and discovery. In the first stage we use MassTag PCR, a PCR platform wherein discrete mass tags rather than fluorescent dyes serve as reporters. This method, which allows simultaneous detection of >20 different pathogens with high sensitivity, has proven useful for differential diagnoses of respiratory disease and viral hemorrhagic fevers (*1–3*). However, it is not sufficient when larger numbers of known pathogens must be considered, when new but related pathogens are anticipated, or when sequence divergence might impair binding of PCR primers. Thus, to address the challenge of more highly multiplexed differential diagnosis, we established an oligonucleotide microarray platform.

Microarrays have potential to provide a platform for highly multiplexed differential diagnosis of infectious diseases (*4,5*). The number of potential features per microarray far exceeds those of any other known technology; hundreds of thousands of features can be printed on 70-mm × 20-mm slides. Furthermore, sequence probes of ≥70 nt are not uncommon. Thus, microbes can be detected when melting temperatures are high enough to allow hybridization, despite a lack of precise complementarity between probe and target. Lastly, microbial and host gene targets can be incorporated, which provides an opportunity to detect microbes and assess host responses for signatures consistent with various classes of infectious agents. Despite these advantages, microbial arrays have not been widely used

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with clinical materials because of limited sensitivity. The primary service of microbial arrays has been characterization of agents propagated to high titer in vitro (*6*).

We report establishment of a microarray platform for pathogen surveillance and discovery, the GreeneChip system. Its key features include a comprehensive microbial sequence database for probe design and protocols for sample preparation, amplification, labeling, hybridization, and analysis. The system has been optimized with cultured viral isolates; tested with blood, respiratory, urine, and tissue samples containing bacterial and viral pathogens; and applied in an outbreak investigation when other methods failed to implicate a microorganism in a fatal hemorrhagic fever case.

Methods

Pathogen Database

A vertebrate viral sequence database (GreeneVrdB) was established by integrating the database of the International Committee on Taxonomy of Viruses (ICTVdB, http://phene.cpmc.columbia.edu), a database that describes viruses at the levels of order, family, genus, and species, and the sequence database of the National Center for Biotechnology Information (NCBI, www.ncbi.nih.gov). Functionally related sequences were clustered by using the protein families (Pfam, http://pfam.janelia.org) database of alignments (*7*). Most viral protein coding sequences in the NCBI database (84%) were represented in the Pfam database; the remainder were mapped by using pairwise BLAST alignments (*8*). The rRNA sequences of fungi, bacteria, and parasites obtained from the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu) or the NCBI database were added to create a panmicrobial database (GreenePmdB). The GreenePmdB comprises the 228,638 viral sequences of the GreeneVrdB that represent complete and partial viral genomes, and 41,790 bacterial 16S rRNAs, 4,109 fungal 18S rRNAs, and 2,626 18S parasitic rRNAs. These sequences represent all recognized 1,710 vertebrate virus species and 135 bacterial, 73 fungal, and 63 parasite genera.

GreeneChip Design and Fabrication

Viral probes were designed to represent a minimum of 3 distinct genomic target regions for every family or genus of vertebrate virus in the ICTVdB. When possible, we chose highly conserved regions within a coding sequence for an enzyme such as a polymerase and 2 other regions that corresponded to more variable structural proteins. We thought that RNAs that encode structural proteins may be present at higher levels than those that encode proteins needed only in catalytic amounts and that use of probes representing noncontiguous sites along the genome might allow detection of naturally occurring or intentionally created chimeric viruses.

Any diagnostic tool based on nucleic acid hybridization is necessarily dependent on the extent to which probes are complementary to their targets. Although sequence databases are increasingly comprehensive, it is unlikely that more than a fraction of the existing microbial sequence space has been explored. Our intent in implementing the GreeneChip was to have the potential to identify known and related agents for which precise sequence information was not available. To assess the extent to which a given probe sequence can hybridize to a nonmatching but related sequence, we analyzed synthetic mismatch controls. Whereas up to 15 terminal mismatches had little effect, strings of $\geq$5 mismatches distributed throughout a sequence, particularly mismatched G/C pairs, resulted in reduced signal; >12 mismatches distributed throughout a sequence resulted in no signal. On the basis of these findings, we pursued a conservative strategy in array design wherein a viral sequence was considered to be covered only if the array included at least 1 complementary probe with $\leq$5 mismatches.

The process for identifying bacterial, fungal, and parasitic probes was similar, although restricted to 16S and 18S rRNA sequences. Viral (GreeneChipVr) and panmicrobial (GreeneChipPm) array platforms were based on the GreeneVrdB and GreenePmdB, respectively. GreeneChipVr version 1.0 contained 9,477 probes to address all vertebrate viruses in the integrated ICTV/NCBI database (1,710 species, including all reported isolates) in 3 gene regions with $\leq$5 nucleotide mismatches. GreeneChipPm version 1.0 contained 29,495 probes that included probes comprising GreeneChipVr version 1.0, as well as 11,479 16S rRNA bacterial probes, 1,120 18S rRNA fungal probes, and 848 18S rRNA parasite probes. A total of 300 host immune response probes were added to arrays as a potential index to pathogenesis.

The 60-mer oligonucleotide arrays were synthesized on 70-mm $\times$ 20-mm glass slides by using an inkjet deposition system (Agilent Technologies, Palo Alto, CA, USA). A slide can accept up to 244,000 different 60-mer probes or 8 arrays, each comprising $\geq$15,000 probes. To facilitate alignment during scanning, 1,000 additional landing-light probes (5′-ATC ATC GTA GCT GGT CAG TGT ATC CTT TTT TTT TTA TCA TCG TAG CTG GTC AGT GTA TCC-3′) were placed in the corners and in a grid on the array. Fluorescently labeled synthetic oligonucleotides complementary to the control probes were included in all hybridizations.

Viruses and Clinical Samples

Sources of viruses and viral reference strains used in this study are shown in Tables 1 and 2. Blood sample

Table 1. DNA virus isolates from tissue culture samples used to test GreeneChip performance

Virus	Genus
Sealpoxvirus 1*	*Parapoxvirus*
Pseudocowpox virus†	*Parapoxvirus*
Orf virus†	*Parapoxvirus*
Cowpox virus†	*Orthopoxvirus*
Human herpesvirus 1*	*Simplexvirus*
Gallid herpesvirus 1†	*Iltovirus*
Human adenovirus E (HAdV-4)‡	*Mastadenovirus*
Human adenovirus C (HAdV-5)‡	*Mastadenovirus*

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†Commonwealth Scientific and Industrial Research Organization, Melbourne, Victoria, Australia.
‡American Type Culture Collection, Manassas, VA, USA.

200501379 (*Lake Victoria marburgvirus*, reference sample from Angola, 2005) and blood sample Angola-460 from a patient suspected of having viral hemorrhagic fever (VHF) were received in containers approved by the International Air Transport Association at either the Centers for Disease Control and Prevention in Atlanta, Georgia, USA or the Public Health Agency of Canada in Winnipeg, Ontario, Canada, respectively.

Sources of clinical samples are shown in Table 3. Nasopharyngeal aspirates (SO4606 and SO5265) were collected by the Instituto de Salud Carlos III in Madrid, Spain, from children with respiratory disease. We also analyzed a nasopharyngeal aspirate (sample 23), a postmortem specimen from a patient who died of infection with severe acute respiratory syndrome coronavirus (SARS-CoV, sample TM-167), urine specimens from 2 patients with urinary tract infections (samples CUMC-NR7 and CUMC-NR9), a urine specimen from an asymptomatic patient (sample CUMC-LO1), and endometrial and lung tissues from a patient infected with *Mycobacterium tuberculosis* (samples CUMC-DL1 and CUMC-DL3).

Sample Preparation and GreeneChip Hybridization

RNA was isolated from blood of VHF patients by using a 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). RNA from virus isolates (culture supernatant) and other clinical samples (blood, nasopharyngeal aspirate, tissue, urine) was isolated by using the Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA). DNA was removed from RNA preparations by treatment with DNase I (DNA-free, Ambion Inc., Austin, TX, USA). First-strand reverse transcription was initiated with a random octamer linked to a specific primer sequence (5′-GTT TCC CAG TAG GTC TCN NNN NNN N-3′) (*5*). After digestion with RNase H, cDNA was amplified by using a 1:9 mixture of the above primer and a primer targeting the specific primer sequence (5′-CGC CGT TTC CCA GTA GGT CTC-3′). Initial PCR amplification cycles were performed at a low annealing temperature (25°C); subsequent cycles used a stringent annealing temperature (55°C) to favor priming through the specific sequence. Products of this first PCR were then labeled in a subsequent PCR with the specific primer

Table 2. RNA virus isolates from tissue culture samples used to test GreeneChip performance

Virus	Genus
Negative-strand virus	
Lake Victoria marburgvirus†	*Marburgvirus*
Zaire ebolavirus‡	*Ebolavirus*
Sudan ebolavirus‡	*Ebolavirus*
Reston ebolavirus‡	*Ebolavirus*
Human respiratory syncytial virus A§	*Pneumovirus*
Human respiratory syncytial virus B§	*Pneumovirus*
Human parainfluenza virus 1§	*Respirovirus*
Human parainfluenza virus 3§	*Respirovirus*
Newcastle disease virus¶	*Avulavirus*
Vesicular stomatitis Indiana virus¶	*Vesiculovirus*
Bovine ephemeral fever virus¶	*Ephemerovirus*
Influenza A virus (H5N1)#	*Orthomyxovirus*
Influenza B virus§	*Orthomyxovirus*
Guanarito virus‡	*Arenavirus*
Machupo virus‡	*Arenavirus*
Junin virus‡	*Arenavirus*
Lassa virus strain Josiah‡	*Arenavirus*
Lassa virus strain Weller‡	*Arenavirus*
Positive-strand virus	
Human enterovirus B (E25)§	*Enterovirus*
Human enterovirus A (HEV71)§	*Enterovirus*
Human enterovirus B (E14)§	*Enterovirus*
Human enterovirus B (E30)§	*Enterovirus*
Vesicular exanthema of swine virus¶	*Vesivirus*
SARS* coronavirus**	*Coronavirus*
Human coronavirus OC43§	*Coronavirus*
Human coronavirus 229E§	*Coronavirus*
Dengue virus 1#	*Flavivirus*
Dengue virus 2#	*Flavivirus*
Dengue virus 3#	*Flavivirus*
Dengue virus 4#	*Flavivirus*
Yellow fever virus#	*Flavivirus*
West Nile virus**	*Flavivirus*
Saint Louis encephalitis virus**	*Flavivirus*
Alfuy virus††	*Flavivirus*
Murray Valley encephalitis virus††	*Flavivirus*
Chikungunya virus#	*Alphavirus*
Sindbis virus¶	*Alphavirus*
Double-strand virus	
Bluetongue virus¶	*Orbivirus*
Epizootic hemorrhagic disease virus-2¶	*Orbivirus*

*SARS, severe acute respiratory syndrome.
†Centers for Disease Control and Prevention, Atlanta, GA, USA.
‡US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA.
§American Type Culture Collection, Manassas, VA, USA.
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Table 3. Clinical samples used to test GreeneChip performance

Pathogen	Genus	Sample
SARS* coronavirus	*Coronavirus*	Lung
Human respiratory syncytial virus A	*Pneumovirus*	Nasopharyngeal
Human enterovirus A (CAV10)	*Enterovirus*	Nasopharyngeal
Lake Victoria marburgvirus	*Marburgvirus*	Blood
Influenza A virus (H1N1)	*Orthomyxovirus*	Nasopharyngeal
Klebsiella pneumoniae†	*Klebsiella*	Urine
Escherichia coli†	*Escherichia*	Urine
Mycobacterium tuberculosis‡	*Mycobacterium*	Lung
Mycobacterium tuberculosis‡	*Mycobacterium*	Endometrial biopsy
Lactobacillus sp.§	*Lactobacillus*	Urine

*SARS, severe acute respiratory syndrome.
†Detected on the array as a gammaproteobacterium.
‡Detected on the array as a mycobacterium.
§Detected on the array as a lactobacillus.

sequence linked to a capture sequence for 3DNA dendrimers containing >300 fluorescent reporter molecules (Genisphere Inc., Hatfield, PA, USA), Products of the second PCR were added to sodium dodecyl sulfate–based hybridization buffer (Genisphere Inc.), heated for 10 min at 80°C, and added to GreeneChip for hybridization for 16 h at 65°C. After 10-min washes at room temperature with 6 × SSC (0.9 mol/L NaCl, 0.09 mol/L sodium citrate, pH 7.0), 0.005% Triton X-100, and 0.1 × SSC, 0.005% Triton X-100, Cy3 3DNA dendrimers were added and incubated at 65°C for 1 h. Slides were washed as before, air dried, and scanned (DNA Microarray scanner, Agilent Technologies).

GreeneChip Analysis

Log-transformed analysis of microarrays using p values (GreeneLAMP) version 1.0 software was created to assess results of GreeneChip hybridizations. A map built from BLAST data was used to connect probe sequences to the respective entries in the GreenePmdB. Each of those sequences corresponds to an NCBI Taxonomy ID (TaxID). Individual TaxIDs were mapped to nodes in a taxonomic tree built based on ICTV virus taxonomy or the NCBI taxonomic classification for other organisms. The program output is a ranked list of candidate TaxIDs.

Probe intensities were corrected for background, $\log_2$-transformed, and converted to Z scores (and their corresponding p values). Where available, control-matched experiments from uninfected samples were used, and spots >2 standard deviations from the mean were subtracted. In instances where control-matched samples were not available, the background distribution of signal fluorescence in an array was calculated by using fluorescence associated with 1,000 random 60 mers (null probes). In both scenarios, positive events were selected by applying a false-positive rate of 0.01 (the rate at which null probes are scored as significant) and a minimum p value per probe of 0.1 in cases with a matching control and 0.023 (2 standard deviations) in cases without a matching control. Candidate TaxIDs were ranked by combining the p values for the positive probes for that TaxID by using the QFAST method of Bailey and Gribskov (*9*). This approach makes the following assumptions: 1) spot intensities are normally distributed; 2) spots represent independent observations (to minimize this effect clustering is used to collapse probes that are 95% identical); and 3) there are relatively few (<100) positive probes for any given TaxID. Probes for each kingdom (bacteria, eukaryotes, fungi, viruses) were analyzed independently to compensate for variations in signal-to-noise levels.

Sequence Recovery from Hybridized Arrays

When a hybridization signal suggests a novel or chimeric agent, or the investigator wants to obtain sequence information, cDNA can be eluted for amplification and sequence analysis. A total of 100 μL of water at 90°C is added to the array and pipetted up and down 10 times. The eluate is recovered, amplified with the specific primer used during the initial amplification, and cloned into a plasmid vector (TOPO TA, Invitrogen, Carlsbad, CA, USA). After transformation into *Escherichia coli*, colonies are screened by sequencing. Primers based on the obtained sequence can be designed for confirmation of the agent or for specific (real-time) PCR screening of other specimens.

Quantitative Real-Time PCR for *Plasmodium falciparum*

A quantitative real-time PCR assay was designed to amplify a 190-bp product from positions 178 to 367 of the 5.8S rRNA sequence eluted from the GreeneChipPm to confirm the presence of plasmodia in the original clinical sample. Reactions were performed in a 25-μL volume by using a commercial SYBR-Green reaction mixture (Applied Biosystems) and performed according to the manufacturer's instructions. The primer sequences were 5′-GGAACGGCTTTGTAACTTGG-3′ and 5′-TGTCCTCAGAGCCAATCCTT-3′. The following cycling con-

ditions were used: 50°C for 2 min and 95°C for 10 min, followed by 45 cycles at 95°C for 15 sec and 60°C for 1 min. To quantitate organism load in the original clinical sample, the targeted sequence region was cloned from the chip-hybridized, eluted nucleic acid. The cloned sequence was used to generate a 7-point standard curve (starting from 5×10^6 copies/assay) for quantitation; each run included negative no-template controls. Thermal cycling was performed in an ABI 7300 real-time PCR system (Applied Biosystems).

Results

Evaluation of GreeneChip Performance

The performance of the GreeneChip system was initially tested in GreeneChipVr hybridizations that used extracts of cultured cells infected with adenoviruses, alphaviruses, arenaviruses, coronaviruses, enteroviruses, filoviruses, flaviviruses, herpesviruses, orthomyxoviruses, paramyxoviruses, poxviruses, reoviruses, and rhabdoviruses (49 viruses). All viruses were accurately identified (Tables 1 and 2). To assess sensitivity, viral RNA extracted from infected cell supernatants (adenovirus, West Nile virus, Saint Louis encephalitis virus, respiratory syncytial virus, enterovirus, SARS-CoV, and influenza virus) was quantitated by real-time PCR, serially diluted, and subjected to analysis with template concentrations ranging from 10 to 1,000,000 copies/assay. The threshold for detection of adenovirus (used as a DNA virus example) was 10,000 RNA copies; the threshold for detection of the RNA viruses tested was 1,900 RNA copies (Table 4).

Array performance was then tested by using samples obtained from patients with respiratory disease, hemorrhagic fever, tuberculosis, and urinary tract infections. In all cases, array analysis detected an agent consistent with the diagnosis obtained by culture or PCR. GreeneLAMP analysis detected human enterovirus A, human respiratory syncytial A virus, influenza A virus, Lake Victoria marburgvirus (MARV), SARS-CoV, lactobacillus, mycobacteria, and gammaproteobacteria (Table 3). Specific real-time PCR analyses indicated viral loads of 6.3×10^5 copies/assay for SARS-CoV (*10*), 1.1×10^3 copies/assay for respiratory syncytial virus (*11*), and 5.46×10^5 copies/assay for enterovirus A (*12*) in clinical specimens. Details of the array analysis process are presented below for the detection of 2 viruses and 2 bacteria in clinical specimens.

Sample 200501379 contained RNA extracted from the blood of a person who died of VHF. In GreeneLAMP analysis, MARV TaxID 11269 was the top prediction by the combined p-value method using QFAST (*9*). The highest relative number of positive probes (10/11, 90.9%) also corresponded to MARV (Figure 1A). In contrast, only 2 of 16 probes were positive for the next best predicted TaxID 11901, bovine leukemia virus. Sequence-based analysis identified GenBank accession no. DQ447653 (Lake Victoria marburgvirus–Angola2005 strain Ang1379c) with 8 positive probes as the best match. The 10 positive probes aligned with all 8 MARV gene motifs represented on the array (Figure 1B). Only 4 (17%) of 23 probes were positive for the next best predicted GenBank entry, AF534225 (*Gorilla gorilla* lymphocryptovirus 1); all aligned with only 1 motif.

Sample TM-167 contained RNA extracted from the lung of a person who died from SARS during the 2003 outbreak in Toronto, Ontario, Canada. In GreeneLAMP analysis, SARS-CoV was the top prediction by the combined p-value method. The highest relative number of positive probes (9/20, 45.0%) also corresponded to SARS-CoV. Sequence-based analysis identified GenBank accession no. AY274119 (SARS-CoV Tor2) with 9 probes representing 9 distinct genome motifs. The next best prediction was for AY738457 (influenza A virus); all influenza virus probes represented only 1 genome motif.

Analyses of bacterial samples were more complex because many rRNA probes are cross-reactive between taxa, and the GreeneLAMP algorithm is not designed to take into account >100 probes positive for 1 TaxID. Thus,

Table 4. GreeneChip sensitivity for detection of various infectious agents*

Agent	Genus	Origin‡	Strain	Sensitivity
Human adenovirus E	*Mastadenovirus*	ATCC VR-1572	HAdV-4 RI-67	1.1×10^4
Human adenovirus C	*Mastadenovirus*	ATCC VR-5	HAdV-5 Adenoid 75	3.2×10^4
Human respiratory syncytial virus A	*Pneumovirus*	ATCC VR-26	Long	1.0×10^4
West Nile virus	*Flavivirus*	GIDL	NY 99	1.9×10^3
Saint Louis encephalitis virus	*Flavivirus*	GIDL	Parton	3.0×10^3
SARS† coronavirus	*Coronavirus*	GIDL	Tor2	4.7×10^3
Human enterovirus B	*Enterovirus*	ATCC VR-184	CBV4 strain JVB	5.2×10^3
Influenza A virus H1N1	*Orthomyxovirus*	MSSM	A/New Caledonia/20/1999	9.8×10^3

*Viral RNA extracted from infected cell supernatants was quantitated by real-time PCR, serially diluted, and subjected to GreeneChip analysis by using template concentrations ranging from 10^6 to 10^1 copies/assay. The threshold level of sensitivity for each virus tested is indicated.
†SARS, severe acute respiratory syndrome.
‡ATTCC, American Type Culture Collection; GIDL, Jerome L. and Dawn Greene Infectious Disease Laboratory, Columbia University, New York, NY, USA.; MSSM, Mount Sinai School of Medicine, New York, NY, USA.

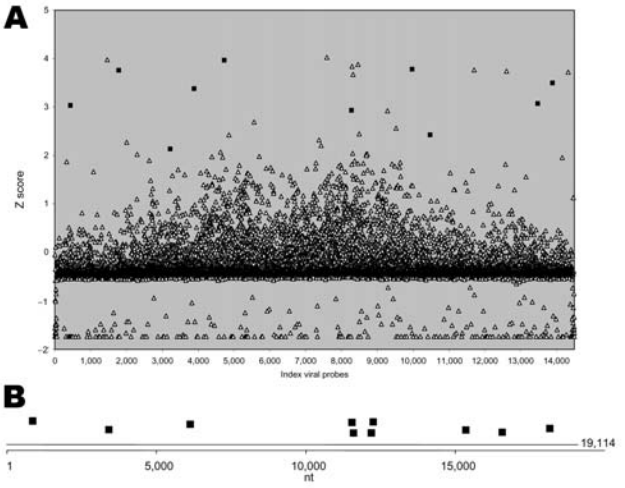

Figure 1. A) Signal intensity for viral probes in blood sample 200501379. Probe intensities were background corrected, $\log_2$-transformed, and converted to Z scores (and corresponding p values). Background distribution of signal fluorescence in the array was calculated by using fluorescence associated with 1,000 random null probes. Positive events were selected by applying a minimum p value per probe of 0.023 (2 standard deviations). Analysis of positive events with GreeneLAMP resulted in prediction of TaxID 11269 as the top prediction by the combined p value. Solid squares indicate Lake Victoria marburgvirus probes; open triangles indicate other probes. Ten of (90.9%) of 11 Lake Victoria marburgvirus probes were positive. B) Genomic location of positive Lake Victoria marburgvirus probes. Sequence-based analysis identified GenBank accession no. DQ447653 (Lake Victoria marburgvirus–Angola2005 strain Ang1379c) with 10 positive probes (all 8 motifs) as the best match.

the program was run considering only probes that reacted with 1 genus-level TaxID. This strategy identified mycobacteria in sample CUMC-DL3 and lactobacilli in sample CUMC-LO1. In sample CUMC-DL3, the sequence-based algorithm identified AY725810 (uncultured *Mycobacterium* sp.) as significant, with 231 positive probes across 6 nonoverlapping regions. In sample CUMC-LO1, AJ853317 (*Lactobacillus vaginalis*) was the most significant result with 87 positive probes. Consensus PCR assays were developed for mycobacteria and lactobacilli. Primers designed by using Greene SCPrimer (http://scprimer.cpmc.columbia.edu/SCPrimerApp.cgi) were Myco_U901: 5′-ATCGAGGATGTCGAGTTGGC-3′ (forward); Myco_L968: 5′-TACTGGTAGAGGCGGCGATG-3′ (reverse); Lacto_817: 5′-CGGTGGAATGCGTAGATATATGGA-3′ (forward); and Lacto_1026: 5′-TCCTTTGAGTTTCAACCTTGCGGT-3′ (reverse). Products obtained after PCR amplification were sequenced and matched the predicted GenBank entries.

Analysis of Unknown Sample from Patient with VHF-like Syndrome

Within 6–8 days of infection, MARV causes an acute febrile illness that frequently progresses to liver failure, delirium, shock, and hemorrhage (*13*,*14*). From October 2004 through July 2005, a MARV outbreak in Angola resulted in 252 cases of hemorrhagic fever; 227 (90%) cases were fatal (*15*). Although most of the putative cases infected with MARV were confirmed by PCR, some were not. During this outbreak, a healthcare worker from a nongovernmental organization had acute fever and liver failure that culminated in death within 1 week. PCR assays of RNA extracted from blood showed no evidence of MARV infection. The same RNA was tested in a multiplex PCR for VHF that used primers for detection of Zaire Ebola, Sudan Ebola, MARV, Lassa fever, Rift Valley fever, Crimean-Congo hemorrhagic fever, Hantaan, Seoul, yellow fever, and Kyasanur Forest disease viruses (*3*) for differential diagnosis of VHF. Because this test did not identify an etiologic agent, the RNA was processed for panviral analysis with GreeneChipVr. Because no significant hybridization was detected, the RNA was assayed with GreeneChipPm. Bioinformatic analysis identified a *Plasmodium* sp. with 21 (62%) of 34 probes positive (Table 5). Chart review showed that the patient had recently arrived in Angola from a country where malaria was not endemic and that he had not taken malaria prophylaxis.

Hybridized cDNA was eluted from the array, cloned, and sequenced. Identified clones contained sequences corresponding to 18S rRNA and 5.8S rRNA of *P. falciparum* (Figure 2, Table 6). Plasmodia contain several alternative 18S-5.8S–28S rRNA genes. The expression of each rRNA set is developmentally regulated, which results in expression of a different set of rRNAs at different stages of the life cycle of the organism (*17*); e.g., S-type rRNA is expressed primarily in the mosquito vector, but A-type rRNA is expressed primarily in the human host (*17*). Only A-type sequences were recovered from the array. Analysis of the original RNA extract in a SYBR Green real-time PCR assay designed to amplify a 190-bp product of the *P. falciparum* 5.8S rRNA gene confirmed the presence of *P. falciparum* ($2 \times 10^6 \pm 8 \times 10^4$ copies/μL blood), and indicated a parasite load >5%. The similarity of the signs and symptoms of severe malarial disease with viral hemorrhagic disease, the detection of a parasite load >5% (*18*), and the origin of this patient from a country nonendemic for malaria are consistent with a diagnosis of infection with *P. falciparum* as the most likely cause of death.

Discussion

Differential diagnosis of hemorrhagic fevers poses challenges for clinical medicine and public health. Syndromes associated with agents are not distinctive, par-

Table 5. Sequences of *Plasmodium*-reactive probes used to predict presence of plasmodia in blood sample Angola-460

Probe	Sequences (5′→3′)	Z score
Eu_5820_309	CGATTAATAGGAGTAGCTTGGGGGCATTTGTATTCAGATGTCAGAGGTGAAATTCTTAGA	3.699
Eu_5820_328	AGGGAGTGAAGACGCTCAGATACCGTCGTAATCTTAACCATAAACTATGCCGACTAGGCT	3.685
Eu_5820_322	ATAGGAGTAGCTTGGGGGCATTTGTATTCAGATGTCAGAGGTGAAATTCTTAGATTTTCT	3.681
Eu_5820_282	TTGTAATTGGAATGGTGGGAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCT	3.672
Eu_5820_269	GCGTAAATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAAGGCCA	3.624
Eu_5820_296	TTAATAGGAGTAGCTTGGGGGCATTTGTATTCAGATGTCAGAGGTGAAATTCTTAGATTT	3.563
Eu_44417_518	ATCGTGATGGGGATAGATTATTGCAATTATTAATCTTCAACGAGGAATGCCTAGTAGGCG	3.558
Eu_5820_277	AACTGCGAAAGCATTTGCCTAAAATACTTCCATTAATCAAGAACGAAAGTTAAGGGAGTG	3.542
Eu_44417_516	GCATCGTGATGGGGATAGATTATTGCAATTATTAATCTTCAACGAGGAATGCCTAGTAGG	3.539
Eu_5820_325	CTTAGTTACGATTAATAGGAGTAGCTTGGGGGCATTTGTATTCAGATGTCAGAGGTGAAA	3.515
Eu_5820_298	GCAATTATTAATCTTGAACGAGGAATGCCTAGTAAGCATGATTCATCAGATTGTGCTGAC	3.507
Eu_5820_285	ATCGTCTTCACTCCCTTAACTTTCGTTCTTGATTAATGGAAGTATTTTAGGCAAATGCTT	3.432
Eu_5820_286	CTAACACAAGGAAGTTTAAGGCAACAACAGGTCTGTGATGTCCTTAGATGAACTAGGCTG	3.407
Eu_5820_311	GTCTAACACAAGGAAGTTTAAGGCAACAACAGGTCTGTGATGTCCTTAGATGAACTAGGC	3.347
Eu_5820_318	AATTATTAATCTTGAACGAGGAATGCCTAGTAGCATGATTCATCAGATTGTGCTGACTAC	3.290
Eu_5820_281	AAGTTTAAGGCAACAACAGGTCTGTGATGTCCTTAGATGAACTAGGCTGCACGCGTGCTA	3.282
Eu_5820_299	TCGATAACGAACGAGATCTTAACCTGCTAATTAGCGGTAAATACAACATATTCTTAAGTA	3.256
Eu_5820_308	TGATTGTAAAGCTTCTTAGAGGAACATTGTGTGTCTAACACAAGGAAGTTTAAGGCAACA	3.255
Eu_5820_324	AGTTTAAGGCAACAACAGGTCTGTGATGTCCTTAGATGAACTAGGCTGCACGCGTGCTAC	3.151
Eu_5820_275	TGATTGTAAAGCTTCTTAGAGGGACATTGTGTGTCTAACACAAGGAAGTTTAAGGCAACA	3.030
Eu_5820_301	CCCTGTTCTACTATAATTTGTTTTTTTTACTCTATTTCTCTCTTCTTTTAAGAATGTACT	2.834

ticularly early in the course of disease. In some instances, including the case presented here, more than 1 agent may be endemic in the region of the outbreak. Outbreaks caused by different agents may also overlap in time and geography. Examples of such coincident outbreaks include monkeypox and varicella-zoster viruses in the Democratic Republic of Congo in 1996 and 2001 (*19*,*20*) and measles and Ebola viruses in Sudan in 2004 (*21*). Furthermore, implicit in globalization is the risk of known or new agents that appear in novel contexts. In 1996, a presumptive diagnosis of Ebola VHF in 2 children who had recently returned to New York City from West Africa resulted in closing a hospital emergency room (*22*). One of the children died of cardiac failure caused by *P. falciparum* parasitemia and hemolysis (*23*). Therapeutic options for treatment of VHF are limited; however, rapid isolation of infected persons is critical to curb contagion. In contrast, whereas human-to-human transmission is not a primary concern with malaria, early specific therapy can have a profound effect on illness and death (*24*).

To address the challenges of emerging infectious diseases and biodefense, public health practitioners and diagnosticians need a comprehensive set of tools for pathogen surveillance and isolation. PCR methods have advantages with respect to sensitivity, throughput, and simplicity, but are limited in potential for multiplexing. Although microarrays have potential to allow highly multiplexed, unbiased surveillance, their use has been limited because of low sensitivity and unwieldy analytical programs. The GreeneChip system introduces sample preparation and

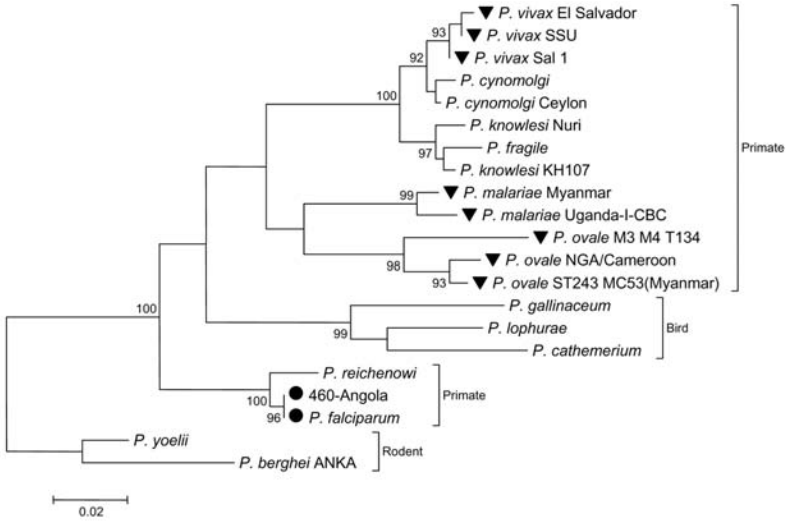

Figure 2. Analysis of 18S rRNA sequence (nt 291,256–292,364) recovered from the array after hybridization of sample Angola-460. The phylogenetic tree was reconstructed with the neighbor-joining method applying a Kimura 2-parameter model with MEGA version 3.1 (*16*). Number of nucleotide substitutions per site are indicated by the scale bar; bootstrap values (percentage of 1,000 pseudoreplicates) are given at relevant branches. Circles indicate *Plasmodium falciparum* sequences; inverted triangles indicate other known plasmodial pathogens of humans.

Table 6. Fragments of *Plasmodium falciparum* sequence recovered after GreeneChip hybridization of blood sample Angola-460

Clone	Position in the genome*	Size, nt	BLAST similarity
B06	286692–286986	295	100% *P. falciparum*, 98% *P. reichenowi*
D09	289685–289784	100	99% *P. falciparum*, 95% *P. berghei*
C01	291256–291624	369	100% *P. falciparum*, 98% *P. berghei*
A09	291521–291631	111	100% *P. falciparum*, 98% *P. berghei*
A08	291521–291614	94	100% *P. falciparum*, 98% *P. berghei*
H10	291521–291616	96	100% *P. falciparum*, 98% *P. berghei*
G02	291601–291637	37	100% *P. falciparum*
A01	291939–292088	150	100% *P. falciparum*, 98% *P. berghei*
J01	292039–292364	326	100% *P. falciparum*, 98% *P. berghei*

*Corresponds to GenBank accession no. AL929354 (*P. falciparum* strain 3D7, chromosome 5, segment 4/4, rRNA).

labeling methods that enhance sensitivity, as well as user-friendly analytical software that we anticipate will facilitate clinical application. The advent of validated highly multiplexed microbiologic assays will afford unprecedented opportunities for unbiased pathogen surveillance and discovery and reduction of illness and death caused by infectious disease.

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Subtypes of *Cryptosporidium parvum* in Humans and Disease Risk

Paul R. Hunter,* Stephen J. Hadfield,† Dawn Wilkinson,* Iain R. Lake,* Florence C.D. Harrison,* and Rachel M. Chalmers†

The 2 main species of *Cryptosporidium* that infect humans are *Cryptosporidium hominis* and *C. parvum*. Here, multilocus fragment analysis of 3 microsatellite loci (ML1, ML2, and gp60) was used to subtype strains from sporadic cases of cryptosporidiosis in Wales and northwest England. Of 72 strains of *C. parvum*, 63 were typeable at all 3 loci, forming 31 subtypes. These strains formed 3 broad clusters, representing 74.6%, 20.6%, and 4.8% of typeable strains. Of 118 *C. hominis* strains, 106 were typeable at all 3 loci, forming 9 subtypes; however, 90% belonged to the same subtype. Analysis with epidemiologic data found an association between strains from case-patients who reported contact with farm animals and individual *C. parvum* microsatellite alleles. The strongest association was with ML1; all strains from case-patients that reported farm animal contact had the same allele (ML1–242). Microsatellite typing of *C. parvum* provides valuable additional information on the epidemiology of this pathogen.

Cryptosporidium species are intestinal parasites that infect a variety of animals; *Cryptosporidium hominis* (synonym: *Cryptosporidium parvum* genotype 1) and *C. parvum* (synonym: *C. parvum* genotype 2) are the 2 most commonly identified species that cause disease (cryptosporidiosis) in humans (*1*,*2*). The main symptom of cryptosporidiosis is diarrhea, which may be accompanied by dehydration, weight loss, abdominal pain, fever, nausea, and vomiting (*3*). In England and Wales, ≈5,000 cases are reported annually (*4*). Disease, although lasting for up to 2 weeks, is usually self-limiting in immunocompetent persons but may be chronic and more severe in immunocompromised patients (*5*). Furthermore, *C. hominis* is associated with increased risk of postinfection symptoms (*6*).

C. hominis primarily infects humans but has recently been reported to infect a dugong and a lamb, and other animals have been infected experimentally (*7*). Rare occurrences of low-level natural infection of cattle by *C. hominis* have also been reported (*8*). By contrast, *C. parvum* naturally infects several animal species that serve as reservoirs for zoonotic infection, including cattle, sheep, goats, and deer (*7*).

Several methods have been described by different research groups to investigate intraspecies variation within the genus *Cryptosporidium*, including microsatellite sequence analysis (*9*–*12*), minisatellite and microsatellite PCR fragment length analysis (*13*,*14*), single-strand conformation polymorphism analysis (*15*), gp60 sequence analysis (*16*,*17*), and telomere sequence analysis (*18*,*19*). A recent study that used minisatellite and microsatellite fragment analysis identified some *C. parvum* clones that may not be zoonotic (*13*,*14*); this study compared isolates from humans and bovines in a single Scottish county. However, no epidemiologic data were presented on case-patients. In the study described here, we investigated the subtypes of *C. parvum* and *C. hominis* and tested the association of subtypes with known epidemiologic factors.

Materials and Methods

Strains

The strains included in this analysis were collected during the case-control study of human cryptosporidiosis in Wales and northwest England (*20*). This study is to date the only case control-study of risk factors for cryptosporidiosis with species identification of infecting strains. Some 427 case-patients and controls were

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surveyed by mail questionnaire. The key findings were that travel abroad and changing diapers of children <5 years of age were associated with risk for *C. hominis* infections. For *C. parvum*, touching farm animals was associated with illness but eating raw vegetables and tomatoes was strongly negatively associated with illness.

As part of that study, clinical laboratories were encouraged to send fecal samples positive for *Cryptosporidium* by microscopy to the UK Cryptosporidium Reference Unit in Swansea. Confirmation that samples were positive by microscopy was performed when required by using a modified Ziehl-Neelsen method as described by Casemore et al. (*21*). To extract *Cryptosporidium* DNA from microscopy-positive feces, oocysts were first separated from fecal matter by saturated-salt-solution centrifugation as described by Elwin et al. (*22*). The oocyst suspension was then incubated at 100°C for 60 min, digested with proteinase K and lysis buffer, and purified by using QIAamp DNA Mini Kit spin columns (QIAGEN Ltd, Crawly, UK) as described previously (*2*). DNA was stored at –20°C before species determination and subtyping, when appropriate.

Identification of Species or Genotype by PCR–Restriction Fragment Length Polymorphism Analysis (PCR-RFLP)

Cryptosporidium sp. was determined by PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) and small subunit (SSU) rRNA genes using methods based on those described by Spano et al. (*23*) and Xiao et al. (*24*), respectively. For PCR-RFLP analysis of the COWP gene, PCR was carried out by using the forward primer 5′-GTAGATAATGGAAGAGATTGTG-3′ and reverse primer 5′-GGACTGAAATACAGGCATTAT CTTG-3′ to produce an amplicon of ≈550 bp. The PCR products were digested by using the restriction enzyme *Rsa*I to differentiate between most *Cryptosporidium* spp.

For nested PCR-RFLP analysis of the SSU rRNA gene, the primary PCR produced fragments of ≈1,325 bp by using the forward primer 5′-TTCTAGAGCTAATA CATGCG-3′ and the reverse primer 5′-CCCATTTC CTTCGAAACAGGA-3′. The secondary PCR, which produced fragments of ≈830 bp, used the forward primer 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and the reverse primer 5′-AAGGAGTAAGGAACAACCTCCA-3′. The products of the secondary PCR were digested with *Ssp*I and *Vsp*I. Digested fragments from SSU rRNA and COWP genes were separated by electrophoresis on 3% agarose gels, visualized by SYBR Green I (Sigma, Gillingham, UK) staining, and images were recorded with a digital imaging system (Alpha Imager, Kodak, Hemel Hempstead, UK).

Confirmation of Species or Genotype by SSU rRNA Gene Sequence Analysis

After PCR-RFLP analysis, unusual species and equivocal samples were confirmed by amplifying a fragment of the SSU rRNA gene and DNA sequencing in both directions. Briefly, amplicons of ≈830 bp were produced from each sample by using the nested primer set described above (*23*), and an ≈298-bp fragment was sequenced (Genetic Research Instrumentation, Braintree, UK) by using the forward primer 5′-AGTGACAAGAAATAACA ATACAGG-3′ and the reverse primer 5′-CCTGCTTTAAGCACTCT AATTTTC-3′ (*25*). The forward and reverse sequences of these fragments were then aligned and analyzed with a CEQ 8000 Genetic Analysis System (Beckman Coulter, High Wycombe, UK) to obtain a consensus sequence. This sequence was then compared with all GenBank, EMBL, DDBJ, and PDB sequences by using the National Center for Biotechnology Information BLASTN tool (available from www.ncbi.nlm.nih.gov/BLAST/).

Analysis of *C. hominis* and *C. parvum* Subtypes

Subtypes were identified by using a multilocus fragment-size–analysis approach to target 3 microsatellite markers (ML1, ML2, and gp60 [synonymous with gp15]) as previously described (*26*). The ML1 fragment was amplified by using the forward primer 5′-CTAAAAATG GTGGAGAATATTC-3′ and the reverse primer 5′-CAACA AAATCTATATCCTC-3′ (*10*,*11*). The ML2 fragment was amplified by using the forward primer 5′-CAATG TAAGTTTACTTATGATTAT-3′ and the reverse primer 5′-CGACTATAAAGATGAGAGAAG-3′ (*11*). The gp60 fragment was amplified by using the forward primer 5′-GCCGTTCCACTCAGAGGAAC-3′ and the reverse primer 5′-CCACATTACAAATGAAGTGCCGC-3′ (*13*). Reverse primers were supplied that were labeled with Beckman Coulter WellRED D3 dye (Proligo**,** Paris, France). The 50-μL PCR mixture for each primer set contained PCR buffer (QIAGEN Ltd), 2.5 mmol/L of $MgCl_2$, 200 μmol/L of each dNTP, 500 nmol/L of each primer, 2.5 U of HotStar *Taq* DNA polymerase (QIAGEN Ltd), and 5 μL of template DNA. The cycling conditions for each PCR were an initial denaturing step of 15 min at 95°C, then 40 cycles of 95°C for 50 s, 50°C (60°C for gp60) for 50 s, and 72°C for 60 s before a final extension of 10 min at 72°C. The fragment sizes of amplified products were then analyzed with a CEQ 8000 Genetic Analysis System (Beckman Coulter). Allele nomenclature was based on the median fragment size of each natural group rounded to the nearest probable base pair number. The combined results of fragment-size analysis at all 3 markers were used to create a multilocus fragment type for subtypes within *C. parvum* and *C. hominis* as described elsewhere (*26*,*27*).

Statistical Analysis

Data analysis was carried out by using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Subclusters were identified by using the SPSS clustering algorithm, a hierarchical algorithm that clusters strains and other clusters together on the basis of their similarity.

χ^2 tests (or Fisher exact test when data were sparse) were used to identify significant trends between *C. parvum* cluster 1 and *C. parvum* clusters 2 and 3 combined, with epidemiologic parameters. A final multivariable model was derived by using logistic regression as previously described (*20*) and including all the different strains of *C. parvum*; the model was recalculated including only the strains that possessed the ML1–242 allele.

The Hunter-Gaston index of discriminatory power was calculated by using StatsDirect (*28*). This index was proposed as a measure of the discriminatory power of microbial typing schemes. By using the typing scheme under investigation, it calculates the probability of randomly picking 2 unrelated strains and finding them to be different.

Results

A total of 190 sporadic strains of *Cryptosporidium* were included in this analysis: 118 were *C. hominis*, of which 106 were typeable at all 3 microsatellite loci; 72 were *C. parvum*, of which 63 were typeable at all 3 loci. The distribution of these types is shown in Table 1.

Of the 106 strains of *C. hominis* typeable at all 3 loci, 95 (90%) were indistinguishable at all 3 loci, having the ML1 allele 233 (ML1–233), ML2–180, and gp60–371. This lack of diversity of *C. hominis* as demonstrated by these 3 markers did not allow further analysis.

Much greater diversity in allele size at all 3 microsatellite loci was displayed by *C. parvum* than by *C. hominis*. The discriminatory power of the 3-loci typing method for *C. parvum* using the Hunter-Gaston index of discriminatory power was 0.957 (95% confidence interval [CI] 0.937–0.977). For *C. hominis*, the discriminatory power was 0.197 (95% CI 0.096–0.298).

The online Appendix Figure (available from www.cdc.gov/ncidod/EID/13/1/82-appG.htm) shows a 3-dimensional scatterplot of the strains of *C. parvum*. Considerable variation can be seen in microsatellite length, and 3 broad subclusters are identifiable. Strains belonging to the 2 smaller clusters had the same ML1–227 allele, whereas all strains belonging to the larger cluster had the ML1–242 allele.

We further looked at the association between polymorphisms at the 3 loci and reported case-patient contact with animals. For this analysis, all strains were included, whether or not they were typeable at all 3 loci. Significantly more persons with strains with ML1–242 (22/52, 43%) had touched or handled farm animals than those with ML1–227 strains (0/14, 0%) (Mann-Whitney U test, p = 0.000 (Figure 1). Similarly, at ML2, significantly more strains with alleles between 223 and 237 (42%, 22/52) were from case-patients who had touched or handled farm animals than were strains with alleles 193 and 197 (0%, 0/13) (Mann-Whitney U test, p = 0.000) (Figure 2). Alleles of gp60 (Figure 3) varied from 311 to 371 bp and peaked at 340 to 341 bp. Case-patients who had contact with farm animals yielded significantly greater product sizes at this locus than those who reported no animal contact before onset of illness (Mann-Whitney U test, p = 0.003).

Table 1. Distribution of multilocus fragment types (MLFTs) for *Cryptosporidim* strains typeable at all 3 loci

Species/MLFT	No. strains	%	ML1 allele	ML2 allele	gp60 allele
C. hominis					
H1	95	89.6	233	180	371
H2	3	2.8	239	180	371
H3	2	1.9	242	180	371
H4	1	0.9	224	180	371
H5	1	0.9	233	180	407
H6	1	0.9	233	180	353
H7	1	0.9	218	180	371
H8	1	0.9	218	180	413
H9	1	0.9	233	180	341
C. parvum					
P1	8	12.7	242	229	341
P2	5	7.9	242	229	338
P3	2	3.2	227	193	329
P4	2	3.2	227	195	338
P5	6	9.5	242	231	341
P6	4	6.3	242	233	338
P7	6	9.5	242	231	338
P8	3	4.8	242	233	341
P9	1	1.6	242	225	341
P10	1	1.6	242	227	338
P11	1	1.6	242	229	332
P12	1	1.6	242	229	359
P13	1	1.6	242	229	347
P14	1	1.6	242	231	356
P16	1	1.6	242	231	344
P17	3	4.8	242	231	347
P18	1	1.6	242	233	347
P19	1	1.6	242	235	338
P20	1	1.6	242	237	341
P21	1	1.6	242	231	350
P22	1	1.6	227	193	320
P23	2	3.2	227	195	326
P24	1	1.6	227	223	332
P25	2	3.2	227	197	311
P26	1	1.6	227	231	341
P27	1	1.6	227	195	353
P28	1	1.6	227	193	326
P29	1	1.6	227	193	329
P30	1	1.6	227	195	332
P31	1	1.6	227	229	326
P32	1	1.6	242	237	338

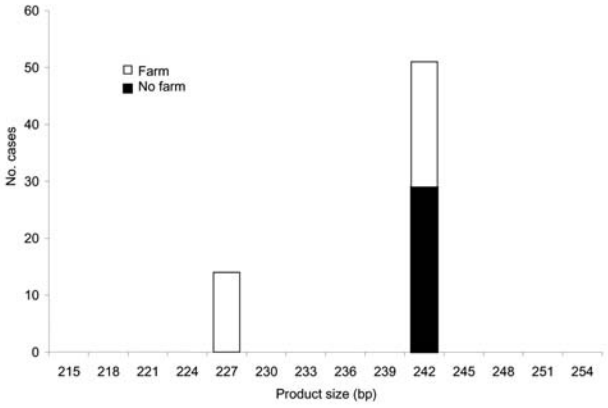

Figure 1. Product size at microsatellite locus ML1 with number of *Cryptosporidium parvum* case-patients who touched or handled farm animals before onset of illness.

To test further the association between the ML1–242 polymorphism and contact with animals, the final logistic regression model for *C. parvum* presented in our earlier article (*20*) was re-run but included only those strains with the 242-bp allele. The positive association with farm animals and the negative associations with eating raw vegetables all are stronger in the model with just ML1–242 allele strains than in the model containing all *C. parvum* strains (Table 2).

Each typeable strain was also categorized by local environment, based on postal code of patient's residence. These categories were urban, town or town fringe, village, and hamlet or isolated dwelling. The attack rates per 100,000 population for each of the 2 ML1 types of *C. parvum* are shown in Table 3. The incidence of ML1–242 strains increased as the home environment became increasingly rural, whereas ML1–227 strains were largely restricted to urban and town environments (Mann-Whitney U test, $p = 0.005$).

Discussion

At these 3 microsatellite loci, much greater genetic diversity was detected among *C. parvum* strains than among *C. hominis* strains. For *C. parvum* the 3 loci were highly discriminatory (Hunter-Gaston index 0.957), but for *C. hominis*, they were poorly discriminatory (0.197). These 3 loci by themselves are unlikely to be sufficient for subtyping *C. hominis* but are adequate for subtyping *C. parvum*.

Using all 3 loci, the typeability for *C. hominis* was 90% and for *C. parvum* 87.5%. The presence of nontypeable strains in any one of the 3 single loci reduced the overall typeability and therefore discriminatory power of the typing method. However, strains that did not type at every locus could still be compared. For example, 70 (96%) strains of *C. parvum* were typed at the ML1 locus, which improved the power of analyses using just this locus. We are unable to say whether nontyping at a particular locus was because of an unusual allele or because of the sensitivity of the method.

The low diversity of *C. hominis* is to be expected because it is a species-specific parasite. Hunter and Fraser (*29*) noted that species adapted to single host species were likely to be less genetically diverse than those with a wider host range, as predicted by the theory of adaptive polymorphism. Greater genetic variation was also found among *C. parvum* (type 2) than *C. hominis* (type 1) isolates in a previous study that used minisatellite and microsatellite loci (*13*). This apparently low genetic diversity among strains of *C. hominis* might make it difficult to develop discriminatory and reproducible typing methods for *C. hominis*. However, recent investigation of isolates from global sources at multiple minisatellite and microsatellite loci showed increased polymorphism, particularly over many minisatellite loci (*30*). On the other hand, the use of only 3 loci gives good discriminatory power for *C. parvum*.

Using just 3 microsatellite loci, we have shown that 3 major groupings of *C. parvum* can be found, which supports the similar findings of Mallon et al. (*13*), who used 7 loci. These researchers reported that the largest cluster contained strains isolated from both humans and animals, while the 2 smaller clusters contained strains isolated only from humans. In our study, all strains isolated from persons reporting contact with animals came from cluster 1, which supports the suggestion of 2 clones of human-adapted strains of *C. parvum*.

The most intriguing finding was that of an association between strains of *C. parvum* that may be human-adapted or zoonotic and particular alleles of the microsatellites. While this association included all 3 loci, the strongest association was with alleles at the ML1 locus. This obser-

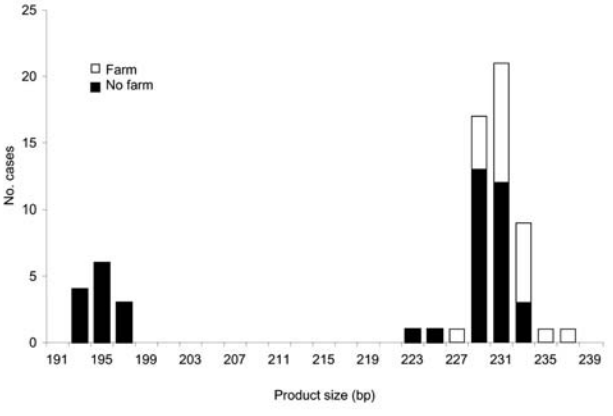

Figure 2. Product size at microsatellite locus ML2 with number of *Cryptosporidium parvum* case-patients who touched or handled farm animals before onset of illness.

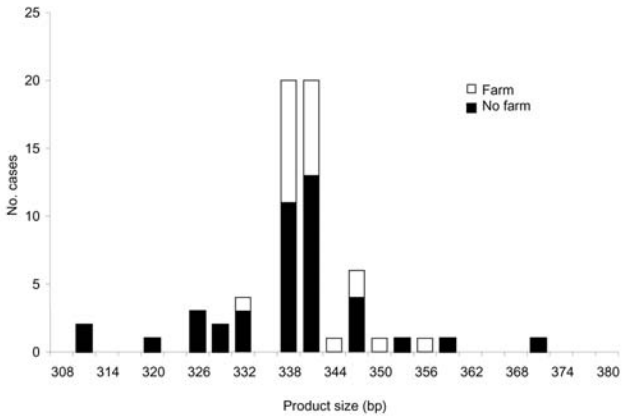

Figure 3. Product size at microsatellite locus gp60 with number of *Cryptosporidium parvum* case-patients who touched or handled farm animals before onset of illness.

vation was even more dramatic, given that only 2 alleles were found at this locus. None of the case-patients whose strains yielded ML1–227 reported contact with farm animals, while 43% of those whose strains yielded ML1–242 reported such contact. This finding is strengthened by the observation that most of the case-patients yielding cluster 2 or 3 strains were more likely to live in urban areas where the possibilities for animal contact are lower than for those yielding cluster 1 strains. In a related study, all 28 strains isolated from animals were ML1–242, which further supports this hypothesis (*27*,*31*).

Although the ML2 locus is more variable than the ML1 locus, the 2 loci correlate very closely. This linkage disequilibrium between the 2 loci has already been noted by other researchers (*11*), although we must emphasize that our results differ from those of Cacciò et al. (*11*), who detected 3 alleles at the ML1 locus (ML1–238, ML1–226, and ML1–220). By sequencing PCR products, these authors also found all 3 alleles in isolates from animals. These discrepancies are not likely to be due to the different methods used for sizing of PCR fragments.

We cannot yet conclude that our findings indicate human-adapted strains of *C. parvum* exist or if all strains are potentially zoonotic. ML1–227 strains do not appear to be zoonotic in the United Kingdom but have been identified as such by other workers in Italy (*11*), for example. If such strains are zoonotic in other countries, they likely would have spread into the UK human population through imported foods or during foreign travel and subsequently spread among humans. However, they may not have yet made the transition to UK animals.

Microsatellite fragment analysis of *C. parvum* would appear to provide a discriminatory and rapid means of distinguishing strains. This technique would be useful in outbreak settings to determine whether outbreaks were due to single or multiple strains and, if the former, may indicate the source of contamination. The microsatellites used in this work would not be discriminatory enough for routine use for *C. hominis*, although others may prove to be of more value.

Table 2. Logistic regression model from case-control study (*19*) showing final model from original study and recalculated using only those strains with the ML1–242 polymorphism as cases*

Cases/variable	Cases, n (%)	Controls, n (%)	Odds ratio	95% CI	p value
All *Cryptosporidium parvum* strains					
Touch or handle any farm animals					
Yes	24 (34)	43 (11)	2.653	1.113–6.323	0.028
No	47	348			
Eat tomatoes					
Yes	24 (36)	249 (50)	0.317	0.140–0.719	0.005
No	43	246			
Eat raw vegetables					
Yes	7 (12)	157 (44)	0.222	0.086–0.572	0.001
No	51	196			
Only ML1–242 strains					
Touch or handle any farm animals					
Yes	21 (43)	43 (11)			
No	28	348	3.810	1.444–10.049	0.007
Eat tomatoes					
Yes	17 (37)	249 (50)	0.425	0.164–1.104	0.079
No	29	246			
Eat raw vegetables					
Yes	4 (10)	157 (44)	0.141	0.042–0.474	0.001
No	37	196			

*CI, confidence interval. Also included in the models were age and Health Authority of residence.

Table 3. Association between subtype number and attack rate per 100,000 population and residential land use

Residential land use	ML1–242		ML1–227	
	No.	Attack rate	No.	Attack rate
Urban	16	0.21	10	0.13
Town and fringe	10	1.31	4	0.52
Village	14	2.72	0	0.00
Hamlet and isolated dwellings	12	3.60	1	0.30

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Blood Transfusion and Spread of Variant Creutzfeldt-Jakob Disease

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Variant Creutzfeldt-Jakob disease (vCJD) may be transmissible by blood. To prevent secondary transmission through blood components, several countries have started to exclude as donors persons who have received a blood transfusion. We investigated the effectiveness of this measure by using a dynamic age-structured model. It is the first such model based on epidemiologic data: 1) blood donor activities, 2) a case-control study on CJD, 3) age distribution of recipients, and 4) death of recipients of blood transfusions. The model predicts that an infection like vCJD, which has been introduced into the population by the alimentary route, could not become endemic by transfusion alone and that only <1% of cases would be avoided by excluding from blood donation those persons who have received a transfusion.

Recent studies of variant Creutzfeldt-Jakob disease (vCJD) indicate that this disease is transmissible by blood. One case of probable transfusion-transmitted vCJD infection has been reported, and 1 case of subclinical infection has been detected (*1*,*2*). On February 9, 2006, a third case was announced by the UK Health Protection Agency (www.hpa.org.uk/hpa/news/articles/press_releases/2006/060209_cjd.htm). Each of the 3 patients had received a blood transfusion from a donor who subsequently developed clinical vCJD, which indicates that transfusion caused the infection. However, a policy to exclude potential donors who had received a transfusion would not have prevented at least the first 2 cases because the corresponding donors had not received any blood transfusion. Diagnostic tools to detect prions in blood are under development (*3*), but no routine test for the presence of the infectious agents of vCJD is available. Therefore, the questions arise as to whether an infection like vCJD could become endemic through blood donation alone and to what extent exclusion of potential donors with a history of transfusion would influence the transmission of such an infection (i.e., how many deaths due to the infection could be prevented?). The following mathematical model is the first to address these questions on the basis of epidemiologic data and realistic and epidemiologically justified assumptions.

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Methods

Model Structure

Figure 1A shows the transitions of a person through the basic states of potential donor activities and receipt of blood transfusion. After birth a person is in the state of not having received any transfusion and not yet being an active donor (S_{00}). The first index refers to the person's state as a transfusion recipient; the second index, to the person's status as a donor. Persons in state S_{00} can change to state S_{01} by becoming a donor or to state S_{100} or S_{101} by receiving a blood transfusion. The third index indicates whether a person with a transfusion history can actually be identified and excluded from donating blood (deferred) (index 1) or not (index 0). The states S_{111} and S_{110} can be reached by either transfusion recipients who start donating blood or active donors who receive a blood transfusion. Blood donors who become inactive are transferred into the states of ex-donors S_{02} and S_{12}, depending on their transfusion history. Ex-donors can also become transfusion recipients; i.e., they are transferred from S_{02} to S_{12}. Donor exclusion transfers a certain proportion of transfusion recipients into

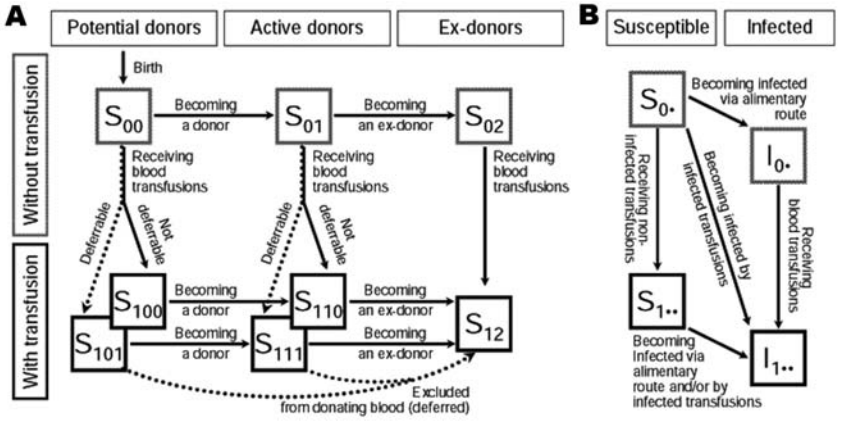

Figure 1. A) States and transitions for the model of blood transfusion in the absence of an infection. B) Routes of infection. The arrows representing deaths out of all states are omitted. Paths of donor exclusion are plotted by dotted arrows. S_{00}, nonrecipients who do not donate; S_{01}, nonrecipients who donate; S_{02}, nonrecipients who are excluded from donating; S_{100}, recipients who do not donate; S_{101}, recipients who become excluded from donating; S_{110}, recipients who donate; S_{111}, recipients who become excluded from donating; S_{12}, recipients who are excluded from donating. Indices replaced by a dot (panel B) represent all other possible states (e.g., $S_{0\cdot}$ represents S_{00}, S_{01}, or S_{02}).

the state of ex-donors. For all susceptible states, Figure 1B shows the transitions to the corresponding infected states. Table 1 provides a list of all input parameters together with descriptions and sources. The details of the model with all the numerical parameter estimates and the equations are given in the online Appendix (available from www.cdc.gov/ncidod/EID/13/1/89-app.htm). The computer program is available upon request. This article summarizes the major features of the model, the data sources, and the estimation of the model parameters.

Demography

To simplify the model, we did not attempt to describe the demographics of the population during the next 150 years. Doing so would involve predicting changes in rates of birth, death, and immigration. It is assumed that in the absence of infection, the population is demographically stationary. We assumed a constant inflow of newborns and an age-specific death rate. The latter was estimated as a weighted mean of the age-specific female and male death rates. Because this study was initiated in Germany, we used the corresponding demographic data. To start the simulation in a demographically stationary state, the model was run for 100 years without infection. Thus, the age distribution of the population was identical to the life table of Germany 2002/2004 averaged over both sexes (www.destatis.de/download/d/bevoe/sterbet04.xls).

Modeling Blood Donors

Blood donors in Germany are ≥18 and <68 years of age. The rates for becoming a new donor and terminating the period as an active donor are age dependent. The corresponding parameters were estimated by using data from 262,071 donors registered with the German Red Cross

Table 1. Summary of input parameters for the model*

Parameters	Description	Source
Age-specific mortality rates	U-shaped, with minimum at age 10.	Federal Statistical Office of Germany
Donor recruitment	Donors ages 18–67 y. Maximum recruitment rate at age 18, lower plateau ages 25–50; further decrease until age 67.	Age-distribution of first-time donors at DRK Blood Service and age structure in population
Proportion of donors	3% of population.	DRK Blood Service West
Duration as active donor	Donors ages 18–40 y, mean duration as active donor 10–14 y, decreases linearly to 0.	Age distribution of active donors at DRK Blood Service West, by age at first donation
Risk of receiving transfusions	Bimodal, with peaks for newborns and aged persons. Multiple transfusions possible.	Data collected from 4,867 patients March 2003, University Hospital Essen, Germany
Transfusion-associated risk for death	Increases according to a sigmoid function, ≈17% at birth to ≈48% in old age. For those with transfusion-associated risk for death, life expectancy is ≈2.5 years at birth and decreases to ≈0.5 y in old age.	Follow-up of ≈3,000 transfusion recipients for ≈7.5 y in Newcastle, UK (*4*)
Alimentary infection	Constant over an initial period of 10 y.	Arbitrary assumption
Incubation period†	Gamma distributed with mean 16 y, SD 4 y. Sensitivity analysis with mean = 50 y and same coefficient of variation.	Models fitted to the UK incidence of vCJD (*5,6*)
Donor exclusion	Either 0 or 95% of those with transfusion history.	Arbitrary assumption

*DRK, German Red Cross; SD, standard deviation; vCJD, variant Creutzfeldt-Jakob disease.
†Time between infection and death, i.e., duration of infection.

(DRK) Blood Service West in Hagen, Germany, including age, sex, age at first donation, number of donations, and date of last donation.

The age-specific prevalence of active donors peaks at ≈24 years of age and subsequently declines monotonically to zero by age 68. The overall prevalence in the population is 3%, i.e., 2.4 million donors in a population of ≈80 million.

Modeling Transfusion Recipients

The model takes into account that persons may receive >1 transfusion throughout their lifetime, but it does not track the number of transfusions received per person. Persons with ≥1 transfusion continue to be at risk for infection from further transfusions. The age-specific risk of receiving a transfusion was estimated from data for all patients hospitalized at the University Hospital in Essen during March 2003. Of 4,867 patients, 1,343 (27.6%) received ≥1 transfusion. The number of persons receiving a blood transfusion in each 5-year age group was divided by the corresponding number of persons in the general population. The observed rates were fitted with a simple model that assumes initially an exponential decline and subsequently a unimodal peak, which is proportional to the density function of the normal distribution. These age-specific ratios were properly scaled to balance the yearly number of transfusions per capita. To limit the complexity of the model, we did not take into account persons in subgroups, such as those with hemophilia, who obtain blood products from pools of donors. Because for medical reasons these subgroups are excluded from donating blood, they cannot contribute to persistence of the infection.

Independence of Receiving and Donating Blood

The events of receiving a blood transfusion and of donating blood are assumed to be independent of each other. This assumption is supported by the results of a case-control study of potential risk factors for CJD, which was coordinated by the Clinical Surveillance Centre for CJD, Department of Neurology in Göttingen, Germany (*7*). Table 2 shows the joint distribution for the control group of having received and donated blood. According to the Fisher exact test, the p value for the hypothesis of no association is 0.43.

Heterogeneity in the risk of receiving a blood transfusion is modeled by the assumption that only a proportion of the population are at risk, whereas the remaining proportion never receives a transfusion. This assumption was introduced to be consistent with data from the case-control study, in which ≈18% of the population reported having ever received a blood transfusion. Without this assumption, the model would predict that eventually 100% of a cohort would receive a blood transfusion because the average annual risk of receiving a blood transfusion is about 5%, i.e., ≈4 million in a population of 80 million.

Table 2. Joint distribution of transfusion history and blood donation

Received blood	Donated blood, no. observed (no. expected if events are independent)		
	No	Yes	Total no. (%)
No	401 (404)	104 (101)	505 (82)
Yes	93 (90)	19 (22)	112 (18)
Total no. (%)	494 (80)	123 (20)	617 (100)

Modeling Transfusion-associated Death Rates

The transfusion-associated death rate has been described in detail by Wallis et al. (*4*). A good fit to the data assumes that at all ages a certain proportion of transfusion recipients have a higher rate of dying and the remaining proportion has a survival rate that corresponds to that of persons of the same age group in the general population. This age-dependent proportion of transfusion recipients with an increased risk for death is described by a generalized logistic function with a positive value at birth and an asymptote <100% for old age. The transfusion-associated death rate increases linearly with age. The increased death rate appears to be concentrated in the first 2 years after a transfusion. Wallis et al. report that 2,888 patients were observed as long as 7.4 years after transfusions received in June 1994 (*4*). The sex-specific rates were averaged for the simulation model.

Modeling the Infection

Usually the incubation period refers to the time between the infection and disease. In the context of CJD, however, disease can refer to onset, diagnosis, or death. Like Bacchetti, we also focused on death rates (*8–10*). The incubation period is assumed to be gamma distributed with a mean duration of 16 years and a standard deviation of 4 years, which conforms to estimates of Valleron et al. and Ghani et al. (*5*,*6*). Because of great uncertainty about the length of the incubation time, we also considered a much higher value of 50 years in the absence of the competing risk for death. The coefficient of variation is assumed to be the same, such that the standard deviation is 12.5 years. Because of competing risks, the actual sojourn in the incubation period is 15.3 for an incubation period of 16 years and 34.0 years for an incubation period of 50 years. The proportions of infected persons who would die with disease symptoms are 79% and 37% for the incubation periods of 16 and 50 years, respectively. This means that for an incubation time of 50 years, nearly two thirds would die without disease symptoms. Hereafter we refer to these values of 15 and 50 years as short and long incubation periods.

We distinguish between 2 modes of transmission. Initially, the infection is introduced into the population by the alimentary route. In the United Kingdom the number of infected animals entering the food supply peaked in 1989; most were concentrated within a period of 10 years (*11*), which we take as the assumed period of alimentary infection. After this period, this mode of transmission was interrupted so that further transmissions are possible only through blood transfusions.

A study to detect the presence of abnormal prion protein in appendix and tonsil tissues has suggested a prevalence of 235 infections per million in the United Kingdom (*12*). We arbitrarily assumed the prevalence of infections in Germany to be ≈1 order of magnitude lower, yielding a cumulative incidence of 25 per million, which was the value used for the simulations.

We made 2 contrasting assumptions about the infectivity of blood preparations and evaluated the results of these 2 simulations: each transfusion (100% infectivity) or no blood transfusion (0% infectivity) from an infected donor leads to infection of the recipient. In the model the infection probability (probability of receiving blood from an infected donor) is proportional to the proportion of infected donors among all donors. Thus, we can calculate the number of infections from blood transfusions compared with the number of infections from alimentary transmission alone.

Modeling Donor Exclusion

The model distinguishes between persons with and without transfusion history, termed recipients and nonrecipients; these terms are applied to persons whether they have or have not donated blood. The model allows recipients to be excluded from donating blood. In modeling the exclusion of recipients, we took into account that this measure may be imperfect and that a certain proportion of recipients may not be excluded.

Results

For the parameter estimates obtained from the sources described above, the infection cannot become endemic (Figure 2). If we assume no further spread through blood transfusions after 10 years of infections by the alimentary route, the maximum prevalence reached is ≈1,860 (1,434 for nonrecipients plus 426 for recipients) because some of the infected persons die of other causes during the incubation period. If transmission is assumed to be possible through blood transfusions (100% infectivity), then the maximum prevalence among recipients is increased by ≈78 infections after 4 more years for the short incubation period and by 193 infections after 23 more years for the long incubation period.

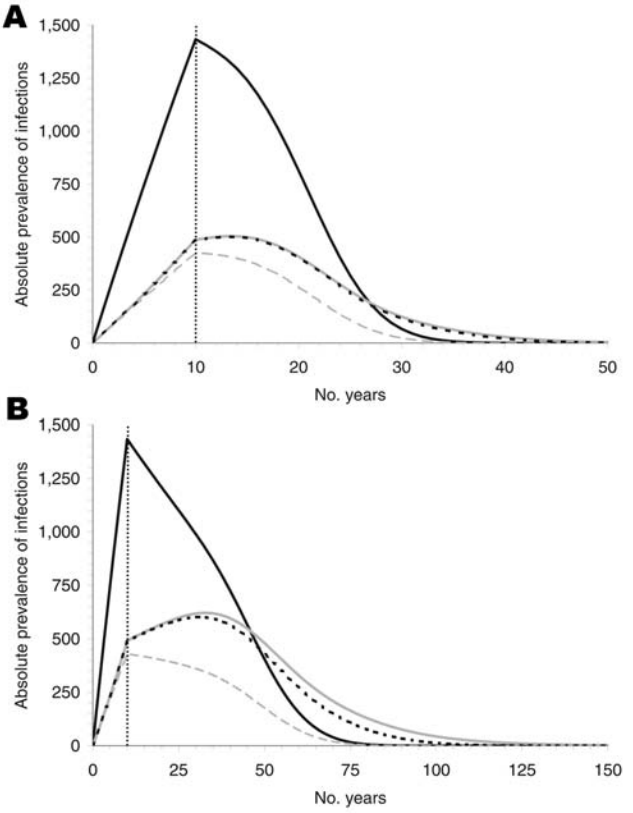

Figure 2. Absolute prevalence of infection for an incubation period of 16 (A) and 50 (B) years, for nonrecipients of blood transfusion (solid, black), recipients under the assumption of no infectivity (dashed, gray), of 100% infectivity without donor exclusion (dotted, black), and 100% infectivity with donor exclusion (solid, gray). The prevalence declines after the alimentary route of transmission is interrupted, i.e., after 10 years. Prevalence differs only slightly if the infection probability of a transfusion from an infected donor is increased from 0% to 100%. Donor exclusion produces negligible reductions.

We assumed that donor exclusion is implemented immediately at the beginning of the alimentary infection risk period, which reduced the original number of 2.55 million donors by ≈20% to a value of 2.05 million donors. Because the model does not account for the stock of blood donations, this reduction in the number of donors must be compensated for with an increased rate of donations per donor to satisfy the demand; i.e., the average number of donations would have to increase from 1.6 to 2 per donor per year. Figure 2A shows that donor exclusion has almost no effect when the incubation period is assumed to be 16 years. The absolute prevalence (i.e., the actual number of infected persons) differs at most by 9. For a long incubation, differences are visible (59 persons at most) but small in view of the long time intervals and the size of the total

population (Figure 2B). The reason for these small differences is described below.

The cumulative numbers of deaths from the infection are given in Table 3. The numbers are considerably smaller for the long than for the short incubation period because a long incubation period implies more deaths from other causes. The numbers are given separately for cases in patients with and without a history of blood transfusion. The route of infection for nonrecipients is alimentary only, whereas the route of infection for recipients is unclear. If we compare the simulations at 100% and 0% infectivity of blood transfusions, we observe 172 and 224 additional cases for the short and the long incubation periods, respectively. These numbers represent 11% of 1,557 and 31% of 725 cases, which would be expected for 0% infectivity for the short and long incubations periods, respectively. For the short incubation period we expect a higher absolute number of alimentary cases but a smaller proportion of transfusion cases than for the long incubation period. The exclusion of donors would prevent only 15 and 50 cases, i.e., ≈15 (0.9%) of 1,729 and 50 (5%) of 949, respectively, at the end of the epidemic. The epidemic lasts for ≈50 or ≈150 years for the short and the long incubation periods, respectively.

The predicted yearly incidence of deaths due to vCJD, separated by transfusion history, is shown in Figure 3. The yearly peak incidence of total deaths would be 128 and 29 for the short and the long incubation periods at 23 and 51 years after the beginning of the epidemic, respectively. For 0% infectivity the peak incidence would be only 5 and 3 cases less for the short and long incubation periods, respectively, which implies that the exclusion of donors with a transfusion history does not effectively prevent infection.

Figure 4 shows the predicted yearly incidence of deaths according to the route of infection. The time lags between the peaks of deaths due to alimentary infection and due to transfusion clearly differ and are 9 and 20 years for short and long incubation periods, respectively.

Finally, we considered the absolute prevalence of infected donors according to their history of blood transfusion (Figure 5). Most infected donors do not have a transfusion history, which explains the negligible effect of a policy excluding transfusion recipients from donation.

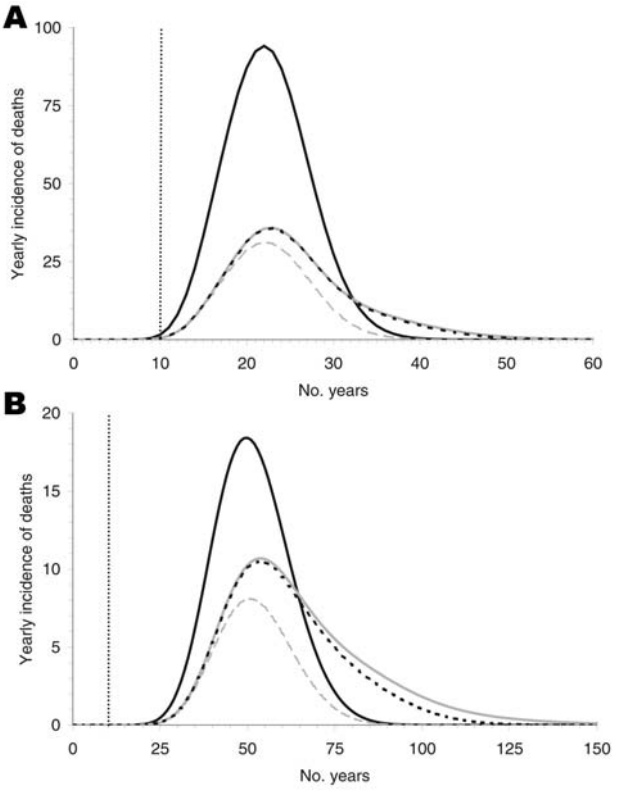

Figure 3. The yearly incidence of deaths for an incubation period of 16 (A) and 50 (B) years. The black curves show nonrecipients of blood transfusion who were infected only by the alimentary route. These curves are independent of the infection probability and the rate of donor exclusion. The lower 3 curves represent the deaths of recipients originating from 0% infectivity of blood transfusions (dashed gray), 100% infectivity without donor exclusion (solid gray), and 100% infectivity of blood transfusions with donor exclusion (dotted black, almost indistinguishable from solid gray line in A). The differences between the solid and dashed gray curves represent the cases due to blood transfusion.

To determine whether the same model could also predict transition into a positive endemic equilibrium of the infection, we made the unrealistic assumptions that the rates of donor recruitment and donor loss are constant between the ages of 18 and 67 and that the rate of receiving a blood transfusion is constant throughout life. Then the model showed an extremely long time (>2,000 years)

Table 3. Cumulative numbers of deaths from variant Creutzfeldt-Jakob disease at the end of the epidemic

Incubation period	Donors excluded	Infectivity (%)	Without transfusion	With transfusion	Total no. cases
Short	No	0	1,167	390	1,557
	No	100	1,167	562	1,729
	Yes	100	1,167	547	1,714
Long	No	0	503	222	725
	No	100	503	446	949
	Yes	100	503	396	899

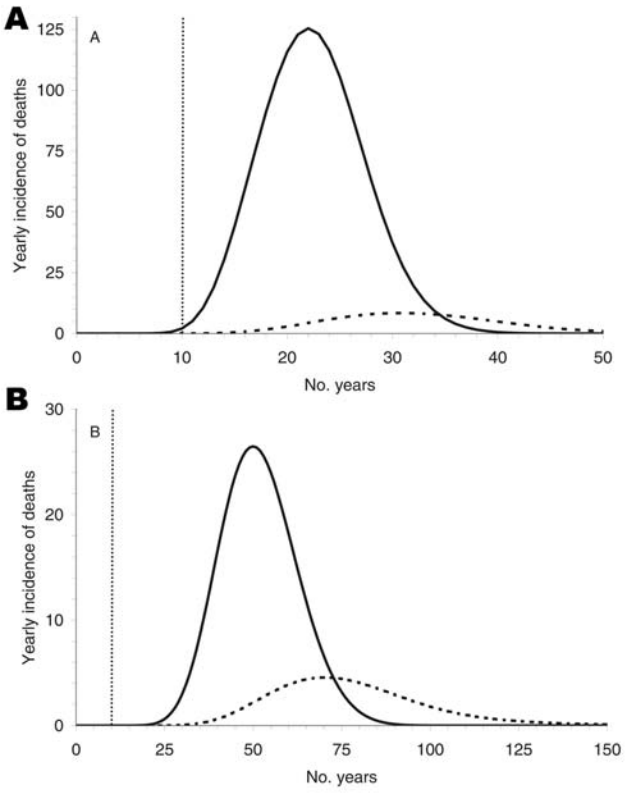

Figure 4. Yearly incidence of deaths caused by alimentary transmission (solid line) and by blood transfusion (dashed line).The 2 peaks differ by 9 and 20 years, depending on the incubation period: 16 (A) and 50 (B) years, respectively.

before positive equilibrium would be reached (results not shown).

Discussion

Our model is the first attempt to describe in a realistic way the transmission of infections through blood transfusions. In 1994, Velasco-Hernández proposed a model for the spread of Chagas disease by vectors and blood transfusion (*13*). His model was used by Roberts and Heesterbeek to introduce their new concept to estimate the effort to eradicate an infectious disease (*14*). Huang and Villasana included transmission through blood transfusion in an AIDS model (*15*). All these models have in common what Inaba and Sekine state about their extension of Velasco-Hernández's Chagas model: "…here we assume that blood donors are randomly chosen from the total population, and so there is no screening and the recipients of blood donations are donating blood themselves at the same rate as anybody else. This is an unrealistic assumption, but we will use it." (*16*). These models implicitly describe transmission through blood transfusion exactly like person-to-person transmission by droplet infections.

The key innovation in our model is the simultaneous incorporation of 6 functions that all depend explicitly on the age of a person: 1) natural death rate, 2) rate of receiving a blood transfusion, 3) rates of donor recruitment, 4) donor loss, 5) death rate associated with transfusions, and 6) proportion of transfusion recipients at increased risk for death. The age-dependent effects of these processes cannot be ignored. Peak ages of donor activity (≈22 years) and of receiving a blood transfusion (≈70 years) are quite distinct and ≈50 years apart. This age pattern does not favor the spread of infection by blood transfusion. Another factor that acts against the infection becoming endemic is the transfusion-associated death rate. The good quality of the follow-up data of nearly 3,000 patients helped to incorporate realistic assumptions about the survival probabilities of transfusion recipients (*4*). The only data available about the joint distribution of blood donor activity and history of a blood transfusion was the CJD case-control study performed in Göttingen, Germany (*7*).

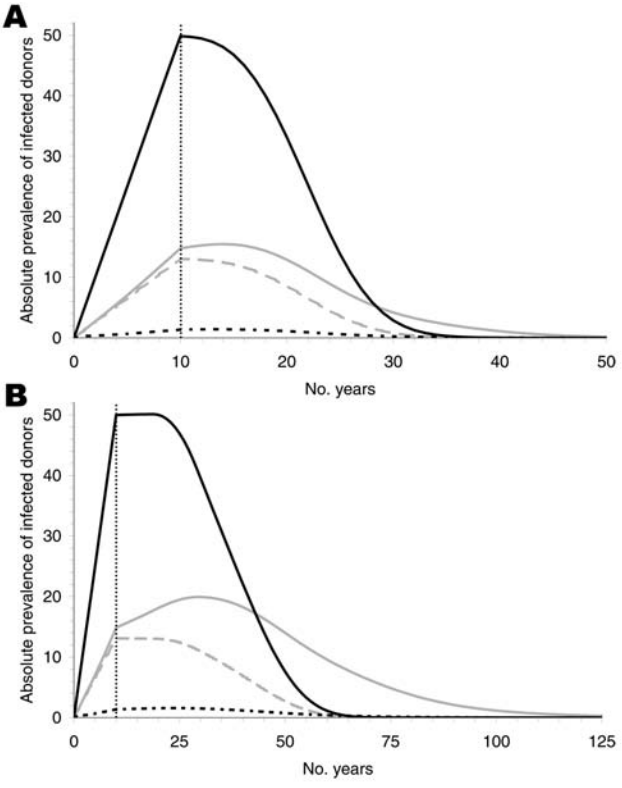

Figure 5. Absolute prevalence of infected donors for an incubation period of 16 (A) and 50 (B) years. The solid black curves show the infected donors without transfusion history. These curves are identical for 0% and 100% infectivity and are independent of donor exclusion. The gray curves show infected donors with transfusion history for 100% (solid) and 0% (dashed) infectivity, respectively, without donor exclusion. The dotted black curves show the effect of donor exclusion starting at the beginning of alimentary risk. Most infected donors have no transfusion history and cannot, therefore, be excluded from blood donation.

The length of the incubation period plays a major role in transmission dynamics and hence was subject to a sensitivity analysis. The model does not account for possible changes of infectivity during the incubation period. The model represents a worst-case scenario because it assumes 100% infectivity throughout the period of infection. Even under this extreme assumption, donor exclusion can prevent only 0.9% (or 5%) of the expected deaths, assuming the incubation period has a mean duration of 16 (or 50) years. The main explanation for this surprising result is that most infected donors have been infected by the alimentary route and never received any blood transfusion and, therefore, are not eligible for donor exclusion.

The present simulations have arbitrarily assumed a cumulative incidence of alimentary infection, about 25 per million (2,000 per 80 million). With pessimistic assumptions, the model predicts either 19.5 deaths per million for the short incubation period or 9 deaths per million for the long incubation period in the absence of spread through blood transfusion. This corresponds to at least 9 (36%) of 25 deaths attributable to the infection, which is ≈2 orders of magnitude higher than expected for vCJD in the United Kingdom. As of July 2006, the number of vCJD cases in the United Kingdom was 160. If we assume that the total number of cases will be 200, then our assumption corresponds to about 3.3 cases per million. Thus, at most, 1.4% of infected persons would die from the infection (unless a second wave of vCJD cases with a long incubation period occurs). According to our model, 0.9% of the deaths could be prevented by donor exclusion under the assumption of the short incubation period. In absolute numbers this would be ≈2 cases.

In France, the total number of vCJD cases recorded through July 2006 is 18. Even under the assumption that this number represents only 35% of the total number of cases (*17*), the absolute expected number of prevented cases would be <1. In 1998, France decided to exclude donors with a transfusion history, primarily to reduce the spread of viruses. The present model could be modified to assess the effectiveness of excluding donors with transfusion history for preventing emerging infections with different modes of transmission and additional epidemiologic states, e.g., latent or immune.

Our worst-case scenario assumptions of the epidemiology might seem similar to the situation in the United Kingdom. In Germany, no case of vCJD has been reported, which indicates that the expected number of cases in Germany is at least 2 orders of magnitude less than that in the United Kingdom. This latter aspect was considered in the interpretation of our model by a working group commissioned by the German Federal Minister of Health, which recommended in April 2006 that persons with a transfusion history not be excluded from donating blood (*18*). Our analysis enables different countries to perform their own risk assessment and choose a strategy according to the absolute number of cases observed or expected.

The German CJD Surveillance study was supported by a grant from the German Ministry of Health (Az 325-4471-02/15 to Inga Zerr and H. A. Kretzschmar). Helpful discussions about previous versions of the model took place with the Working Group Overall Blood Supply Strategy with regard to vCJD, Germany (Chairman R. Seitz).

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Multidrug-resistant *Acinetobacter* Infection Mortality Rate and Length of Hospitalization

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Acinetobacter infections have increased and gained attention because of the organism's prolonged environmental survival and propensity to develop antimicrobial drug resistance. The effect of multidrug-resistant (MDR) *Acinetobacter* infection on clinical outcomes has not been reported. A retrospective, matched cohort investigation was performed at 2 Baltimore hospitals to examine outcomes of patients with MDR *Acinetobacter* infection compared with patients with susceptible *Acinetobacter* infections and patients without *Acinetobacter* infections. Multivariable analysis controlling for severity of illness and underlying disease identified an independent association between patients with MDR *Acinetobacter* infection (n = 96) and increased hospital and intensive care unit length of stay compared with 91 patients with susceptible *Acinetobacter* infection (odds ratio [OR] 2.5, 95% confidence interval [CI] 1.2–5.2 and OR 2.1, 95% CI 1.0–4.3] respectively) and 89 uninfected patients (OR 2.5, 95% CI 1.2–5.4 and OR 4.2, 95% CI 1.5–11.6] respectively). Increased hospitalization associated with MDR *Acinetobacter* infection emphasizes the need for infection control strategies to prevent cross-transmission in healthcare settings.

Acinetobacter species are aerobic gram-negative bacilli that can cause healthcare-associated infections and can survive for prolonged periods in the environment and on the hands of healthcare workers (*1–3*). The proportion of healthcare-associated infections caused by *Acinetobacter* spp. has increased over the past decade in the United States (*4*). Furthermore, *Acinetobacter* infections have become increasingly difficult to treat because of the emergence of strains resistant to all drugs or all but 1 commonly prescribed antimicrobial drug (*5–7*). These multidrug-resistant (MDR) strains are sometimes susceptible only to polymyxins (colistin and polymyxin B), a class of antimicrobial drugs that has not been in widespread use for several decades and is more toxic than most currently used antimicrobial drugs. Outbreaks caused by MDR *Acinetobacter* have been reported in hospitals all over the world; more recently, they have become a serious problem in military medical facilities (*7–9*).

Although drug resistance of *Acinetobacter* is a recognized problem, the effect of MDR *Acinetobacter* infections on patient outcomes remains controversial. Previous studies on clinical outcomes of patients infected with *Acinetobacter* have yielded conflicting results and are limited by methodologic challenges that include small sample sizes, failure to control for severity of illness before infection, and failure to exclude patients colonized with *Acinetobacter* (*10–14*). Furthermore, most studies do not differentiate outcomes of patients infected with MDR *Acinetobacter* from those infected with drug-susceptible *Acinetobacter*. This finding leads to questions about the merits of targeting infection control measures to control MDR *Acinetobacter.* To determine the effect of MDR *Acinetobacter* infection on mortality rates, length of hospital stay, and length of intensive care unit (ICU) stay, the University of Maryland Medical Center, The Johns Hopkins Hospital, the Maryland Department of Health and Mental Hygiene, and the Centers for Disease Control and Prevention collaborated to perform a retrospective, matched, cohort study in Baltimore, Maryland. This is the first study that directly examines the effect of multidrug

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resistance on outcomes of *Acinetobacter* infections while controlling for severity of illness.

Methods

Study Design

A retrospective, matched cohort investigation was performed by using patient records from January 2003 through August 2004 from 2 tertiary care hospitals in Baltimore to examine outcomes of hospitalized patients with MDR *Acinetobacter* infection (exposure) compared with 2 unexposed reference groups: patients with susceptible *Acinetobacter* infection (susceptible references) and patients without *Acinetobacter* infection (uninfected references). We chose 2 reference groups to explore the effects of multidrug resistance and MDR *Acinetobacter* infection on patient outcomes (*15*). We defined MDR *Acinetobacter* as organisms resistant to all or all but 1 antimicrobial drug classes commonly prescribed to treat gram-negative infections. Susceptibility to polymyxins was not considered in these criteria because susceptibility testing for these drugs is not routinely performed. We defined susceptible *Acinetobacter* as organisms susceptible to ≥3 antimicrobial classes. Those patients infected with *Acinetobacter* that was susceptible to only 2 antimicrobial drug classes were excluded.

A computer-generated list was used to identify all persons from whom MDR *Acinetobacter* had been recovered from January 2003 through August 2004. Charts were then reviewed to determine, on the basis of criteria set forth by the National Nosocomial Infection Surveillance System (NNIS) (*16*), if the patient had 1 of the following infections caused by MDR *Acinetobacter*: bloodstream, pneumonia (respiratory tract), surgical site, urine, or sterile site other than blood. Admitted patients with both healthcare-acquired (i.e., infection was diagnosed >48 h after hospital admission) and community-acquired *Acinetobacter* infections (i.e., infection was diagnosed within 48 h of hospital admission) were included.

For the selection of matched susceptible references, microbiology records were reviewed to identify patients from whom susceptible *Acinetobacter* had been recovered from January 2003 through August 2004 at each institution. NNIS definitions were then applied to identify *Acinetobacter*-infected patients who were included in the study (*16*). To ensure that susceptible references and their matched MDR *Acinetobacter* patients had a similar exposure time, the susceptible references had to have a preinfection length of hospital stay within 5% of the matched MDR *Acinetobacter* patient's preinfection length of stay. Patients infected with MDR *Acinetobacter* were matched to susceptible references from the same institution.

The second reference group, uninfected patients, included patients without *Acinetobacter* infection who had a length of hospital stay (time between admission and discharge) at least as long as the preinfection length of stay of the respective matched patient infected with MDR *Acinetobacter*. The matched uninfected patient also had to be present in the ward where the patient infected with MDR *Acinetobacter* was located within 30 days of becoming infected with MDR *Acinetobacter*.

Data abstracted from medical records included demographic information; presence of prior and concurrent medical conditions; dates of admission and discharge to the ICU and hospital; date and time of *Acinetobacter* culture; *Acinetobacter* antimicrobial susceptibility pattern; length of stay before infection (exposure time); presence or absence of concordant antimicrobial therapy on the day of the *Acinetobacter* culture (based on the susceptibility pattern of the organism); patient status on day of discharge; and date and cause of death if applicable. Data were collected on the following outcomes: in-hospital mortality rates and total number of days in hospital and ICU after the index day (i.e., length of stay after the exposure). We defined the index day as either the day of hospitalization on which *Acinetobacter* infection was diagnosed for patients infected with MDR *Acinetobacter* and for susceptible reference patients or the same day of hospitalization for uninfected patients. For example, if an MDR *Acinetobacter* infection was diagnosed on hospital day 15, that would be the index day for the MDR *Acinetobacter* patient and for the matched, uninfected patient.

To control for severity of illness before *Acinetobacter* infection, data were collected to calculate a modified Acute Physiology and Chronic Health Evaluation III (APACHE) score (*17*,*18*) ≈48 h before the index day. Our modified APACHE score did not include blood pH, pulmonary arterial oxygen saturation, pulmonary arterial gradient, urine output, or scoring for neurologic abnormalities. These parameters were excluded because they were not uniformly available for all patients in the study, particularly for those not in the ICU. To control for underlying disease, a Charlson comorbidity index (*19*) was calculated by using data from the medical history recorded on the chart.

Statistical Analysis

All data were collected on standard forms, entered into an Access database (Microsoft, Redmond, WA, USA) and analyzed with SAS software (SAS Institute, Cary, NC, USA). Demographic data were analyzed with Mantel-Haenszel relative risks and 95% confidence intervals (CIs) to compare categorical variables and the Wilcoxon 2-sample test with t approximation to compare continuous variables. Matched univariate analysis was performed by using conditional logistic regression. Multivariable analysis con-

trolling for severity of illness with the APACHE score and underlying diseases with the Charlson index was performed by using conditional logistic regression to evaluate in-hospital mortality rate and hospital and ICU length of stay.

To create a dichotomous variable for ICU and hospital lengths of stay, we compared the number in each comparison group that had a length of stay greater than the combined mean of the 2 groups being compared. For example, the combined group of MDR *Acinetobacter*–infected and susceptible *Acinetobacter* references had a mean hospital length of stay of 23 days after the index day (day of infection). The number of MDR *Acinetobacter* patients who had a hospital length of stay >23 days was then compared with the number of susceptible references who had a hospital length of stay >23 days, while controlling for severity of illness and associated underlying diseases in the multivariable model. We chose to compare against the mean rather than the median length of stay to account for outliers. Unmatched MDR *Acinetobacter*–infected and reference patients were excluded from the groups before the mean was calculated. A linear regression model and an ordinal logistic regression model were also attempted; however, these models could not be used because the outcome data were not normally distributed and could not be transformed appropriately for linear regression and because the assumptions for the ordinal logistic regression model could not be satisfied.

To examine the effect of discordant empiric antimicrobial drug therapy on clinical outcomes, multivariable analysis was performed on the MDR *Acinetobacter* patients alone; the exposure evaluated was concordant versus discordant empiric antimicrobial drug therapy. Outcomes included mortality rate, length of hospital stay, and ICU stay. We defined discordant empiric antimicrobial drug therapy as the administration of antimicrobial drug(s) to which the *Acinetobacter* strain was not susceptible. Concordant empiric antimicrobial drug therapy is defined as the administration of antimicrobial drug(s) to which the *Acinetobacter* strain was susceptible. APACHE and Charlson index scores were included in the model to control for their effect on outcomes.

Effect modification between MDR *Acinetobacter* reference groups and APACHE and Charlson index variables was evaluated by testing appropriate interaction terms for statistical significance. All statistical tests were 2-tailed; a p value ≤ 0.05 was considered statistically significant.

Results

From January 2003 through August 2004, a total of 166 patients had cultures that grew MDR *Acinetobacter*, and 96 (58%) met the NNIS criteria for an MDR *Acinetobacter* infection (*16*). Of the MDR isolates, 88 (92%) were not susceptible to carbapenems.

MDR *Acinetobacter*–infected Patients Compared with Susceptible References

We identified 91 reference patients infected with susceptible *Acinetobacter* who had similar lengths of hospital stay before the index day as MDR *Acinetobacter*–infected patients. Five MDR *Acinetobacter*–infected patients were excluded from the analysis because reference patients could not be identified due to a lack of susceptible references who were hospitalized for long periods. MDR *Acinetobacter* patients and susceptible references were similar in age and sex; however, MDR *Acinetobacter* patients had higher baseline mean APACHE and Charlson index scores than susceptible references (Table 1). The distribution of culture sites among MDR *Acinetobacter* and susceptible reference patients was similar; ≈50% of each group had respiratory infections, 31% in each group had bloodstream infections, and <10% of both groups had surgical wound, urinary tract, or other sterile site infections (p not significant for all comparisons). A total of 78 (81%) MDR *Acinetobacter* infections and 73 (80%) susceptible *Acinetobacter* infections were identified >48 h after hospital admission and thus met criteria for nosocomial infection.

Matched univariate analysis of patient outcomes showed that MDR *Acinetobacter*–infected patients had higher mean lengths of hospital stay and ICU stay after the index day than susceptible and uninfected references (Table 2). In-hospital mortality rates for patients with MDR *Acinetobacter* infections (26%) were higher than for susceptible references (18%) and uninfected references (11%). However, only the difference between MDR *Acinetobacter*–infected patients and uninfected patients was statistically significant (Table 2). When controlling for severity of illness with the APACHE score and for underlying disease with the Charlson index in a conditional logistic regression model, association with a longer hospital and ICU length of stay was approximately twice as likely for patients with MDR *Acinetobacter* infection as for susceptible references (Table 3). Multivariable analysis controlling for severity of illness with the APACHE score and underlying diseases with the Charlson index showed a trend toward more deaths associated with infection with MDR *Acinetobacter* than with infection with susceptible *Acinetobacter*, but the difference was not statistically significant (relative risk 2.6, 95% CI 0.3–26.1) (Table 3).

Discordant antimicrobial drug therapy was more common for MDR *Acinetobacter*–infected patients than for susceptible references (91% vs. 65%, $p<0.001$). When we controlled for severity of illness and underlying diseases, we found that initial discordant antimicrobial drug therapy was not associated with increased mortality rates or hospital length of stay among patients infected with MDR *Acinetobacter* (Table 4). However, patients infected with MDR *Acinetobacter* who were treated with initial discor-

Table 1. Comparison of baseline characteristics of patients with multidrug-resistant (MDR) *Acinetobacter* infection with those with susceptible *Acinetobacter* infection and those without *Acinetobacter* infection, Baltimore hospitals, 2003– 2004*

Characteristic	MDR *Acinetobacter*, n = 96	Susceptible *Acinetobacter*, n = 91	p values for MDR *Acinetobacter* vs. susceptible, n = 187	Uninfected, n = 89	p values for MDR *Acinetobacter* vs. uninfected, n = 185
Median age, y	54	52	0.61	52	0.97
Age range, y	14–83	12–85		17–90	
Sex, % male	67	57	0.78	65	0.96
Mean modified APACHE III score (median)	41 (42)	36 (33)	0.02	32 (28)	<0.001
Mean Charlson index (median)	3.9 (3.0)	2.8 (2.0)	0.01	2.5 (2.0)	<0.01

*APACHE III, Acute Physiology and Chronic Health Evaluation III.

dant antimicrobial drug therapy were >5× as likely to be associated with an increased ICU length of stay.

MDR *Acinetobacter*–infected Patients Compared with Uninfected References

Uninfected reference patients were identified for 89 of 96 patients with MDR *Acinetobacter* infections. Reference patients were not identified for 7 MDR *Acinetobacter* patients because there were not enough uninfected patients with extensive hospital lengths of stay. MDR *Acinetobacter*–infected patients and uninfected references were similar in age and sex. However, patients with MDR *Acinetobacter* infection had higher baseline mean APACHE and Charlson index scores than references (Table 1). Matched univariate analysis of patient outcomes showed that patients with MDR *Acinetobacter* had higher in-hospital mortality rates (26% vs. 11%, p<0.01) and mean hospital and ICU lengths of stay after the index day than uninfected references (Table 2). When we controlled for severity of illness and underlying conditions, we found that MDR *Acinetobacter*–infected patients were more likely to have both longer hospital and ICU lengths of stay than uninfected references (Table 3).

Discussion

Acinetobacter is emerging as an important pathogen in traditional and nontraditional healthcare settings. Its ability to infect healthy hosts and its propensity to develop antimicrobial drug resistance have caused concern among the infectious diseases community. Our study assessed the clinical outcomes of patients infected with MDR *Acinetobacter* compared with outcomes of patients infected with susceptible *Acinetobacter* strains and patients without *Acinetobacter* infections among a large cohort. We demonstrated that patients infected with MDR strains of *Acinetobacter* have longer lengths of stay in both the hospital and ICU than patients infected with drug-susceptible *Acinetobacter* and patients without *Acinetobacter* infection when we controlled for severity of illness. We found a trend toward increased mortality rates among patients with MDR *Acinetobacter* infection. However, the difference was not statistically significant when we controlled for severity of illness.

According to NNIS, *Acinetobacter* species caused 7% of ICU healthcare-associated pneumonias in 2003 compared with 4% in 1986 (p<0.001) (*4*). The proportion of ICU healthcare-associated urinary tract infections and surgical site infections caused by *Acinetobacter* also increased significantly from 1986 to 2003 (p<0.001) (*4*). Furthermore, the proportions of *Acinetobacter* isolates reported to NNIS that were resistant to ceftazidime, amikacin, and imipenem all increased significantly during that period (p<0.001). Healthcare-associated outbreaks of MDR *Acinetobacter* infection have been reported in Asia, Europe, North America, and among US service members injured in the Middle East (*7*–*9*). These findings have brought control of MDR *Acinetobacter* infections to the forefront of discussion.

Investigating the effect of multidrug resistance on clinical outcomes presents multiple methodologic challenges that have been explicitly addressed in our study design. Confounding risk factors associated with mortality rates and antimicrobial drug resistance, such as age, severity of illness, and underlying disease (*18*–*21*) must be controlled for in the study design or analysis. Our results differ from those of researchers who examined outcomes of *Acinetobacter* infections without controlling for these confounders (*14*,*22*,*23*), which makes their findings

Table 2. Matched univariate analysis comparing outcomes of patients with multidrug-resistant (MDR) *Acinetobacter* infection with those with susceptible *Acinetobacter* infection and those without *Acinetobacter* infection, Baltimore hospitals, 2003–2004

Outcome evaluated	MDR *Acinetobacter*, n = 96	Susceptible *Acinetobacter*, n = 91	p values for *MDR Acinetobacter* vs. susceptible, n = 182	Uninfected, n = 89	p values for *MDR Acinetobacter* vs. uninfected, n = 178
Mean length of stay after index day, d	27.5	19.8	0.02	18.6	<0.01
Mean intensive care unit length of stay after index day, d	13.3	6.7	0.04	7.3	<0.01
Mortality rate (%)	26.0	17.6	0.21	11.2	<0.01

Table 3. Multivariable analysis of outcomes of patients with and without multidrug-resistant (MDR) *Acinetobacter* infections, Baltimore hospitals, 2003–2004*

Outcome evaluated	MDR *Acinetobacter* vs. susceptible† OR (95% CI)	MDR *Acinetobacter* vs. uninfected† OR (95% CI)
Length of stay, d	2.5 (1.2–5.2)	2.5 (1.2–5.4)
Intensive care unit length of stay, d	2.1 (1.0–4.3)	4.2 (1.5–11.6)
Mortality rate (%)	2.6 (0.3–26.1)	6.6 (0.4–108.3)

*OR, odds ratio; CI, confidence interval.
†Models include modified Acute Physiology and Chronic Health Evaluation III score to control for severity of illness and Charlson index to control for underlying disease.

difficult to interpret. We assessed and controlled for severity of illness and underlying disease with 2 measurements: the APACHE score, which included age, physiologic parameters, and selected underlying diseases; and a separate Charlson index, which included a broader range of underlying diseases. Both measurements have been validated, although the APACHE score has only been studied in its original form (*19*,*21*,*24*). Because patients infected with *Acinetobacter* have worse clinical outcomes than those who are colonized with the organism (*11*), we separated *Acinetobacter* infection from colonization on the basis of standardized, validated Centers for Disease Control and Prevention (Atlanta, GA, USA) NNIS definitions for nosocomial infection (*16*,*25*) and applied them uniformly to MDR *Acinetobacter* and susceptible references. We compared outcomes of MDR *Acinetobacter* infections with those of 2 reference groups and showed an association of MDR *Acinetobacter* infection with both increased hospital and ICU lengths of stay, regardless of the reference group selected. As one would predict on the basis of results of a study by Kaye et al., the effect of multidrug resistance was greater compared with uninfected than susceptible references (*15*).

Because of the lack of a standard definition for multidrug resistance in the literature, we defined multidrug resistance as resistance to all or all but 1 antimicrobial drug class commonly prescribed for treatment of patients with gram-negative infections, with the exclusion of polymyxins (*26*). This definition has 2 advantages. First, it is a strict standard and is readily accepted by clinicians as representative of multidrug resistance. Second, it allows for a clear distinction between susceptible and MDR *Acinetobacter* strains because we excluded isolates that showed intermediate resistance (strains resistant to all but 2 commonly prescribed antimicrobial drug classes).

The association of MDR *Acinetobacter* infections with worse clinical outcomes could be related to discordant empiric antimicrobial drug therapy. Previous studies that examined the effects of delayed concordant antimicrobial therapy on patient outcomes have shown conflicting results (*27*–*29*). We examined this issue and found that patients with MDR *Acinetobacter* infections who received discordant empiric antimicrobial drug therapy were not more likely to die or have a longer hospital length of stay than patients who received concordant empiric drug therapy; however, they were more likely to have a longer ICU length of stay. On the basis of these results, to what extent discordant empiric antimicrobial drug therapy affects clinical outcomes of MDR *Acinetobacter* infection is not clear.

Determining optimal infection control approaches to MDR *Acinetobacter* has been complicated by the lack of agreement on the clinical significance of *Acinetobacter* infections. The Hospital Infection Control Practices Advisory Committee guideline for isolation precautions in hospitals recommends targeting increased infection control efforts toward "resistant bacteria judged by the infection control program to be of special clinical and epidemiologic significance" (*30*). We found that MDR *Acinetobacter* infections are independently associated with increased hospital and ICU lengths of stay. This finding, combined with increased risk for in-hospital transmission of the organism (*31*), supports recommendations to implement aggressive control measures to limit the transmission of MDR *Acinetobacter* in healthcare settings.

Several limitations of this study merit discussion. Because of the lack of available data to calculate a standard APACHE III score for non-ICU patients, we modified the APACHE III score by excluding variables that were unavailable for non-ICU patients. However, our findings

Table 4. Multivariable analysis of discordant versus concordant empiric antimicrobial drug therapy in patients with multidrug-resistant *Acinetobacter* infections, Baltimore hospitals, 2003–2004*

Outcome evaluated	Discordant vs. concordant empiric antimicrobial drug therapy† OR (95% CI)	p value
Length of stay, d	1.6 (0.4–6.5)	0.54
Intensive care unit length of stay, d	5.8 (1.2–27.1)	0.03
Mortality rate (%)	0.7 (0.1–4.5)	0.74

*OR, odds ratio; CI, confidence interval.
†Model includes modified Acute Physiology and Chronic Health Evaluation III score to control for severity of illness and Charlson index to control for underlying disease.

support the validity of this scoring system as a measure for severity of underlying illness. Mean APACHE scores were higher in MDR *Acinetobacter*–infected patients than in both reference groups and progressed stepwise from no infection to MDR infection. These findings are expected because drug-resistant infections reportedly occur in sicker patients (*24*,*32*,*33*). Univariate analysis showed that a modified APACHE score was also associated with mortality rates ($p<0.001$), which further supports its validity as a measure of illness severity.

The lack of available reference patients with similar exposure times to several of the case-patients (5 susceptible references and 7 uninfected references) was a second limitation because we were obligated to exclude unmatched MDR *Acinetobacter*–infected patients from our analysis. These excluded patients typically had prolonged exposure times and tended to have long hospital and ICU lengths of stay after infection. Exclusion of these patients decreased the power of our study and likely biased our results toward showing no difference in hospital or ICU length of stay between the groups. Finally, the lack of a difference in mortality rates, according to multivariable analysis, could mean that MDR *Acinetobacter* are not more virulent than nonresistant strains or that the sample size in this study lacked the power to show a difference.

Our study indicates that infections with MDR *Acinetobacter* are independently associated with the adverse clinical outcomes of prolonged hospital and ICU lengths of stay compared with the outcomes for uninfected patients and those infected with drug-susceptible *Acinetobacter*. This is the first study evaluating length of stay and mortality rates associated with MDR *Acinetobacter* infection while controlling for important confounders such as severity of illness and underlying disease. These data emphasize the need for aggressive infection control strategies to prevent MDR *Acinetobacter* infection and its adverse effects on hospitalized patients.

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Clusters of Hantavirus Infection, Southern Argentina

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Person-to-person transmission of a hantavirus was first confirmed during a 1996 outbreak of hantavirus pulmonary syndrome in southern Argentina, where Andes virus is endemic. To identify other episodes of secondary transmission, we reviewed reports of 51 cases of hantavirus infection from this region (November 1993–June 2005). Nine clusters involving 20 cases (39.2%) were found. Two patients, who had symptoms 3 weeks after they shared risks for rodent exposure, were considered a cluster. The other 8 clusters each began with an index case, which was almost always fatal, followed 19–40 days later by the illness of at least 1 person who had close and prolonged contact with the index case-patient. Person-to-person transmission was considered the probable source of these 8 clusters. The probability of initiating secondary cases was 41% for patients who died versus 4% for those who survived (p = 0.005). Interpersonal transmission of Andes virus infection should be considered even when rodent exposure cannot be definitively excluded.

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) are rodentborne, zoonotic, lipid-enveloped RNA viruses. Old World hantaviruses are associated with hemorrhagic fever with renal syndrome (HFRS), whereas New World hantaviruses cause hantavirus pulmonary syndrome (HPS) (*1*). Transmission to humans is thought to occur predominately by inhalation of infected rodent excreta (*2*). Person-to-person transmission was first documented in 1996, when an HPS outbreak due to Andes virus in southern Argentina provided reliable evidence for person-to-person transmission of a hantavirus (*3*–*5*). In this region, HPS was recognized in 1995 when an outbreak of respiratory illness in a family was investigated, and Andes virus was identified from autopsy tissues of one of the case-patients (*6*,*7*). Until now, Andes virus (reservoir *Oligoryzomys longicaudatus*) was the only hantavirus associated with human infections in this region and with most HPS cases reported in Chile (*8*,*9*).

The 1996 HPS outbreak in southern Argentina showed unique characteristics. The cases occurred in 3 cities over an 11-week period, and each case-patient had proven contact with another HPS case-patient. This unusual circumstance made it possible to identify the epidemiologic chain (*3*,*4*). An identical viral nucleotide sequence in HPS case-patients linked by interpersonal contact supported the hypothesis of person-to-person transmission (*5*). Serious outbreaks such as this are often fully investigated. However, investigation resources are usually limited for small outbreaks in HPS-endemic rural areas, so the epidemiologic diagnosis not easy to establish. To investigate the possibility of other episodes of person-to-person transmission, first proved in the 1996 outbreak, we reviewed the epidemiology of HPS and cluster formation in our region.

Materials and Methods

The HPS-endemic southern area in Argentina is located in the western Patagonia region bordering Chile (Figure 1). The area consists of the western strip of Neuquen, Rio Negro, Chubut, and Santa Cruz provinces. Since 1995, information about all cases of hantavirus infection from Rio Negro and most of Neuquen has been collected by M.E. Lázaro. Systematic registry used standardized forms, including surveillance case reports, results of environmental and epidemiologic case investigations, and clinical data. This registry was used to identify clusters

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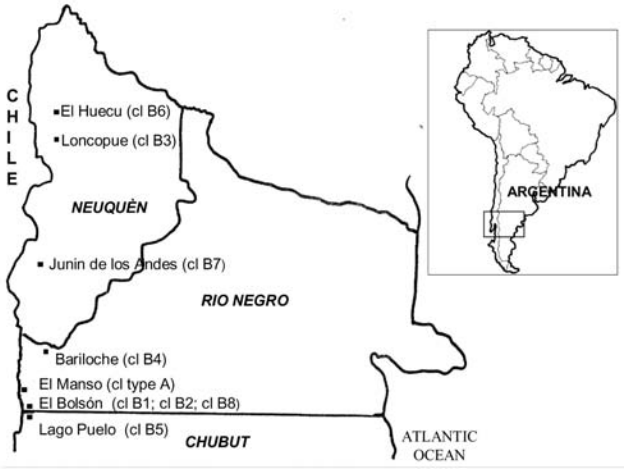

Figure 1. Geographic origin of clusters (cl) of Andes virus cases, southern Argentina.

of hantavirus infections from November 1993 through June 2005. Case-patients who undoubtedly acquired the infection in the region (resided in the area ≥45 days before the onset of symptoms, had molecular evidence of Andes virus infection, or both) were considered in this review.

A cluster was defined as the association of a patient with confirmed HPS (index case-patient) with ≥1 contacts who showed laboratory evidence of hantavirus infection within 6 weeks of the onset of symptoms. Diagnostic confirmation was performed in referral centers (Instituto Nacional de Enfermedades Virales Humanas "Dr. Julio Maiztegui" and Instituto Nacional de Enfermedades Infecciosas "Dr. Carlos Malbran"). Serologic specimens were tested by ELISA for immunoglobulin M (IgM) and IgG antibodies. RNA was extracted from tissue, blood clots, or serum samples and amplified by reverse transcription (RT)–PCR. Viral genotype was characterized by sequencing the RT-PCR products.

When any HPS case was confirmed, an epidemiologic investigation of the places where the patient had lived, worked, or visited within the 6 weeks before the onset of symptoms was immediately conducted. To determine the most probable site of a patient's exposure, we favored those where rodents were trapped, handled, or seen, or where rodent infestation was clearly evident (presence of excrements, nests, or gnawed food). As soon as a case was confirmed, rodent traps were set in the potential exposure sites. When a linked patient became ill, a new search that focused on identifying common or persistent rodent sources and possible interpersonal transmission was performed. A cluster that occurred in 1994 was studied retrospectively.

HPS case-patients who needed mechanical ventilation and hemodynamic support were considered to have a severe clinical form, while those that did not require such support were considered to have a moderate form. Infections without pulmonary involvement were considered mild forms.

Fisher exact test and Student *t* test were used to compare independent variables; $p < 0.05$ was considered significant. Epidemiologic records of 43 case-patients from Rio Negro, 21 from Neuquen, and 3 from Chubut were reviewed.

Of the 67 total cases, 16 belonged to the 1996 outbreak and were excluded from this study. Of the 51 patients whose cases were reviewed, hantavirus infection was confirmed in 49. Acute infection was confirmed by detection of specific IgM antibodies in 47 patients. In 15 of them, the diagnosis was also confirmed by RT-PCR. Andes genotype was characterized for all 15 cases. For the other 2 cases, specific IgG confirmed past infection. The remaining 2 were potential HPS case-patients who died without confirmed diagnosis because of lack of samples but who were linked to close contacts with persons with confirmed infection.

Among the 51 cases, 9 clusters involving 20 patients (39.2%) were identified. Seven clusters met the strict cluster definition of confirmed acute infection because specific IgM was detected for 16 case-patients in these clusters. Each of the remaining 2 clusters was composed of a potential HPS case-patient (without confirmation due to lack of samples) who died and a household contact of that patient with confirmed infection.

Results

Two types of cluster were observed and depended on the interval between cases (onset of symptoms). Type A consisted of infections with <1 week between cases; type B were those infections with >2 weeks between cases.

Type A Clusters

Only 1 type A cluster was identified; it occurred in August 2002. It involved a 10-year-old male student from El Bolson and a 17-year-old male student from Bariloche. They became ill 21 and 23 days, respectively, after returning from a holiday week in El Manso, Rio Negro province, a rural area bordering Chile, where they had both participated in high-risk activities: hunting excursions, games in wilderness areas, and visits to stables. Both exhibited moderate forms of HPS and survived. No risks were identified in their respective houses in Bariloche and El Bolsón.

Type B Clusters

Eight type B clusters, comprising 18 patients, were identified. Each was composed of an index case-patient, followed 19–40 days later by the disease in ≥1 household contact (Figure 2).

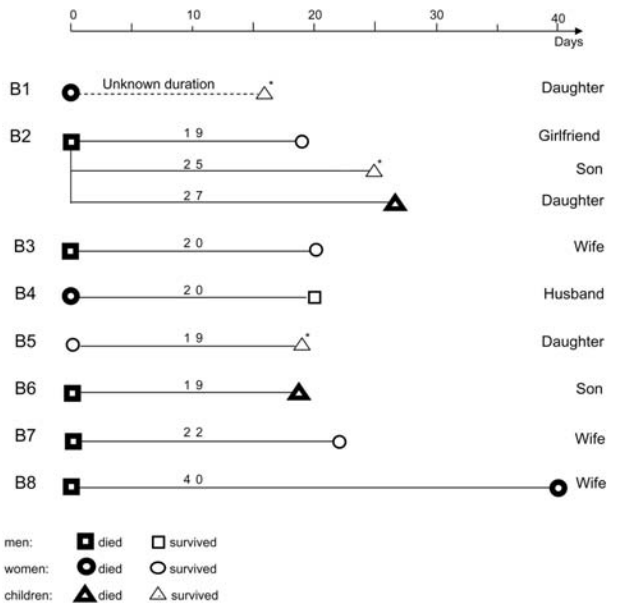

Figure 2. Type B clusters of Andes virus infection, southern Argentina. Mild forms of infection are indicated with an asterisk (*). Horizontal lines join linked cases. Values above the lines indicate the interval in days between the onset of symptoms of linked cases. The relationship of secondary case-patients to index case-patients is indicated at the far right.

Cluster B1 took place in El Bolson, Rio Negro province, in September 1994. A 21-year-old housewife, who lived in a rural area and was breast-feeding her 7-month-old daughter, died after she experienced a flulike syndrome with acute respiratory distress. No samples were available for etiologic diagnosis. Eight months later, during a retrospective research study of contacts of persons with suspected HPS, the baby was studied because her mother fulfilled criteria for potential HPS. Antihantavirus IgG without specific IgM was detected in the baby, so a retrospective epidemiologic/ecologic study was performed. A second sample obtained 2 years and 9 months later confirmed past infection. Recollections by family members supported asymptomatic infection.

Cluster B2 occurred in El Bolson, Rio Negro province, in March and April 1995. A 38-year-old-man, who worked as soft drink distributor, died of HPS. Three members of his family showed laboratory evidence of acute infection with different degrees of clinical severity: 1) his 25-year-old pregnant girlfriend, a cashier in a supermarket, who did not live with him (moderate form, survived); 2) his 9-year-old son, a student, who lived with him (mild form, nonspecific fever syndrome); and 3) his 15-year-old daughter, a student, who lived with him (severe form, died). They became ill on the 19th, 25th, and 27th day respectively, after the onset of symptoms in the index case-patient. Some weeks before becoming ill, the man had gone on a fishing excursion with his girlfriend and son.

Cluster B3 took place in Loncopue, Neuquen province, in November and December 1997. A 41-year-old man, a rural worker with suspected HPS, died while being transferred to the hospital. Hantavirus infection was not confirmed because of lack of samples. He had captured and handled rodents and had observed rodent nests and excrement at home and work. He had also cleaned several unoccupied cabins. His wife, who was 31 years old, experienced symptoms 20 days later (severe form, survived).

Cluster B4 occurred in Bariloche, Rio Negro, in April and May 2000. A 26-year-old woman, who worked as gardener, died of HPS. She lived and worked in a rural area and had cleaned an uninhabited house 6 or 7 weeks before becoming ill. Her husband, a 63-year-old gardener, had onset of symptoms 20 days later (severe form, survived).

Cluster B5 happened in Lago Puelo, Chubut province, in May 2000. A 36-year-old woman was admitted to a hospital in Bariloche with a moderate form of HPS (survived). Her 3-year-old daughter, who was breast-feeding when her mother became ill, showed symptoms 19 days later (fever, vomiting, myalgia, and nasal congestion) but had no clinical or radiologic signs of pulmonary involvement (mild form). Seroconversion confirmed acute infection.

Cluster B6 occurred in Paraje El Morado, El Huecu, Neuquen province, in May and June 2000. A 46-year-old male farmer died of HPS. He was exposed to rodents at work and home. His 10-year-old son became ill 19 days later (severe form, died).

Cluster B7 occurred in Junin de los Andes, Neuquen province, in April and May 2001. A 42-year-old male veterinarian died of HPS. During the previous weeks he had been conducting ecologic research in wilderness areas in Neuquen and southern Chile. His wife, a 44-year-old teacher, became ill 20 days later (moderate form, survived). They were separated and lived in neighboring houses, but during the man's illness he moved to his wife's home where she took care of him.

Cluster B8 took place in El Bolson, Rio Negro province, in October and November 2003. A 31-year-old male construction worker died of HPS. During the previous weeks he had cleared weeds from a wilderness area and started building a cottage. His wife, a 28-year-old housewife, became ill 40 days later (severe form, died). She had not participated in the same potentially risky activities as her husband.

Type B clusters involved family groups. Clusters B2, B4, B5, B6, and B7 occurred during fall; clusters B1, B3, and B8 occurred in spring. All patients lived in rural or semirural areas. Each cluster was composed of 2 members, except B2, which comprised 4 (Figure 2). Ten (55.5%) patients were female. The average age was 28.3 ± 16.3

years (median 29.5 years; range 1–63 years); the baby of B1 was 1 year old when specific IgG was detected, but she had likely became infected at the age of 7 months, when her mother was sick with the disease. Children <16 years of age (28%) had secondary cases.

Andes genotype was identified in patients of cluster B2 (1 case), B3 (1 case), and B4 (2 cases). Index case-patients of clusters B3, B4, B6, B7, and B8 had occupational exposure. Exposure could have been either occupational or recreational for the index case-patient of B2. In B5, peridomestic and occupational exposure overlapped. In B1 (mother-baby), the study was retrospective without any information about rodent exposure.

Secondary cases occurred in household or intimate contacts of the respective index case-patients. Children had daily direct contact (e.g., kissing, touching, hugging, droplet spread) with their infected parents (B1, B2, B5, B6). Sexual intercourse was another route to be considered for persons in clusters B2, B3, B4, and B8. In B1 and B5, the mother was breast-feeding her child when she became ill. Airborne transmission cannot be excluded for any cluster.

When clusters B8 and B9 were studied, no evidence of rodents was found in or around the patients' houses, and no small mammals were captured. All trapped rodents related to clusters B1 and B2 (49 rodents), B4 (9 rodents), and B5 (11 rodents) were seronegative for hantavirus. Information about captured rodents associated with clusters B3 and B6 was not available. In 6 of the 8 clusters, no evidence for rodent exposure by secondary case-patients was found. In the other 2 clusters (B3 and B6), rodent exposure was probable.

In 7 clusters, the average interval between symptom onset in index case-patients and in secondary case-patients was 23.4 ± 6.8 days (median 21 days; range 19–40 days) (Figure 2). In B1, diagnosis was retrospective, and infection was subclinical in 1 of the 2 members, so determining the interval was not possible.

Tested serum specimens from asymptomatic contacts of HPS index case-patients showed no evidence of hantavirus infection. The baby from cluster B1, who exhibited IgG antibodies, would be the exception. Contact surveillance identified 2 acute infections (mild forms) in children, by the presence of IgM antibodies (cluster B2) or by seroconversion (cluster B5).

All index case-patients had typical HPS symptoms. Among patients with secondary cases, 7 of 10 fulfilled the HPS definition. The remaining 3 were 2 children with mild forms (clusters B2 and B4) and a baby with asymptomatic infection (cluster B1). Index case-patients had a higher death rate than patients with secondary cases (87.5% vs. 30%, p = 0.023). The death rate was also higher in index-case-patients than in patients not included in type B clusters (those with sporadic cases and cases in type A clusters) (87.5% vs. 30%, p = 0.005) (Table).

Among the 51 patients whose cases were initially reviewed, 41 had undoubtedly acquired the infection from rodents (secondary case–patients excluded). Seventeen of these 41 died. Of the 17 patients who died, 7 (41%) had become an index case-patient of a cluster. In contrast, only 1 (4%) of the 24 patients who survived became an index case-patient (p = 0.005). These results indicate that the risk of initiating secondary cases was associated with the most severe manifestations.

Among the patients with sporadic cases and index patients who died, no differences in clinical characteristics were found. The mean number of days between onset of symptoms and death (6.78 ± 2.23 vs. 6.86 ± 1.46, respectively; p = 0.9) was similar for both groups.

Discussion

Both Old and New World hantavirus infections usually occur as sporadic cases (*10*). Although several persons frequently are exposed to the same risks, they rarely become infected. In a review of cases in the United States, where most infections are caused by Sin Nombre virus, 12 (7.5%) of 160 patients were grouped in clusters. The pattern of case manifestations and the fact that the members of each cluster were exposed to sites with large infestations of rodents induced the authors to conclude that these clusters originated from exposure to common rodent sources (*11*).

In our study, 39.2% of the cases were grouped in clusters; in addition, the 1996 outbreak was an extremely large cluster (*4,5*). Patients in our cluster A lived in different towns and became sick almost simultaneously, 3 weeks after coming back from a wild area where they shared high-risk activities. These characteristics suggest simultaneous exposure to a rodent source. Unlike the previous category, type B clusters were characterized by an index case-patient, who almost always died, followed 19–40 days later by the illness of ≥1 more close contacts of that patient.

Although the incubation period for human hantavirus illnesses is 8–45 days, it usually lasts 2–3 weeks (*2*). If the members of each cluster type B were infected after a common exposure, the incubation period of the patients with secondary cases would result from adding the estimated incubation period for the index patient (2–3 weeks) to the intervals between index and secondary cases. Under this hypothesis, the incubation period for secondary cases to develop would be abnormally long and rarely probable. On the other hand, the average interval between index and secondary cases in type B clusters (23.4 days) was similar to the average incubation period accepted for hantavirosis. The former also matched the average interval among 1996

Table. Case-fatality rate according to the type of cluster

Cluster	No. deaths/ no. cases	Case-fatality rate, %
Type B clusters		
All cases	10/18	55.6
Index cases	7/8	87.5
Secondary cases	3/10	30
Sporadic and type A cluster	10/33	30

outbreak case-patients (22.8 days) that was associated with the incubation period for person-to-person transmission. Therefore, intervals between cases in type B clusters suggest person-to-person transmission. In these clusters no transmission from secondary case-patients was detected, in contrast with the 1996 outbreak in which up to 4 link chains were identified (*4*,*5*).

Another explanation for the long intervals could be that infected peridomestic rodents contaminate the environment, resulting in multiple transmissions over an extended period. However, this possibility seems unlikely for 4 reasons. First, for clusters B7 and B8, occupational exposures, considered as the most likely risk for the index case-patients, were excluded for the secondary case-patients. In cluster B2, 3 of the patients shared a possible common exposure (fishing excursion), but the fourth (index case-patient's daughter) did not. Second, immediate actions to eliminate risk sources were taken when a patient was detected, which lowered the risk for persisting domestic or peridomestic rodent sources. Third, evidence of domestic rodent infestation was absent for some clusters. Fourth, no serologic reactivity was detected in rodents captured in peridomestic areas. Overall, although in any other hantavirus outbreaks, multiple virus introductions to humans from the environment are possible, clusters like type B are the exception.

In any case, in the southern region of Argentina, rodent exposure risks are difficult to exclude. Even in urban zones, wild vegetation is intermingled with settlements, and close contact with rural areas allows the circulation of *Oligoryzomys longicaudatus*. This fact delayed the suspicion for interpersonal transmission in the 1996 outbreak because the first case-patients lived in or visited towns with these characteristics. Person-to-person transmission was first strongly suspected when 1 patient was transferred to a hospital outside of the Andes virus–endemic area. The admitting physician, who had no other risk factor for exposure, became ill with HPS 3 weeks later (*3*). Recently, in a reported cluster caused by Andes virus infection, the journey of the index case-patient out of the Andes virus–endemic area was also the key to suspecting person-to-person transmission (*12*,*13*). Therefore, person-to-person transmission is evident only when special circumstances converge, as happens when an infected patient spreads the virus out of the disease-endemic area. In this situation, molecular studies are especially useful because identical sequences in geographically separated but linked cases support interpersonal transmission (*5*,*12*,*13*). However, when all case-patients remain in the disease-endemic area, infection may be attributed to other sources, hiding the interpersonal transmission. In this situation, molecular dissimilarities rule out person-to-person transmission, but identical sequences do not support it. The interval length may be helpful: a 2- to 4-week interval among linked patients supports interpersonal transmission, whereas a shorter interval suggests simultaneous exposure. Reported clusters of Andes virus infection in southern Chile showed the 2 patterns; some cases had intervals of 2–5 days, and others had intervals of >2 weeks between cases (*14*,*15*).

An infection with high levels of virus replication might correlate with the severity of the patient's illness and result in increased shedding of virus, which would initiate secondary cases. Subsequent spread of the virus through human hosts might induce a reduction of the initial virulence, which would explain the lower death rate and mild forms observed among patients with secondary cases. The 1996 outbreak may be explained by the circulation of a uniquely virulent and transmissible virus strain or an unusually high viral replication in a particular patient. However, the human-to-human spread may show idiosyncratic behavior of Andes virus as well as an extraordinary situation. From this point of view, the 1996 outbreak can be considered the maximum expression of person-to-person spread. Human infections by Andes virus have also shown distinctive clinical characteristics with more hemorrhagic, renal, hepatic, and muscular impairment than those reported for Sin Nombre virus infection (*16*–*20*).

Although the mechanisms of person-to-person transmission are still not clear, direct contact and aerosol transmission must first be considered. Direct contact was always taking place between family members (type B clusters); because cough appears at the end of the febrile phase, saliva may play an important role in transmission during the early stages (as suggested by the detection of virus antigen in rodents' saliva glands) (*21*). For infected couples, sexual contact should also be considered, and breastfeeding cannot be excluded in mother-baby clusters. Aerosol infectivity can be suspected between persons because the natural spread from rodents to persons is by the aerosol route (*22*). Respiratory secretions may be sources of infection because pulmonary involvement is essential in HPS, viral antigen is present in pulmonary endothelium (*23*,*24*), and Andes virus RNA has been reported in tracheal secretions of infected persons (*25*).

Although in the 1996 outbreak, hospitals played a key role in amplification, nosocomial transmission of Andes virus seemed to be the exception (*4*,*5*). Under usual

circumstances, the period of transmission is probably short and limited to the early phase. Close and prolonged contact may be necessary for interpersonal transmission. Generally, the infection has already progressed to the cardiopulmonary phase at the time of patient's admission, so healthcare workers are exposed during a late stage and do not have close contact with the patient if they take adequate biosafety measures. These facts may explain the absence of HPS cases in healthcare workers during the period reviewed and the low seroprevalence reported in this group, which is similar to that of the general population of the region (*26*,*27*). Seroprevalence in healthcare workers may not be a sensible indicator of the need to investigate person-to-person transmission of Andes virus because such transmission mostly occurs in the patient's domestic circle during the early stages of the illness. In any situation, universal precautions should be strictly followed. Surgical masks with visor, gowns, and gloves should be routinely worn and, whenever possible, additional measures such as using HEPA respirators and providing private rooms should be used to protect against inspired particles.

Even though virus characterization was not performed for all the cases reviewed, Andes virus is the unique genotype identified since 1995 in infected persons and rodents in the southern region of Argentina. For this reason, Andes virus is the most probable etiologic agent in those cases not characterized.

The number of clusters identified during the period reviewed is high compared with the low incidence of HPS in the region. This finding suggests that person-to-person transmission of Andes virus is not exceptional and must always be suspected when the onset of symptoms of $\geq$2 case-patients linked by contact are separated by an interval of $\approx$3 weeks. Case-patients with an ultimately fatal disease have an increased risk of initiating secondary cases.

Surveillance of household contacts is useful for identifying mild symptoms. Contacts should be encouraged to seeking immediate medical care if febrile symptoms appear, and specific diagnostic tests must be performed.

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Emergence of Arctic-like Rabies Lineage in India

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A collection of 37 rabies-infected samples, 10 human saliva and 27 animal brain, were recovered during 2001–2004 from the cities of Bangalore and Hyderabad in southern India and from Kasauli, a mountainous region in Himachal Pradesh, northern India. Phylogenetic analysis of partial N gene nucleotide sequences of these 37 specimens and 1 archival specimen identified 2 groups, divided according to their geographic (north or south) origins. Comparison of selected Indian viruses with representative rabies viruses recovered worldwide showed a close association of all Indian isolates with the circumpolar Arctic rabies lineage distributed throughout northern latitudes of North America and Europe and other viruses recovered from several Asian countries.

An estimated 55,000 people, mostly in Asian countries, die of rabies each year (*1*). The etiologic agent of this disease is rabies virus or a closely related member of the *Lyssavirus* genus; various rabies virus variants, which circulate widely in many dog populations throughout Asia (*2*), are responsible for most human infections. Although rabies is preventable, the high cost of postexposure prophylaxis, compounded by the lack of education and awareness about rabies, limits use of postexposure prophylaxis in many developing countries. Moreover, visitors to these countries are also sometimes unaware of the rabies risk posed by dog bites and thus may not seek appropriate medical attention for such bites. The occasional cases of rabies reported in industrialized countries, such as the United Kingdom, are often the result of exposure while traveling in developing countries such as India (*3*,*4*). In Germany, a recent case of rabies in a person who had visited India remained unidentified until after the patient's death; soft tissue transplantation from this patient resulted in rabies transmission to several organ recipients (*5*).

Despite the availability of techniques to improve the global rabies situation, limitations in surveillance and epidemiologic investigations impede the institution of such measures (*6*). In industrialized countries, diagnosis of rabies in animals is achieved by using rabies-specific fluorescein-conjugated antibody to detect viral antigen in brain smears; however, antemortem diagnosis in humans must rely on less-invasive methods. The utility of PCR-based methods to detect rabies virus sequences in saliva and other body fluids has been reported (*7*), and PCR is being used in many industrialized countries (*8*,*9*). An additional component of rabies control in such countries is the application of viral typing methods to identify viral variants that circulate in specific host reservoirs (*10*). Knowledge of the association of specific variants with animal hosts has led to increasingly effective control measures that target the hosts responsible for spreading this disease (*11*). Moreover, molecular epidemiologic approaches have enabled study of the spread of certain rabies virus variants and their incursion into new geographic regions (*12*). Adaptation of such methods in developing countries would help provide reliable data on the true extent of rabies in such countries, provide epidemiologic data about the spread of rabies, and justify allocation of increased resources.

Recently, a national rabies survey in India, based on clinical diagnosis and sponsored by the World Health Organization, found that 20,000 persons died of rabies each year (*13*). These observations indicate a great need to strengthen laboratory diagnostic capabilities for rabies in India and to use genetic typing to improve knowledge of the nature of the viruses that circulate in India. The resulting increase in disease surveillance would help justify subsequent control measures. Accordingly, molecular methods for rabies virus detection have been introduced to

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the National Institute for Mental Health and Neurosciences in Bangalore, India. Using several positive samples identified by this method, we studied the epidemiologic origins of rabies from multiple areas of the country.

Materials and Methods

Sample Collection

Rabies was diagnosed by direct fluorescent antibody (DFA) test (*14*) in 27 animal brains (Table) recovered from 2 locations in India: the city of Bangalore (and its surrounding 15 km) and the northern community of Kasauli in the state of Himachal Pradesh. An archival bovine sample from an unknown location in India was included in the study.

Antemortem saliva samples were obtained from 37 human patients with clinical signs consistent with a diagnosis of rabies. All patients were located at 1 of 3 hospitals in Bangalore or at 1 hospital in Hyderabad. The molecular methods described below confirmed 10 of these samples (Table) as rabies infected.

Molecular Characterization of Viruses

Total RNA was recovered from each specimen by using TRIzol reagent (for animal brain tissue) or TRIzol LS reagent (for human saliva samples) as recommended by the supplier (Invitrogen, Burlington, Ontario, Canada). Standard reverse transcription–PCR (RT-PCR) was used to amplify the complete N gene of rabies virus as previously

Table. Rabies samples from India examined in this study

Sample	Date of isolation	Source	Location	GenBank accession no.
Animal brain				
IND01-1	2001	Dog	Bangalore	DQ521215
IND01-18	2001	Dog	Bangalore	DQ521216
IND01-29	2001	Dog	Bangalore	DQ521217
IND01-56	2001	Dog	Bangalore	DQ521218
IND01-63	2001	Dog	Bangalore	DQ521219
IND01-85	2001	Dog	Bangalore	DQ521220
IND01-90	2001	Dog	Bangalore	DQ521221
IND01-91	2001	Dog	Bangalore	DQ521222
IND02-08	2002	Dog	Bangalore	DQ521223
IND02-55	2002	Dog	Bangalore	DQ521224
IND02-62	2002	Dog	Bangalore	DQ521225
IND02-84	2002	Dog	Bangalore	DQ521226
IND02-85	2002	Dog	Bangalore	DQ521227
IND03-08	2003	Dog	Bangalore	DQ521228
IND03-20	2003	Dog	Bangalore	DQ521229
IND03-24	2003	Dog	Bangalore	DQ521230
IND03-37	2003	Dog	Bangalore	DQ521231
IND03-38	2003	Dog	Bangalore	DQ521232
IND04-53	2004	Dog	Bangalore	DQ521233
IND04-57	2004	Dog	Bangalore	DQ521234
IND04-66	2004	Dog	Bangalore	DQ521235
IND04-72	2004	Dog	Bangalore	DQ521236
INDN1	2004	Dog	Kasauli	DQ521237
INDN2	2004	Dog	Kasauli	DQ521238
INDN3	2004	Dog	Kasauli	DQ521239
INDN5	2004	Cat	Kasauli	DQ521240
INDN6	2004	Bovine	Kasauli	DQ521241
V458IND*	1991	Bovine	(Unknown)	AY854599
Human saliva (antemortem)				
INDH10	12 Feb 2004	Female, 40 y	Bangalore	DQ521242
INDH13	20 Feb 2004	Male, 13 y	Hyderabad	DQ521243
INDH14	21 Feb 2004	Male, 30 y	Bangalore	DQ521244
INDH19	17 Mar 2004	Female, 21y	Hyderabad	DQ521245
INDH20	17 Mar 2004	Male, 14 y	Hyderabad	DQ521246
INDH26	3 Apr 2004	Female, 20 y	Hyderabad	DQ521247
INDH27	15 Apr 2004	Male, 8 y	Hyderabad	DQ521248
INDH28	14 Apr 2004	Female, 12 y	Hyderabad	DQ521249
INDH33	25 Apr 2004	Female, 23 y	Hyderabad	DQ521250
INDH34	27 Apr 2004	Male, 27 y	Bangalore	DQ521251

*Archival specimen from unrecorded location.

described (*15*). Universal primers RabNfor/RabNrev, shown to be useful for amplification of a wide range of rabies virus strains, were used to perform a nested second round of PCR (*15*). In initial trials, when DFA-positive samples from dogs in Bangalore were used, most samples (17 of 22) required 2 rounds of PCR to generate a visible amplicon; hence, all subsequent analyses routinely incorporated a nested protocol, and samples were scored for presence of rabies only after the second round of PCR. For nucleotide sequencing, 5 μL of the nested PCR product was spotted onto Whatman (Brentford, UK) no.1 filter paper, air-dried, and transferred to the laboratory in Canada. Each PCR product was eluted from the filter paper into 50 μL of RNase-free water and reamplified by using the nested primer set. Final products were purified by using a Wizard PCR Preps Purification System (Promega, Madison, WI, USA). Nucleotide sequencing was performed with an NEN model 4200L automated sequencing system (Li-Cor Biosciences, Lincoln, NE, USA) and IR700/800-labeled primers (Li-Cor Biosciences), based on either the universal primers or the internal N gene sequence, together with a Thermosequenase cycle sequencing kit (Amersham Biosciences, Baie d'Urfé, Quebec, Canada).

Nucleotide sequences were aligned by using CLUSTALX v1.8 (*16*), and phylogenetic analysis was accomplished by using the neighbor-joining algorithm of the PHYLIP 3.61 software package (*17*). Trees were displayed using TREEVIEW (*18*).

Results

All 38 samples (Table), including the archival specimen (V458IND), were confirmed rabies positive by using nested PCR to amplify a portion of the viral N gene. The nucleotide sequence of a 500-base segment in all amplicons was determined for each. These aligned sequences were subjected to phylogenetic analysis using a neighbor-joining algorithm with the CVS strain of rabies included as an out-group. The Indian samples formed 2 main clades (Figure 1). The 5 samples from northern India (IN-1), which were identical over the portion of genome characterized (100% homology), clearly segregated from the main cluster (IN-2) that comprised the more heterogeneous southern isolates. Members of IN-2 exhibited homologies ranging from 94.8% to 100% and showed no segregation according to location of origin. However, 2 specimens, the archival isolate V458IND and a recent human specimen INDH33, were clearly the most distinctive of the group and formed a strongly supported subgroup within this cluster. Intergroup (IN-1 and IN-2) sample homologies ranged between 91.2% and 93.6%.

Because extensive N gene sequence information for rabies viruses is available in publicly accessible databases, similar phylogenetic methods could be used to compare selected Indian isolates with rabies viruses representative of many strains that currently circulate throughout the world (online Appendix Table, available from www. cdc. gov/ncidod/EID/13/1/111-appT.htm). The tree in Figure 2 was generated by using a shortened sequence window to accommodate variants for which only partially overlapping sequences were available. As shown in Figure 2, all the Indian isolates of this study clustered within a clade designated as Arctic/Arctic-like and were well separated in evolutionary terms from the cosmopolitan lineage as well as other lineages that circulate in various parts of Southeast Asia. One cluster in particular (ASIA1), composed of 2 specimens from dogs of Sri Lanka and Madras (INDIA-Dog), clearly segregated independently of the isolates examined in this study.

Within the Arctic/Arctic-like clade, 3 main groups were strongly supported by bootstrap analysis. Group 1 comprised all North American specimens (from Ontario, northern Canada, and Alaska), specimens from Greenland, and 2 specimens from the former Soviet Union (from

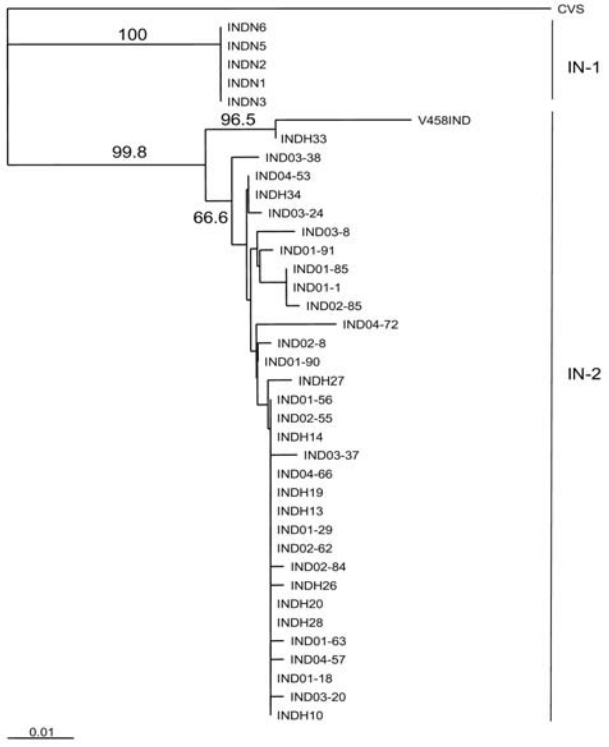

Figure 1. Neighbor-joining tree for 500 bp of nucleoprotein gene sequence for 38 rabies virus samples from India, as described in the Table, using the CVS sequence as an out-group. The sequence window used corresponded to positions 279 to 778 of the CVS reference sequence. Bootstrap values >65% for 1,000 resamplings of the data are shown on branches to the left of the corresponding sample clusters. The 2 main Indian clusters identified by this analysis (IN-1 and IN-2) are indicated to the right of the tree. A genetic distance scale is indicated at bottom left.

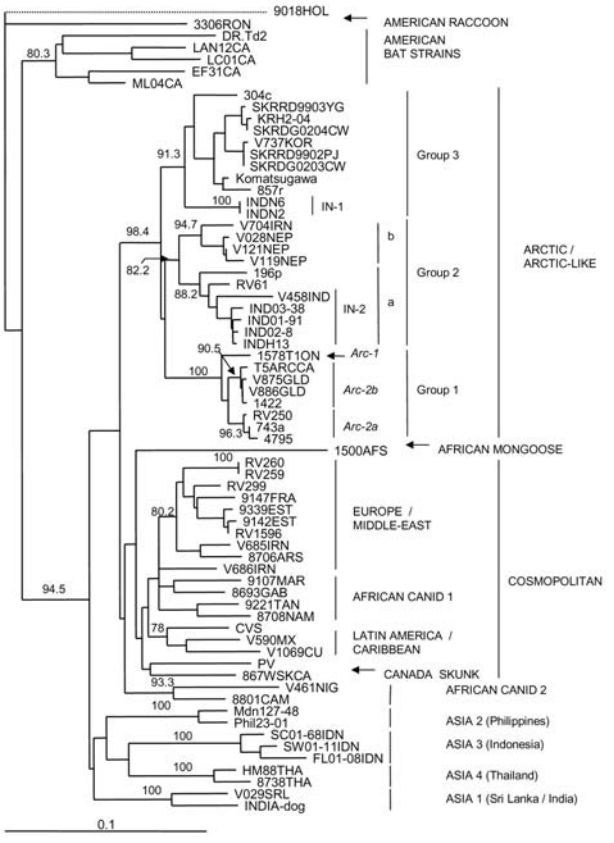

Figure 2. Neighbor-joining tree for 460 bp of nucleoprotein gene sequence for 67 rabies viruses, including representative samples from India, and a European bat lyssavirus type 2 (EBLV-2) specimen, 9018HOL, used as an out-group. The latter branch is shown as a dotted line to indicate that its length has been shortened to permit more detailed illustration of the rest of the tree. All additional rabies viruses used in this analysis are described in the online Appendix Table (www.cdc.gov/ncidod/EID/13/1/111-appT.htm). Bootstrap values >70% for 1,000 resamplings of the data are shown on branches corresponding to the sample clusters. Strains and variants described in the text and in the online Appendix Table are illustrated to the right of the tree. A genetic distance scale is indicated at bottom left. Subdivision of the Arctic group 1 into 3 subgroups—Arc-1, Arc-2a, and Arc-2b as described previously (*19*)—is shown in italics.

Yakutia in the north and Tuva in the south). A prior analysis of Arctic specimens (*19*) strongly supported further division of this group into 3 subgroups designated here as Arc-1, Arc-2a, and Arc-2b. Close association was noted among specimens in subgroup Arc-2a, which originated from Alaska (4795) and the former Soviet Union (RV250 and 743a).

Group 2 comprised specimens from the northeast corner of Iran, where incursion of Arctic-like lineage rabies was recently discovered (*20*); Nepal; Pakistan (sample 196p); and members of the Indian group IN-2 together with 1 additional Indian isolate (RV61) that was recently described (*21*). Support for further subdivision of this group, by which the Indian and Pakistani specimens (subgroup 2a) segregated from the Iranian and Nepalese specimens (subgroup 2b), was strong (bootstrap values of 88.2 and 94.7 for each group, respectively).

Group 3 included the Komatsugawa strain recovered in Japan some years ago; 6 isolates from Korea; and 2 specimens from different regions of the former Soviet Union, Chita (304c), and Chabarovsk (857r). Two members of the northern Indian group (IN-1) of this study formed an outlying branch closely associated with this group.

Discussion

This study benefited from an initiative to explore the utility of PCR technology for antemortem diagnosis of rabies in human saliva samples. Of 37 suspected rabies cases, 10 were confirmed positive by this technique. Unfortunately, no subsequent follow-up of these patients or postmortem analysis of brain material by DFA was possible. At least some of these patients for whom the saliva test was negative for rabies had likely contracted rabies but had no detectable shedding of virus in saliva during the period of saliva collection. Thus, using these data to infer rabies incidence in humans is difficult. Further application of this method, together with improved follow-up of patient outcome, is needed.

Previous reports (*19*,*21*,*22*) indicated that rabies viruses belonging to the Arctic/Arctic-like lineage are widely dispersed throughout the Northern Hemisphere and are not limited to Arctic regions. Indeed, of the very few genetically characterized isolates originating from India and neighboring countries such as Pakistan (e.g., RV61 and 196p, which are included for comparison in this report), most appeared to be related to the Arctic lineage. However, most of the characterized isolates have been recovered from travelers after their return to developed countries. Ours is the first comprehensive genetic analysis of substantial numbers of isolates directly recovered from several locations in India; our study confirms extensive circulation of the Arctic-like rabies virus lineage in 3 geographically separate areas of India.

Phylogenetic analysis identified 3 groups of viruses belonging to the Arctic/Arctic-like rabies virus lineage. A map showing the known distribution of all 3 of these groups throughout Asia is illustrated in Figure 3. This map was compiled from data generated in this study and from previous reports (*4*,*19*–*23*). Because some common specimens were incorporated in many of these analyses, we could surmise the phylogroup membership of many previously described isolates according to the group classification described here (Figure 3).

The viruses that can be considered as the true Arctic strain (group 1) circulate extensively in northern areas of

Figure 3. Map of mainland Asia. The locations from which Arctic and Arctic-like variants of the rabies virus have been recovered are shown in circles or ovals with the group designation (1, 2a, 2b, or 3) indicated in the center. Generation of this map was achieved in part by compiling data from 2 previous publications (*19*,*21*). Viral phylogroups previously designated as A and B (*21*) are equivalent to groups 1 and 3, respectively, in this study. B, Bangalore, G, Goa; H, Hyderabad; K, Kasauli; M, Madras.

Russia but have also been found in the Tuva region just north of western Mongolia. These viruses are closely related to all the Arctic strain viruses recovered from the Americas. With the exception of the sample from Madras, all Indian isolates recovered from the south of the country, including 1 from a tourist visiting Goa (*4*), belonged to subgroup 2a and were thus closely related to the few characterized viruses recovered from Pakistan. A related but slightly more distant group of viruses (subgroup 2b) was recovered from Nepal and northeastern Iran. Group 3 viruses of the Arctic/Arctic-like lineage circulate extensively in northeastern Asia, Korea (*20*,*23*), and parts of Russia (*21*), and were present in Japan (Komatsugawa isolate) before rabies was eradicated from the country. Perhaps our most surprising finding was that the northern Indian samples were more closely related to these group 3 viruses than to the viruses circulating in southern India and neighboring Nepal. These patterns of viral variant distribution may reflect migrations, recent and historic, and movements of humans and their animals throughout the region. The incursion of 2 separate variants into northern India and Nepal might be a consequence of difficult access between these 2 areas due to the regional terrain. Moreover, it appears likely that group 3 viruses circulate more extensively throughout Asia than is presently documented; further analysis of specimens from the region, especially from China, Mongolia, and Russia, will be needed to form a more complete picture of the spread of this variant throughout the region. A recent study of several Chinese isolates indicated that the circulating virus variants were related to those of Southeast Asia (e.g., ASIA 4) and to the cosmopolitan lineage, but no representatives of the Arctic lineage were found (*24*). Because that study examined rabies viruses recovered only from the southeastern region of China, the possibility remains that the northern regions of this country harbor Arctic-like variants.

The evolutionary mechanisms underlying these phylogenetic patterns can only be speculated upon at this time. Observations made in Canada throughout the 20th century (*12*,*25*,*26*) have documented frequent movement of the Arctic rabies lineage from northern regions to the south, by transmission among populations of red and arctic foxes. Similarly, this lineage could have moved southward from Siberia or other northern latitudes of the former Soviet Union into Nepal, India, and other Asian countries by means of a species jump from the fox to the dog at some point during this spread. However, the tree in Figure 2 provides some argument against this hypothesis. First, within the Arctic/Arctic-like clade, all specimens from temperate and Arctic regions are restricted to group 1 and exhibit more limited genetic variation than that observed for the Asian specimens that are represented in all groups. Although no a priori reason exists to rule out the possibility that rabies can jump from wild-life species to dogs, recent surveillance reports suggest that successful rabies species jumps most often occur from dogs to wildlife (*27*,*28*). Thus, consideration should be given to the possibility that the "Arctic" lineage of rabies first emerged in southern Asia in dogs and that it subsequently spread to northern climes, where it is now maintained by fox populations. The future acquisition of additional data on rabies viruses from Asia should provide the dataset required for a robust molecular clock analysis to explore these hypotheses. Transmission of rabies from a wild fox to a human has been documented in central India (*29*). Given the relatively close phylogeny between rabies virus variants of the Indian dog and arctic fox, further consideration might be given to the role of wildlife in maintaining rabies in India.

A single Indian rabies specimen, INDIA-dog recovered from Madras on the southeastern Indian coast (*30*), clustered with an isolate (V029SRL) typical of a distinct variant found in Sri Lanka (*31*) rather than with the other Indian isolates described in this study. Movement of humans and their animals between Sri Lanka and India, particularly within the southeastern coastal area of the mainland, may have resulted in the movement of this variant between these 2 geographically separate regions. Further studies may show regional circulation of this or other rabies virus variants within India.

We hope that this report will encourage further studies that apply these molecular approaches to the diagnosis of additional rabies cases and the characterization of viruses

recovered from other parts of India. Increased knowledge of the complexity of the rabies situation in India should spur efforts to improve public awareness and to better control this disease. Moreover, the data presented here promise to alter current paradigms about the emergence of Arctic rabies.

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Tickborne Relapsing Fever Diagnosis Obscured by Malaria, Togo

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Given the prevalence of relapsing fever (RF) in Senegal, this disease may cause illness and death in other areas of West Africa. We performed a cross-sectional, clinic-based study to investigate the presence of RF in Togo during 2002–2004. Blood samples from patients with fever were examined for RF spirochetes by microscopy, PCR, and DNA sequencing of amplicons and for antibodies to the glycerophosphodiester phosphodiesterase antigen. Although no spirochetes were seen in blood smears, ≈10% of the patients were positive by PCR and ≈13% were seropositive for spirochetes. DNA sequencing demonstrated that *Borrelia crocidurae* and *B. duttonii* were present. Most patients were treated for malaria whether or not plasmodia were observed. Thus, many RF patients originally had a misdiagnosis of malaria, which resulted in ineffective treatment. The inability of microscopic analysis to detect spirochetes compared with PCR demonstrates the need for tests with greater sensitivity.

Spirochetes of the genus *Borrelia* are known to cause 2 major types of human disease, Lyme disease, which occurs primarily in temperate regions, and relapsing fever (RF), which occurs in both temperate and tropical regions. Many vertebrates serve as enzootic hosts for the bacteria, and borreliosis is related to climatic and other environmental parameters required for the vectors and reservoir hosts (*1*,*2*). *Borrelia*-related disease is endemic in tropical and subtropical regions; *B. hermsii* and *B. turicatae* cause tick-borne RF (TBRF) in North America. In Europe, TBRF is uncommon; *B. hispanica* is the causative agent in Spain, Portugal, Greece, and Cyprus (*3*,*4*).

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TBRF in Africa is caused primarily by *B. duttonii*, which is transmitted by *Ornithodoros moubata* ticks in East and Central Africa, and by *B. crocidurae*, which is transmitted by *O. sonrai* in West Africa. Humans are the only known vertebrate host for *B. duttonii*. *B. crocidurae* is maintained in enzootic cycles in rodents and other small mammals. African TBRF is associated with proximity to tick-infested burrows and huts (*4*–*6*).

The primary clinical manifestations of RF are recurrent high fever interrupted by afebrile periods, hepatomegaly, splenomegaly, and anemia. These signs are similar to those of malaria. The fever peaks are associated with high spirochetemias, and antigenic variation leads to new antigenic variants of a major surface protein and the recurrence of high numbers of borreliae in the blood (*7*–*10*). When patients have high fever, spirochetes may achieve sufficiently high cell densities in the blood to be observed directly by microscopy when wet mounts of blood or Giemsa-stained blood smears are examined. Between peaks, the bacteria are too scarce to be visualized in the blood. Treatment with various antimicrobial drugs is effective (*5*,*6*); however, borreliae may rapidly invade the brain, and infection of the central nervous system may persist if not treated or if treated with antimicrobial drugs that do not readily penetrate the blood-brain barrier (*5*).

Research on RF in Africa has been limited, and little is known regarding the presence and geographic distribution of the spirochetes and tick vectors. Studies in Senegal indicate that RF is widely distributed and prevalent in this country; investigators speculate that RF may cause illness in rural areas throughout much of West Africa (*11*). A 2-year prospective investigation in a rural community in the Senegalese savanna showed that 10% of the study population became infected during the study period, resulting in an incidence of 5.1% (*12*). A recent 14-year longitudinal

study demonstrated an average TBRF incidence of 11/100 person-years in Delmo, Senegal, and suggested that TBRF is a common cause of fever in most rural areas of Senegal as well as in some regions of Mauritania and Mali (*13*). In other areas of West Africa, where RF has not been identified, the disease is generally not considered in the diagnosis when patients have a fever.

We hypothesized that RF caused by *B. crocidurae* may be present in other areas of West Africa where the climate and environment are similar to that of Senegal. However, because of the lack of knowledge, diagnostics, and the high prevalence of malaria in these areas, RF remains undetected (*14*). Therefore, we conducted a study in Togo to determine if patients with fever might have RF. The study included examination of blood by direct microscopy, molecular methods, and serologic analysis.

Methods

Setting

Clinics participating in the study were located in the northern dry savannah and the southern tropical high plateau of Togo. The clinics were at the children's hospital, Hopital d'Enfants, in Dapaong (urban/semiurban) in northern Togo and rural clinics in southern Togo, including the Centre Medico-Social de Sodo in Sodo, Hopital Bethesda, Agou Clinic in the Agou area, and the general hospital in Kpalimé, a town with ≈50,000 inhabitants (Figure).

Participants

Interviews and sampling were conducted from March 2002 through September 2004; sampling was performed from August through October in 2003 and 2004 in northern and southern Togo, respectively. Trained laboratory personnel in various clinics obtained blood samples, conducted interviews, and performed microscopic analyses. A total of 244 persons with fever were randomly selected; 14 persons without fever were included as controls.

Procedures

A questionnaire that contained information on demography, living conditions such as building materials that may be favorable for nesting ticks and rodents, and occupation was used in interviews. Axillary temperature of each patient with fever was measured. Blood was collected from the arm by venipuncture or from a finger by lancet stick and applied to glass microscope slides. Thick and thin blood smears were stained with Giemsa and analyzed by microscopy at a magnification of 1,000× for plasmodia and at 400× for spirochetes. Microscopic examination for spirochetes was used at the clinics to enable diagnosis on site by the method routinely used in Senegal (*14*,*15*). Approximately 300 fields were examined to detect malaria parasites and borreliae. For malaria diagnosis, samples were examined for trophozoites and gametocytes. Blood and serum samples were stored at −20°C and shipped frozen to Sweden for further testing.

Figure. Locations of clinics in Togo involved in the study: 1, Dapaong; 2, Sodo; 3, Kpalimé; 4, Agou; 5, Bethesda; 6, sites of the community study in the Sodo region (adapted from www.maps.com).

Plasmid Cloning and Protein Expression

Genomic DNA from *B. crocidurae* was used as a template for amplification of the glycerophosphodiester phosphodiesterase (*glpQ*) gene and subsequent sequence analysis (Table 1). The PCR amplification product was digested with *Bam*HI and *Xho*I and cloned into the pET-15b vector (Novagen, Madison, WI, USA) as previously described (*16*). The resulting recombinant plasmid was transformed into the Rosetta strain of *Escherichia coli*. The heterologously produced histidine (His)–tagged GlpQ fusion protein was purified by using Ni-NTA spin columns (Qiagen, Valencia, CA, USA), and the protein concentration was determined with Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

ELISA with Recombinant GlpQ Protein

An ELISA was used to detect anti-GlpQ immunoglobulin G (IgG) antibodies in patient sera and was performed as described by Porcella et al. (*16*). Briefly, His-GlpQ protein was adsorbed onto microtiter well surfaces of Ni-NTA HisSorb plates (Qiagen). Wells were blocked with diluent to inhibit nonspecific binding and washed. Serum samples were tested at a 1:100 dilution by incubating 100 μL/well

Table 1. Primers used for cloning, PCR, and DNA sequencing

Primer	Sequence (5′→3′)*	Reference
Br_GlpQ_3_*Bam*HI	GGC**GGATCC**GCTTGACCAGTTGCTCCTCCGC	(*16*)
Br_GlpQ_5_XhoI	GCCG**CTCGAG**GAAAAGAAAATGCAAAAATAAATAAA	(*16*)
GlpQ For	GGTATGCTTATTGGTCTTC	Present study
GlpQ Rev	TTGTATCCTCTTGTAATTG	Present study
GlpQ1	CAAATCACTAAGCCTTAGCGAAAGAT	Present study
GlpQ2	ATCTGTTGGTGCTTCTTCCCAGT	Present study
GlpQ3	CAGGGAAAATTGATAATGCTTGTTGG	Present study
GlpQ4	CTGCTAATGTGAAATCGACGGAATA	Present study
Nested_1_F	AGAGTTTGATCCTGGCTTAG	(*15*)
Nested_1_R	CTTGCATATCCGCCTACTCA	(*15*)
Nested_2_F	GGCTTAGAACTAACGCTGGCA	(*15*)
Nested_2_R	CTGCTGGCACGTAATTAGCC	(*15*)

*Sequences in **boldface** indicate restriction sites for *Bam*H1 and *Xho*I.

for 1 h at room temperature. After 3 washes, 100 μL of a 1:2,500 dilution of goat anti-human IgG (heavy and light chains) conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added to each well and incubated for 1 h. After 3 washes, 50% 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonate) substrate was added and incubated for 25 min before analysis at 405 nm with a Multiskan microtiter plate reader (Labsystems, Vantaa, Finland). Each serum sample was tested in triplicate, and the mean absorbance value was determined. Samples were considered positive if their mean absorbance was greater than the mean plus 3 standard deviations of the absorbance of control sera tested at the same dilution. Serum samples from Ethiopian patients with RF were included as positive controls (*16*).

Blood Screening by Nested PCR

Blood samples that were positive or borderline positive by ELISA were tested by a nested PCR for *Borrelia* DNA. DNA was purified from the blood samples and 4 primers (Table 1) were used for detection of the 16S rRNA gene of borreliae. The first PCR amplified a 584-bp region of the gene with the primers Nested_1_F – Nested_1_R. The second PCR amplified a 498-bp region with the primers Nested_2_F – Nested_2_R. The amplicons obtained from the nested PCRs were sequenced to identify the *Borrelia* species because the primers were not able to amplify DNA from specific species of RF spirochetes.

PCR and Sequencing

For amplification of the complete coding sequence of *glpQ* gene from *B. duttonii*, 2 primers were designed by using *B. hermsii* noncoding sequences flanking the *glpQ* gene and 4 primers were designed by using sequences within the gene (Table 1). Nested PCR primers (Table 1) were designed to target the 16S rRNA gene. The PCR product for the *B. duttonii glpQ* gene was sequenced, and data were deposited in GenBank (accession no. DQ909058).

Ethics

The study was approved by the Ethics Committee at Umeå University (Dnr 04–050 M). Informed consent was obtained at clinics from all patients or from the accompanying parent if the patient was a child.

Statistical Analysis

Proportions were compared with a 2-tailed χ^2-corrected (Yates) analysis and Fisher exact test. p values <0.05 were considered significant.

Results

No patients were positive for borreliae by microscopic examination of Giemsa-stained blood smears. Among patients with fever, 9 (10%) of 90 children in northern Togo, and 5 (9.8%) of 51 children and 16 (16.3%) of 98 adults in southern Togo were seropositive by ELISA. A total of 12.6% of patients with fever were positive by ELISA (Table 2). For those patients without fever, 2 (14.3%) of 14 were seropositive. Because the *glpQ* gene is present in RF spirochetes but not in Lyme disease spirochetes, the positive serologic results strongly suggest that patients were infected with RF spirochetes (*11*). However, this serologic test is not species specific and cannot distinguish current from past infections.

Current *Borrelia* infections were detected by PCR and 16S rRNA gene sequence analysis in blood samples of patients from both northern and southern Togo. DNA sequencing identified both *B. crocidurae* and *B. duttonii*, but *B. duttonii* was found only in patients from northern Togo (Table 2). All 81 patients from northern Togo who were seronegative were also negative by PCR. In contrast, 8 (88.9%) of 9 patients who were positive by ELISA were also positive by PCR ($p<0.05$). All patients who were positive by PCR had a fever when their blood samples were collected.

A total of 28 patients from southern Togo were tested for current spirochetemias and included all ELISA-positive and some ELISA-negative patients. The negative

Table 2. Prevalence and identification of *Borrelia* infections in patients with fever at clinics in northern and southern Togo, 2002–2004

Region, age group, y	Seropositive for relapsing fever *Borrelia*,* no. positive/no. tested (%)	*Borrelia* in blood,† no. positive/no. tested (%)	Infected with *B. crocidurae*,‡ no. positive/no. tested (%)	Infected with *B. duttonii*,‡ no. positive/ no. tested (%)
Northern				
0–4	6/60 (10)	4 (6.7)	3 (5)	1 (1.7)
5–14	3/30 (10)	4 (13.3)	3 (10)	1 (3.3)
Total	9/90 (10)	8 (8.8)	6 (6.7)	2 (2.2)
Southern				
0–4	2/16 (12.5)	1 (6.3)	1 (6.3)	0
5–14	3/35 (8.6)	2 (5.7)	2 (5.7)	0
Total	5/51 (9.8)	3 (5.9)	3 (5.9)	0
15–24	7/43 (16.3)	3 (7)	3 (7)	0
≥25	9/55 (16.4)	7 (12.7)	7 (12.7)	0
Total for adults	16/98 (16.3)	10 (10.2)	10 (10.2)	0
Total	21/149 (14.1)	13 (8.7)	13 (8.7)	0
All	30/239 (12.6)	21 (8.8)	19 (7.9)	2 (1.2)

*Determined by ELISA for glycerophosphodiester phosphodiesterase antigen.
†Determined by 16S rRNA gene PCR amplification from blood of patients with high ELISA values divided by all ELISA- tested patients.
‡Determined by genomic sequence of 16S rRNA in blood samples.

samples chosen were those with the highest values below the cut-off value as well as randomly chosen samples with lower values. The 13 PCR-positive patients included 11 (55%) of 20 ELISA-positive patients and 2 (25%) of 8 ELISA-negative patients. Two samples from ELISA-positive patients could not be tested by PCR because the DNA was degraded. Both patient samples that were PCR positive and ELISA negative had ELISA values just below cut-off value used in the study.

All patients from northern Togo were children (age range <1–14 years). For children ≤4 years of age, 6 (10%) of 60 were seropositive. Of these children, 5 had a fever and 4 had an active *Borrelia* infection detected by PCR. DNA sequence analysis showed that 3 children were infected with *B. crocidurae* and 1 with *B. duttonii*. In southern Togo, 1 (9.1%) of 11 children <1–4 years of age were seropositive. The overall male-to-female ratio among study patients was 1.3:1. Serologic and PCR results did not differ by sex, ethnic background, or profession, with the exception of cow herders. The prevalence of seropositive adults was 62.5% (5/8) among cow herders compared with 12.2% (11/90) among those in other professions ($p<0.05$); 11 (78.6%) of 14 adults in the Peuhl ethnic group were cow herders (95% confidence interval [CI] 49.2%–95.3%), and 4 of 5 ELISA-positive Peuhl were cow herders. A total of 10.8% (95% CI 5.9%–17.8%) of all adults studied were cow herders. More *Borrelia*-infected patients lived in houses made of mud rather than cement than persons without RF infection ($p = 0.008$, data not shown).

The prevalence of malaria among patients with fever was 63.1%. Of 21 patients with PCR-confirmed *Borrelia* infections, malaria was diagnosed for 7 on the basis of a positive blood smear. Therefore, 7 (4.5%) of 154 patients were coinfected with malaria parasites and *Borrelia* (Table 3). In the youngest children (≤4 years of age), 4 of 5 *Borrelia*-infected children also had malaria.

In northern Togo, patients infected with malaria and *Borrelia* were treated primarily with chloroquine, artemether, quinine, and amoxicillin. *Borrelia*-infected, malaria-negative patients were treated primarily with chloroquine, quinine, or amoxicillin. In southern Togo, patients with malaria and *Borrelia* infections were treated primarily with quinine and chloramphenicol for typhoid fever, metronidazole for amebiasis, and albendazole for digestive parasitosis. *Borrelia*-infected, malaria-negative patients were treated with chloroquine and antimicrobial drugs, such as amoxicillin, which are ineffective against RF *Borrelia* (Table 3) (*14*).

Discussion

Although microscopic analysis of Giemsa-stained blood smears for *Borrelia* showed negative results, current infections were demonstrated by PCR and gene sequencing in 8.8% of patients with fever. Therefore, no RF infections would have been detected if only microscopic examination of stained blood smears had been performed. Our results emphasize the low sensitivity of microscopy in the diagnosis of RF, as has demonstrated by others (*11*,*14*,*17*). Another problem in diagnosis is that the borreliae are detectable only during the short peaks of fever (*18*). Microscopy might have shown positive results if dark-field or fluorescent microscopy had been used, but this was not possible in the clinics in this study. However, 1 of the primary objectives of this study was to improve the diagnosis of RF borreliosis by using molecular and serologic techniques.

Table 3. Coinfection with malaria and relapsing fever caused by *Borrelia* and treatment in patients in northern and southern Togo with fever, 2002–2004

Region, group	Malaria infection,* no. positive/ no. tested (%)		*Borrelia* infected† and treated for malaria, no. positive/no. tested (%)		*Borrelia* infections effectively treated, no. positive/no. tested (%)
	All patients	*Borrelia* infected	Malaria positive	Malaria negative‡	
Northern					
Children	34/96 (35.4)	4/8 (50)	4/4 (100)	1/4 (25)	0/8
Southern					
Children	46/68 (67.6)	2/3 (66.7)	1/2 (50)	0/1	1/3 (33.3)
Adults	35/80 (43.8)	1/10 (10)	0/1	1/6 (16.6)	0/7
Total	81/148 (54.7)	3/13 (23.1)	1/3 (33.3)	1/7 (14.3)	1/10 (10)
All	154/244 (63.1)	7/21 (33.3)	5/7 (71.4)	2/11 (18.2)	1/18 (5.6)

*Determined by microscopy of Giemsa-stained blood smears.
†Determined by positive PCR result and *Borrelia* species identification by sequence in blood. Three malaria-negative adults were not included because treatment information was not available.
‡Values represent patients treated only for malaria. Two of these 4 patients were treated for malaria in combination with drugs for treating helminth infections.

A study of rodents in Senegal showed that 57.7% of *Borrelia* infections were false negative by microscopy (*11*). Microscopy is the method routinely used to diagnose *B. crocidurae* infections in Senegal, where the disease is common (*14*,*15*). Thus, methods that provide greater sensitivity are needed to determine more accurately the presence of RF throughout West Africa. Since the GlpQ antigen is present in RF *Borrelia* but absent in Lyme disease *Borrelia*, positive antibody test results strongly indicated that infections with RF *Borrelia* were occurring or had occurred (*16*). However, the infecting species and the time of infection could not be determined by this method. Positive serologic test results correlated with current infection, as demonstrated by PCR and sequence analysis. Thus, serologic tests may be more adequate for diagnosis, although ELISA procedures would have to be modified for use in small rural clinics.

Our finding of TBRF in Togo demonstrates that the geographic distribution of this disease in West Africa is greater than previously thought. Trape et al. suggested that RF caused by *B. crocidurae* might be spreading to new areas because of the sub-Saharan drought, which might allow vector ticks to colonize new areas in the savannas of West Africa (*2*). Our results suggest that this might be true. We also found RF caused by *B. crocidurae* in the tropical region of southern Togo, where the climate may be less favorable for its tick vector. Thus, habitats believed to be preferred by *O. sonrai* may need to be reconsidered. Southern Togo has been subjected to deforestation, periods of drought, and slash-and-burn agriculture. During the rainy season, the average temperatures in Dapaong and Atakpame are 24°C and 25.8°C, respectively, compared with dry season mean temperatures of 28.9°C and 26.8°C, respectively (*19*). The dry wind or harmattan from the Sahara Desert during winter can cause periodic droughts in northern Togo. We propose that areas with similar climate in West Africa are likely to have TBRF.

Of particular interest was our finding of *B. duttonii* in northern Togo, which extends the known distribution of this species in Africa. Additional work is needed to determine if its vector *O. moubata* is present in northern Togo or if *B duttonii* can be transmitted by *O. sonrai*. More studies are needed to determine the distribution of RF spirochetes and their vectors in Africa and what effect these infections have on human health.

Many of the primary symptoms of malaria and RF are similar, such as recurrent fever, chills, anemia, hepatomegaly, splenomegaly, and possible neurologic symptoms. Thus, a considerable risk for misdiagnosis of TBRF as malaria exists in countries in which RF is not recognized but in which malaria is prevalent. Occasional reports of European tourists returning home from Senegal with *B. crocidurae* infections have implied that the disease may be misdiagnosed as malaria, which leads to incorrect treatment (*14*,*15*,*20*). Because RF has not been investigated in most West African countries including Togo, the disease is generally not considered in a differential diagnosis for fever patients. Also, malaria is often diagnosed on the basis of only clinical symptoms, not examination for parasites in the blood, which increases misdiagnosis. Increased knowledge of the geographic distribution and epidemiology of RF in West Africa should improve the recognition and treatment of this disease. Prompt diagnosis of RF would also reduce the number of people in whom chronic symptoms such as neuroborreliosis later develop when bacteria cross the blood-brain barrier and infect the central nervous system (*5*,*21*,*22*).

We used a questionnaire to determine possible risk factors associated with RF. Some children <1–≤4 years of age were infected with RF *Borrelia*. Since *O. sonrai* is nocturnal, feeding mostly at night, these children may have been infected at home. However, RF among infants may also reflect infection from mothers before or during birth, as occurs with *B. duttonii* in East Africa (*18*). We also

found that persons with current *Borrelia* infections more often lived in houses made of mud rather than cement. This observation is similar to ones in Senegal, where *Ornithodoros* ticks feed primarily at night inside mud or in adjacent areas where small mammals are present (*2*,*6*,*20*).

Before our study, we trapped 66 rodents and insectivores in or near houses in Togo. Four species were identified: musk shrew (*Crocidura* spp.), Nile rat (*Arvicanthis niloticus*), multimammate rat (*Mastomys natalensis*), and brown rat (*Rattus norvegicus*); 3 are known reservoirs for *B. crocidurae* in Senegal. These findings showed the presence of these potential reservoirs in northern and southern Togo (unpub. data). We found that mud huts were associated with entrances of rodent burrows, which might increase the risk for exposure to ticks (data not shown). In the present study, cow herders were also at a greater risk of acquiring RF. However, more studies are required to investigate potential risk groups. A higher proportion of cow herders who were ELISA positive were also Peuhl, who are nomadic people. Their behavior and sleeping outside at night may put them at greater risk of being bitten by nocturnal soft ticks.

We report TBRF in Togo with a prevalence of 8.8% among the patients studied. For those with fever, 63.1% had malaria and 4.5% were coinfected with RF *Borrelia*. Among those with RF, 33.3% were coinfected with malaria parasites. Given the retrospective finding of spirochetes by PCR, only 1 of 18 patients with TBRF received treatment effective against this disease at the time she was seen in the clinic. Thus, TBRF is obscured by the high incidence of malaria in Togo, and this problem likely occurs in other regions in West Africa. In rural health centers without laboratory facilities, diagnosis of malaria is based only on clinical symptoms. The potential risk for misdiagnosis and ineffective treatment of patients with RF rather than malaria needs to be addressed. Our findings demonstrate the need for improved diagnostic procedures to detect TBRF in West Africa. We consider the high prevalence of RF among febrile children and the lack of correct treatment as important health concerns, particularly with regard to the severity of untreated neuroborreliosis and women infected during pregnancy.

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Clinical Diagnosis and Geographic Distribution of Leptospirosis, Thailand

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We defined the positive predictive accuracy of a hospital-based clinical diagnosis of leptospirosis in 9 provinces across Thailand. Of 700 suspected cases, 143 (20%) were confirmed by laboratory testing. Accuracy of clinical diagnosis varied from 0% to 50% between the provinces and was highest during the rainy season. Most confirmed cases occurred in the north and northeast regions of the country.

Leptospirosis is an emerging infectious disease in Thailand (*1*). Before 1996, the number of cases reported to the Thailand Department of Disease Control (DDC) was ≈200 per year. Leptospirosis was sporadic and reported mainly in central and southern regions. A marked change occurred in the decade thereafter, with an increase from 358 cases in 1996 to a peak of 14,285 cases in 2000. This was followed by a continual decline to 2,868 cases in 2005 (*1*). Most cases (90%) throughout this period were reported in northeast Thailand. A study of >600 adults who sought treatment for fever at 1 hospital on the Thai-Myanmar border provided further evidence of the importance of leptospires as a pathogen in this region, with serologic evidence for leptospirosis found in 17% of the patients (*2*). The true extent of the disease is likely considerable in Thailand, which illustrates the need for accurate epidemiologic tools for its evaluation. An essential part of this process is understanding the mechanisms of reporting and their inherent inaccuracies.

Reporting of leptospirosis to the DDC in Thailand is voluntary. During a review of the national surveillance system for leptospirosis in 2 northeastern provinces, interviewed physicians said the national case definition was difficult to understand and apply (*3*). Investigators concluded that the lack of a standardized case definition for leptospirosis; the infrequent use of confirmatory laboratory testing; and the inability to link clinical, epidemiologic, and laboratory data hindered the system's utility (*3*). These results imply that both underreporting and diagnostic inaccuracy of reported cases may be occurring. We conducted a prospective multicenter study to define the accuracy of clinical diagnoses of suspected leptospirosis in Thailand and to describe the geographic distribution of laboratory-confirmed cases.

The Study

From March 2003 though November 2004, admitting physicians in district and provincial hospitals within 9 provinces of Thailand in the north, northeast, central, and southern regions were invited to recruit patients of all ages suspected on clinical grounds to have leptospirosis. Clinical features considered were those specifically referred to in the national guidelines (e.g., fever, headache, muscle pain, meningism, conjunctival suffusion, and jaundice), together with hemoptysis, hepatomegaly, diarrhea, hypotension, and reduced urine output. From each patient, a 5-mL serum sample was taken to be cultured for *Leptospira*, another 5-mL serum sample was taken for serologic testing, and a third sample was taken 2 weeks later for serologic testing. Serum was stored at –80°C until analysis.

Microscopic agglutination test (MAT) was performed at the World Health Organization (WHO)/United Nations Food and Agriculture Organization (FAO)/World Animal Health Organisation (OIE) Collaborating Center for Reference and Research on Leptospirosis, Brisbane, Queensland, Australia (*4*). A positive MAT was defined as a single titer of ≥1:400 or a 4-fold rise in titer between acute and convalescent phase samples. For *Leptospira* culture, 100 μL of whole blood, 500 μL of plasma, and 500 μL of serum were each injected into 3 mL of Ellinghausen, McCullough, Johnson, and Harris (EMJH) medium and supplemented with 3% rabbit serum and 0.1% agarose, then incubated aerobically at room temperature (25°C–30°C) for 6 months and examined every week for 2 months, every 2 weeks during months 3 and 4, and once a month during months 5 and 6. Examination was done by placing 1 drop of culture onto a microscopic glass slide and viewing by dark-field microscopy at 200× magnification. Positive cultures were referred to the WHO/FAO/OIE Collaborating Center for Reference and Research on Leptospirosis for identification by using the cross-agglutination absorption test (*4*).

A total of 700 patients with a clinical diagnosis of leptospirosis were recruited during the study period. All patients had blood samples collected at the hospital for lep-

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tospire culture and serologic testing; convalescent-phase serum samples were obtained during follow-up for 509 (73%) patients.

The median age of patients with suspected leptospirosis was 38 years (range 2–95 years, interquartile range [IQR] 28–49 years); 504 (72%) were men. The number of clinically diagnosed leptospirosis cases by month in the north, northeast, central, and southern regions is shown in Figure 1. Most cases (597, 85%) were recorded in 4 provinces in the north or northeast (Table). Cases were predominantly identified during the rainy season (June–October) in the north and northeast in 2003, with a second peak in the northeast, but not the north, during the rainy season of 2004. Little variation occurred over time in the central and southern regions.

Of the 700 patients who received a clinical diagnosis of leptospirosis, 143 (20%) received a confirmed diagnosis of leptospirosis based on *Leptospira* isolation, MAT testing, or both (Table). The median age of patients with confirmed leptospirosis was 35 years (range 10–68 years, IQR 27–45 years); 121 (85%) were men. The diagnosis was confirmed after isolation of leptospires from 15 (11%) patients; the geographic distribution is shown in the Table. The serovars of cultured *Leptospira* were *L. interrogans* serovar (sv.) Autumnalis (7), *L. interrogans* sv. Bataviae (2), *L. interrogans* sv. Pyrogenes (2), *L. borgpetersenii* sv. Javanica (1), *L. interrogans* sv. Hebdomadis (1), *L. interrogans* sv Grippotyphosa (1), and an unidentified serovar (1). An additional 128 patients with culture-negative samples had been exposed to *Leptospira* as determined by MAT; results for 96 (75%) were based on a 4-fold rising titer and for 32 (25%), on a single raised titer of ≥1:400.

The geographic distribution of the 143 laboratory-confirmed cases is summarized in the Table. Most of these patients (124, 87%) lived in the 4 provinces found in the north and the northeast. The month of diagnosis for confirmed cases is shown in Figure 2; most were during the rainy season.

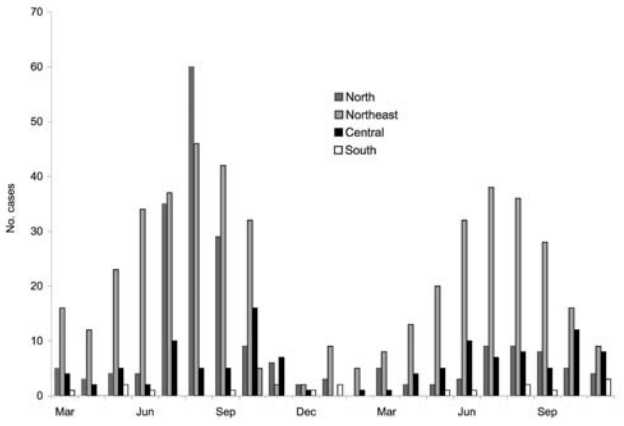

Figure 1. Cases of clinically suspected leptospirosis by month for each geographic region, Thailand, March 2003–November 2004.

The positive predictive accuracy of a clinical diagnosis is defined by the number of laboratory-confirmed cases divided by the number of clinically suspected cases. Results for each of the 9 provinces are shown in the Table. When only data from centers that reported at least 10 cases were used, positive predictive accuracy ranged from 3% to 29%. Positive predictive accuracy by month of study is shown in Figure 2.

Conclusions

Diagnosing leptospirosis at the point of care is notoriously difficult in the tropical setting, where several common infectious diseases are often hard to differentiate. Positive predictive accuracy for leptospirosis was highest during the rainy season, an observation that is likely related to the higher disease incidence and pretest probability. Variability in positive predictive accuracy was seen among the 3 provinces with the highest number of both suspected and true cases. The reason for this is unclear but may relate to perceived risk to the community, local policy, or other factors.

Table. Distribution of suspected and confirmed cases of leptospirosis according to province, Thailand

Province	Geographic region	Clinically suspected cases (%)*	Laboratory-confirmed cases (%)†	Positive predictive accuracy (95% CI)‡	Culture-positive cases
Lumpang	North	161 (23)	28 (20)	17% (12–24)	2
Udon Thani	Northeast	223 (32)	64 (45)	29% (23–35)	10
Maha Sarakham	Northeast	181 (26)	26 (18)	14% (10–20)	1
Ya Sothon	Northeast	32 (5)	6 (4)	19% (7–36)	1
Chainut	Central	13 (2)	3 (2)	23% (5–54)	0
Rayong	Central	45 (6)	13 (9)	29% (16–44)	1
Chanthaburi	Central	4 (0.6)	2 (1)	50% (7–93)	0
Prachuap Khiri Khun	South	33 (5)	1 (0.7)	3% (0.1–16)	0
Phattalung	South	8 (1)	0	0% (0–37)	0
Total		700	143	20%	15

*Percentage of total suspected cases.
†Percentage of total confirmed cases.
‡CI, confidence interval.

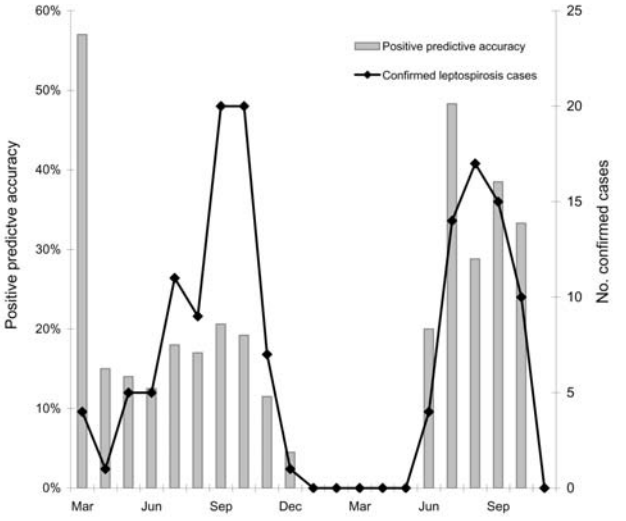

Figure 2. Cases of laboratory-confirmed leptospirosis and positive predictive accuracy of clinical diagnosis by month, Thailand, March 2003–November 2004.

The finding that both clinical and confirmed cases of leptospirosis were more common in the north and northeast is consistent with DDC reports. Increased incidence in this region may have resulted from an event such as an increase in the rodent population, a natural reservoir for this pathogen, and a population in which around one third are positive for *Leptospira* in northeast Thailand (*5*). Alternatively, 1 clone or a small number of bacterial clones may have become adapted for persistence at greater numbers within the natural host or in the environment. These factors could increase the leptospire count in contaminated water. It is also possible that 1 clone or a small number of clones have become adapted for enhanced invasion of the human host. The most prevalent serovar isolated was *L. interrogans* serovar Autumnalis (7/15 [47%] isolates), 6 of which were from cases in the north or northeast. Further genomic analysis is required to determine whether clonality exists among these isolates.

The effect of the low level of accuracy of hospital-based clinical diagnosis of leptospirosis in rural Thailand is not known. A common disease in this setting that is easily confused with leptospirosis is scrub typhus; both diseases would be predicted to respond to doxycycline, an antimicrobial drug often prescribed for undifferentiated fever. Further studies are required to define the implications of our findings and determine whether routine laboratory testing for leptospirosis should be implemented in Thailand.

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Indigenous Case of Disseminated Histoplasmosis, Taiwan

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We report the first indigenous case of disseminated histoplasmosis in Taiwan diagnosed by histopathology of bone marrow, microbiologic morphology, and PCR assay of the isolated fungus. This case suggests that histoplasmosis should be 1 of the differential diagnoses of opportunistic infections in immunocompromised patients in Taiwan.

Histoplasma capsulatum, a dimorphic fungus that causes human disease, is endemic in North and Central America, particularly in the region of the Ohio and Mississippi River valleys. Humans are infected by inhalation of the mycelial fragments and microconidia of the organism. After the emergence of HIV infection, histoplasmosis has become 1 of many troublesome opportunistic infections among patients with AIDS. Patients receiving immunosuppressive agents are also predisposed to *H. capsulatum* infection (*1*). Although the organism is found worldwide, cases of histoplasmosis are rarely encountered in Taiwan; only a few, imported, cases have been reported in this decade (*2–7*). We report the first indigenous case of disseminated histoplasmosis in Taiwan.

The Case

In November 2005, a 78-year-old man with underlying rheumatoid arthritis was sent to the emergency department with generalized weakness and poor appetite of several weeks' duration. He had received oral therapy with prednisolone (5 mg twice per day), hydroxychloroquine (200 mg twice per day), sulfasalazine (1,000 mg twice per day), and methotrexate (MTX) (15 mg per week) for 4 months. The patient's body temperature was 38.5°C, blood pressure was 129/80 mm Hg, pulse rate was 76 beats/min, and respiratory rate was 20 breaths/min. Physical examination disclosed mild icteric sclera and multiple ecchymoses on the extremities.

A complete blood cell count showed a leukocyte count of 6,110/μL (4% bands, 77% segmented neutrophils, 7% lymphocytes, 6% normoblasts, and 3% myelocytes), hemoglobin level of 11.5 g/dL, and platelet count of 3,000/μL. Biochemical testing showed total bilirubin level of 3.89 mg/dL (normal 0–1.3 mg/dL) and alkaline phosphatase level of 480 U/L (60–220 U/L). Renal function, liver enzymes, and electrolyte levels were all within normal limits. The rheumatoid factor level was 76.3 IU/mL (normal <15 IU/mL). The erythrocyte sedimentation rate and C-reactive protein level were 30 mm/hour (0–20 mm/hour) and 100 mg/L (0–5 mg/L), respectively. Test results for HIV and antinuclear antibody were negative. A chest radiograph showed bilateral interstitial micronodules and a fibrocalcified pattern. Abdominal ultrasonography indicated splenomegaly. The patient was admitted with the tentative diagnosis of MTX-induced thrombocytopenia.

Bone marrow aspiration (Figure 1) and biopsy were performed because of refractory thrombocytopenia and the presence of young blood cells on the peripheral blood smears. Intravenous amphotericin B (0.7 mg/kg/day) was administered because disseminated histoplasmosis was highly suspected because of the bone marrow findings. Four weeks later, the fungal culture of the bone marrow showed growth of mold (Figure 2A and B). The microorganism was subsequently identified as *H. capsulatum* by PCR assay (Figure 2C) (*8*). No *H. capsulatum* was cultured from the patient's peripheral blood or sputum.

The patient's general condition improved after administration of a total dose of 1 g of intravenous amphotericin B, and the patient was discharged and treated with oral itraconazole, 200 mg once a day. Two weeks later, itraconazole therapy was suspended because impaired liver function was found. The patient was closely monitored for

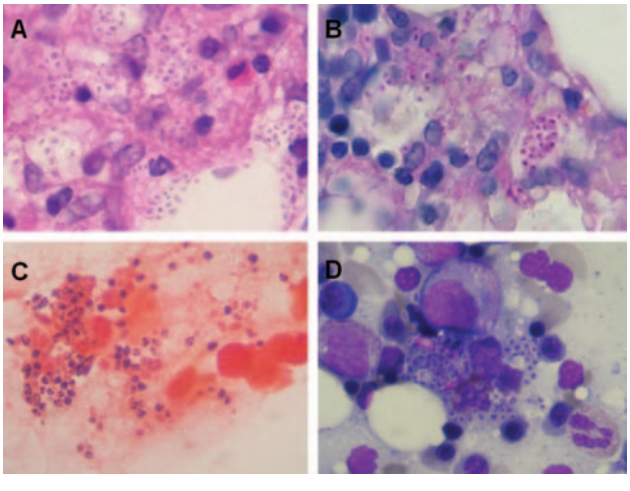

Figure 1. A bone marrow biopsy specimen showing numerous oval-shaped intracellular and extracellular microorganisms (A and B). A bone marrow aspiration smear showed numerous intracellular yeastlike microorganisms (C and D). A) hematoxylin and eosin stain, ×1,000; B) periodic acid-Schiff stain, ×1,000; C) Gram stain, ×1,000; and D) Wright stain, ×1,000.

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3 months, and no clinical evidence of histoplasmosis relapse was noted.

Conclusions

A variety of laboratory tests for diagnosis of histoplasmosis, including fungal culture, histopathology, serologic tests, antigen detection, and molecular methods, have different sensitivities based on clinical manifestations and host status (*9*). The standard for diagnosis is isolation of *H. capsulatum* from culture. However, it usually requires ≈4 weeks to grow and has a low sensitivity rate (15%) in self-limited histoplasmosis. The knobby appearance of macroconidia and microconidia indicated by Lactophenol Cotton Blue Stain (Hardy Diagnostics, Santa Maria, CA, USA) of the mold form from fungal cultures is characteristic of *H. capsulatum* (Figure 2B) (*10*). In histopathology, histoplasmosis is impressive, with its numerous ovoid-shaped microorganisms in infected tissue (Figure 1). However, without sufficient experience, one could misidentify *Blastomyces dermatidis*, *Candida glabrata*, *Cryptococcus neoformans*, *Penicillium marneffei*, *Leishmania* spp., *Pneumocystis jirovecii*, or *Toxoplasma gondii* as *H. capsulatum* (*11*). Despite a characteristic form, specific DNA probing is usually applied for faster definitive identification (*12*).

In areas where histoplasmosis is not endemic, including Taiwan, serologic tests, antigen detection reagents, and specific DNA probes for diagnosis of histoplasmosis are not universally available. Among serologic tests, immunodiffusion and complement fixation for anti–*Histoplasma* antibody detection are widely applied; sensitivity rates are 95%–100% and 82%–90% for pulmonary and disseminated histoplasmosis, respectively. Some limitations occur, including the need for 2 to 6 weeks for antibody production after infection, impaired production of antibodies in immunosuppressed patients, and presence of cross-reaction mainly due to paracoccidioidomycosis, blastomycosis, and aspergillosis (*9*). Antigen detection in serum and urine is the most useful method of diagnosing histoplasmosis because it provides early diagnosis before culture and antibody production, monitors response of therapy, and detects relapse. The sensitivity rates of antigen detected are 25%–75% in pulmonary histoplasmosis and 82%–95% in disseminated histoplasmosis. Cross-reactions may occur in cases of paracoccidioidomycosis, blastomycosis, African histoplasmosis, and *P. marneffei* (*9*).

Although the environment in Taiwan is suitable for *H. capsulatum* to grow, histoplasmosis has been rarely encountered. In a survey of histoplasmin skin tests conducted in Taiwan in the 1950s, only 7 (0.19%) of 3,589 schoolchildren tested positive, and the author concluded histoplasmosis probably does not exist in Taiwan or is very rare (*13*). The first possible case of histoplasmosis in Taiwan was reported in 1977; however, the diagnosis was doubtful because it was based only on histopathologic findings in a cervical lymph node biopsy specimen, without definitive fungal culture or molecular identification (*2*). No further cases of histoplasmosis were reported in Taiwan until this decade, when 6 cases were reported (*3*–*7*). The clinical characteristics of these cases are summarized in the online Appendix Table (available from www.cdc.gov/ ncidod/ EID/ 13/1/127-appT.htm). All cases include a history of travel or residence outside Taiwan, where the patients might have acquired the infection; furthermore, most of the patients had underlying HIV infections. Diagnoses were mostly based only on histopathologic or morphologic findings in fungal cultures because the serologic tests, antigen detection reagents, and commercial DNA probes for diagnosis of histoplasmosis were not available in Taiwan. In contrast to these cases, our patient had never traveled outside Taiwan and did not have an HIV infection. To confirm the identification of *H. capsulatum* on the basis of histopathologic and fungal form findings (Figures 1, 2A, and 2B), we applied a specific PCR assay as previously described (*8*), and the results were definitive (Figure 2C). The fibrocalcific nodules found on the chest radiograph imply that either 1) the patient might have had histoplasmosis for years and it became disseminated because of immunosuppressive

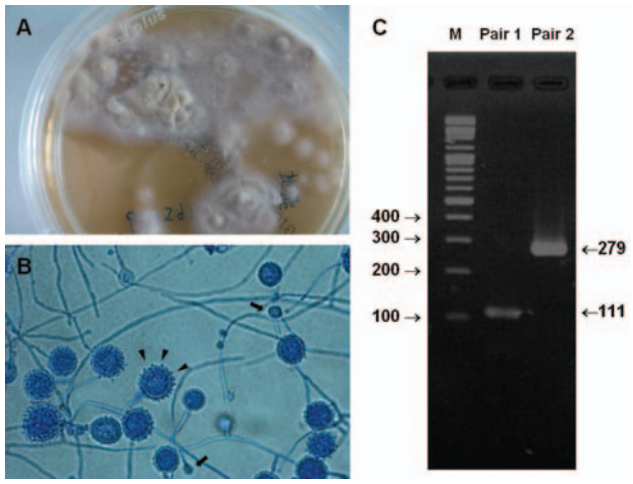

Figure 2. A) Colony of the mold from the patient is white-brown with a cottony appearance on Sabouraud dextrose agar. B) Lactophenol Cotton Blue Stain (Hardy Diagnostics, Santa Maria, CA, USA) of the isolated mold showing thick-walled and tuberculate macroconidia (arrowheads) and microconidia (arrows). C) PCR assay for identification of *Histoplasma capsulatum* based on the nucleotide sequence of the M antigen. PCR products included 111-bp and 279-bp fragments amplified with primers Msp1F-Msp1R (pair 1) and Msp2F-Msp2R (pair 2), respectively, which confirmed the identification of *H. capsulatum* (8). M, molecular mass marker.

therapy for rheumatoid arthritis or 2) the patient was reinfected with the calcified lesions that resulted from prior histoplasmosis. The results indicate that this is the first definitive indigenous case of disseminated histoplasmosis in Taiwan. Nonetheless, we were unable to monitor our patient's response to treatment by antigen tests, as is recommended (*9*,*14*), because they are not available in Taiwan.

The rarity of diagnosed histoplasmosis cases in Taiwan could be explained in several ways. First, the diagnostic rate of histoplasmosis might be markedly decreased because of the lack of serologic and antigen testing kits and reagents, which are useful for diagnosis of self-limited and nondisseminated histoplasmosis. Second, pulmonary histoplasmosis might be misdiagnosed as tuberculosis, which is prevalent in Taiwan. Third, physicians are unfamiliar with histoplasmosis and may consider that histoplasmosis is absent in Taiwan. With increasing immunocompromised hosts resulting from immunosuppressive therapy and HIV infections, as well as improved diagnostic tests, histoplasmosis might be an emergent infectious disease in Taiwan in the future.

In summary, although an indigenous case of histoplasmosis had never been encountered, it should be 1 of the differential diagnoses of opportunistic infections in immunocompromised patients in Taiwan. The true prevalence of histoplasmosis in non-disease–endemic regions might be underestimated because of the paucity of diagnostic tools and familiarity with histoplasmosis.

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Interaction Between Humans and Poultry, Rural Cambodia

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Because avian influenza H5N1 infection risks are associated with exposure to infected poultry, we conducted a knowledge, attitudes, and practices survey of poultry-handling behavior among villagers in rural Cambodia. Despite widespread knowledge of avian influenza and personal protection measures, most rural Cambodians still have a high level of at-risk poultry handling.

The circulation of the highly pathogenic H5N1 avian influenza (AI) strain throughout Asia since late 2003 (*1*), and more recently in Europe and Africa, has resulted in considerable concern for the potential of a new pandemic. In Cambodia, outbreaks of HPAI A/H5N1 infection were first reported in poultry in early 2004 (*2*). Since 2005, 6 human cases have occurred (100% fatal); the 2 most recent cases occurred in early 2006 (*3*,*4*).

Most Cambodians live in rural areas and raise animals for consumption (*2*), typically keeping poultry, swine, or cattle close to the home. Because H5N1 infection has been associated with exposure to infected poultry (*5*–*10*) and little is understood of the perceptions of rural farmers regarding AI (*11*), we conducted a knowledge, attitude, and practices survey of poultry handling in rural Cambodia to estimate the extent of interactions between humans and poultry, to understand practices in poultry handling among villagers, and to develop interventions designed to increase reports of poultry deaths and safe poultry handling.

The Study

We conducted a 2-stage household based cluster survey (*12*) with a goal of 500 participants: 20 persons ≥15 years of age in each of 25 villages from Prey Veng and Kampong Cham Provinces. The sampling frame of eligible villages within these provinces were those located in H5N1 high-risk communes, as defined by the Food and Agriculture Organization of the United Nations training program for village animal health workers. The villages were selected with probability proportional to size. For the second stage, we randomly selected the first household within each village. Subsequently, households were selected by proximity until 20 eligible participants were enrolled in each cluster.

Verbal consent was obtained from all participants. All were interviewed by using a structured questionnaire designed to collect information on demographics, basic hygiene practices, quantity of poultry owned, poultry death reporting, practices when deaths occurred, knowledge and attitude of sick and dead poultry, and knowledge of AI.

Twenty-three villages were included in Kampong Cham (*11*) and Prey Veng (*12*) Provinces (Figure 1). Four hundred sixty respondents from 269 households completed the questionnaire. Most were women (60%), farmers (88%), and persons who had completed less than primary schooling (57%). The median number of household members was 5 (range 1–16), and 77% of all households included children <15 years of age.

Many households owned chickens (97%) and ducks (39%) (Figure 2), although the size of most poultry flocks was small (Table). Almost all poultry were free ranging (100% of chicken flocks; 96% of duck flocks), and mixing of the poultry with pigs and other domestic animals was common. Respondents reported that they use poultry feces for manure (77%), touch sick/dead poultry with bare hands (75%), eat poultry that died from illness (45%), eat wild birds (33%), let children touch sick/dead poultry with bare hands (20%), and gather dead wild birds for consumption (8%).

During the previous 6 months, of the 260 households that owned poultry, 162 (62%) experienced poultry deaths;

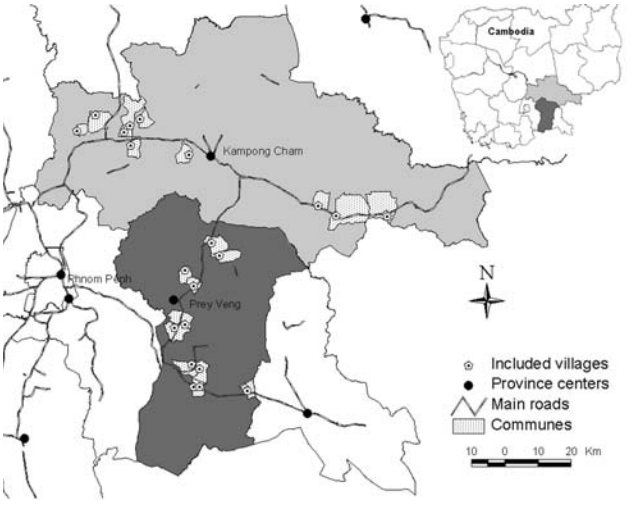

Figure 1. Distribution of selected communes in Kampong Cham and Prey Veng provinces, Cambodia, 2006.

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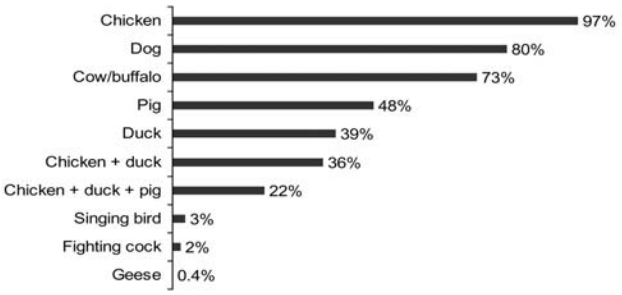

Figure 2. Proportions of animals raised in the household (n = 269), rural Cambodia.

however, only 18 (7%) reported these deaths to local authorities. Half of the respondents (n = 231) believed that it was important to report any poultry deaths because the death may be due to AI (61%) or because the poultry owners may receive management advice from the village veterinarians (39%). Among these 231 respondents, many did not report poultry deaths because they did not know how (41%), were in the habit of not reporting poultry deaths (31%), believed they would have a problem selling poultry if they reported deaths (18%), did not know the risks of AI (7%), or feared poultry culling (5%). Among those respondents who did not believe reporting deaths was important, the reasons provided included the following: "the number of poultry deaths were too few" (62%), "poultry are not as important as cattle" (18%), "no help would be provided from veterinary staff or authorities" (13%), or "because mortality was similar to previous years" (7%). Of respondents that experienced poultry deaths, 62% buried or burned dead poultry, 53% prepared them for food, 22% threw away the dead poultry, 3% used them to feed other animals, and 2% prepared them for sale or gave them to their neighbors.

Participants had learned about AI from television (81%) and radio (78%). Thirty-one percent of respondents were able to describe AI symptoms in humans, and 72% believed that AI is a fatal disease among poultry that can be transmitted to humans. Most respondents believed it is unsafe to touch sick or dead poultry with bare hands (67%), eat wild birds (70%), let children touch sick or dead birds with bare hands (83%), and eat meat or eggs that are not fully cooked (86%). Sixty-one percent of respondents mentioned at least 1 of the recommended behavioral practices that protect against AI infection.

Table. Poultry raising and flock characteristics, rural Cambodia

Flock characteristics	Chickens (n = 261)	Ducks (n = 97)
Median number per flock (range)	10 (1–110)	6 (1–800)
1–25	83%	93%
26–50	15%	3%
51–100	2%	0%
>100	0.4%	4%
Animal age when raising begins (months)	0 (0–15)	0 (0–12)
Type of raising		
Free ranging	100%	96%
Raising purpose (noncumulative)		
Household needs	73%	70%
Meat for sale	54%	42%
Eggs for sale	1%	16%

Conclusions

General media reports about AI through radio and television broadcasts appear to have been effective at reaching rural people. However, despite high awareness and widespread knowledge about AI and personal protection measures, most rural Cambodians still often practice at-risk poultry handling. Anecdotally, we also reported that family members of H5N1-infected patients, who knew about AI risks, still prepared dead or sick poultry for household consumption during massive die-offs, because they observed that neighbors with the same behavior did not become sick (Institute Pasteur in Cambodia, unpub. data). These findings provide evidence that high awareness does not necessary lead to behavior change. Behavior change involves comprehensive and multidisciplinary intervention, which combines risk perception communication and feasible and practical recommendations, including economic considerations. We speculate that it is hardly feasible to sustain good poultry-handling practices if access to personal protective equipment is cost prohibitive, particularly when disease occurrence poultry die-offs are common. Further studies are needed to determine appropriate behavior change strategies in Cambodia.

We did find that many of the villagers were willing to report poultry deaths but did not know how. However, this finding should be interpreted in light of some limitations. We observed difficulties and frustrations among farmers whose flocks underwent culling after identification of H5N1 viruses in their flocks because compensation has not yet been approved by the government of Cambodia. In contrast, Thailand and Vietnam have introduced compensation along with the introduction of poultry vaccination in Vietnam and the reduction of backyard poultry ownership in Thailand in an effort to protect the commercial poultry industry. Thus, it is difficult to envision effective control strategies in Cambodia based exclusively on culling. Coincidentally, Vietnam has reported far fewer H5N1 outbreaks in poultry and humans since the introduction of the vaccination program, while Cambodia detected 4 outbreak sites in domestic poultry and 2 unrelated human cases in 2006. The real effect of a no-compensation policy on willingness to report poultry deaths needs to be assessed.

Not surprisingly, direct contact with poultry and poultry products was common among household members. Transmission of H5N1 from poultry to humans, even in circumstances in which human–poultry interactions are regular and intense has been limited; however, as the virus continue to circulate and evolve among poultry, bird-to-human transmission may increase. In this context, improvement in risky practices can only be achieved through relentless behavior change efforts. Because lack of knowledge does not appear to be a factor, intervention programs must include feasible options for resource-poor settings that have limited materials for personal protection (water, soap, rubber gloves, masks) and must offer farmers alternative methods to safely work with poultry on a daily basis.

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Sapovirus in Water, Japan

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Sapoviruses are etiologic agents of human gastroenteritis. We detected sapovirus in untreated wastewater, treated wastewater, and a river in Japan. A total of 7 of 69 water samples were positive by reverse transcription–PCR. Phylogenetic analysis of the viral capsid gene grouped these strains into 4 genetic clusters.

The family *Caliciviridae* contains 4 genera, *Sapovirus*, *Norovirus*, *Lagovirus*, and *Vesivirus*, which include sapovirus (SaV), norovirus (NoV), rabbit hemorrhagic disease virus, and feline calicivirus strains, respectively. SaV and NoV are agents of human gastroenteritis. The most widely used method of detection is reverse transcription–PCR (RT-PCR), which has a high sensitivity and can also be used for genetic analysis. Only a limited number of SaV studies have been conducted, although most studies have shown that SaV infections are more frequent in young children than in adults and that nearly all children are infected by 5 years of age.

NoVs have been detected in oysters (and other shellfish), water from drinking fountains, ice, and community drinking water (*1–4*). Environmental studies of SaV have not been conducted. SaV strains can be divided into 5 genogroups (GI–GV), among which GI, GII, GIV, and GV infect humans; GIII infects porcine species. Phylogenetic studies have also designated SaV clusters or genotypes to further describe strains that differ by ≈10% in nucleotide or amino acid sequences. The purpose of this study was to identify and describe SaV strains in environmental samples, namely, untreated wastewater, treated wastewater, a river, and seawater, in Japan.

The Study

Water samples were obtained at different locations once a month in Miyagi Prefecture, Japan, from March 14, 2004, through February 16, 2005 (*5*). A total of 69 samples were obtained, which included 12 untreated wastewater samples, 12 treated wastewater samples, 23 river samples (2 different locations), and 22 seawater samples (2 different locations) (Figure 1). Untreated wastewater and treated wastewater were obtained from a wastewater treatment plant that processes domestic wastewater from residents living in a nearby city (Matsushima City). The treated wastewater is chlorinated at the wastewater treatment plant and then discharged into the Takagi River. The river runs directly into Matsushima Bay and then into the Pacific Ocean. River water was obtained from 2 locations upstream from the wastewater treatment plant, and seawater was obtained from 2 locations outside Matsushima Bay in the Pacific Ocean.

The methods of viral concentration were different for each location, as previously described (*5*). For untreated wastewater, 1 L was centrifuged for 15 min at 9,000 × *g* and concentrated with polyethylene glycol (resuspended in 4 mL distilled water). For treated wastewater and river water, 1 L was directly concentrated with polyethylene glycol. For seawater, 10 L was filtered, viruses were absorbed to a filter (type HA negatively charged membrane with a 0.45-μm pore size, Nihon Millipore, Tokyo, Japan) and eluted in 40 mL alkali buffer, and 40 mL buffer was further concentrated by ultracentrifugation to give a final volume of 500 μL (*6*).

RNA was extracted as previously described (*7*). Nested RT-PCR was used to detect all human genogroups (*8*). For the first PCR, primers F13, F14, R13, and R14 were used. For the nested PCR, primers F22 and R2 were used. All RT-PCR products were analyzed by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. RT-PCR products were excised from

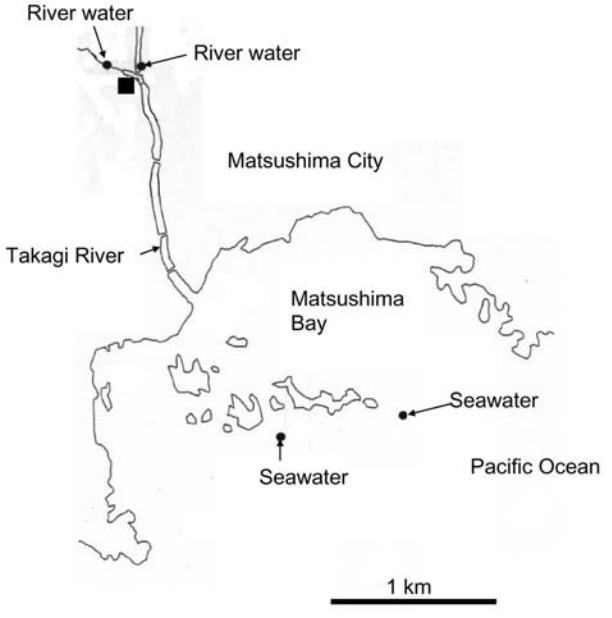

Figure 1. Locations in Miyagi Prefecture, Japan, from which water was isolated. The solid square shows the location of the wastewater treatment plant (sampling site of untreated and treated wastewater).

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the gel and purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). Nucleotide sequences were determined with the terminator cycle sequence kit (version 3.1) and the ABI 3130 Avant sequencer (PerkinElmer Biosystems, Wellesley, MA, USA). Sequences were aligned with Clustal X (*9*), and distances were calculated by using the Kimura 2-parameter method as previously described (*10*). Nucleotide sequence data from this study have been deposited in GenBank under accession nos. DQ915088–DQ915094.

SaV was detected in 7 (10%) of 69 concentrated water samples. Negative controls were included in the RT-PCR and showed negative results (data not shown). Genetic analysis of the positive samples showed 4 distinct genetic clusters, which included 3 GI clusters and 1 GV cluster (Figure 2). Three GI sequences were identical (strains 16, 24, and 42), 2 of which were obtained from treated wastewater 3 months apart (strains 24 and 42), and 1 was obtained from the river water (strain 16). The other 2 GI sequences grouped into 2 different clusters (strains 29 and 64) and were isolated from untreated wastewater. The 2 GV sequences were identical (strains 5 and 6). These 2 GV-positive samples were obtained on the same day, although they were obtained from different locations, i.e., untreated wastewater and treated wastewater (Figure 1). Comparison of SaV sequences detected in this study with sequences in GenBank indicated that all 7 isolates closely matched previously reported SaV sequences (Figure 2). Positive SaV samples were obtained in both hot (summer) and cold (winter) months.

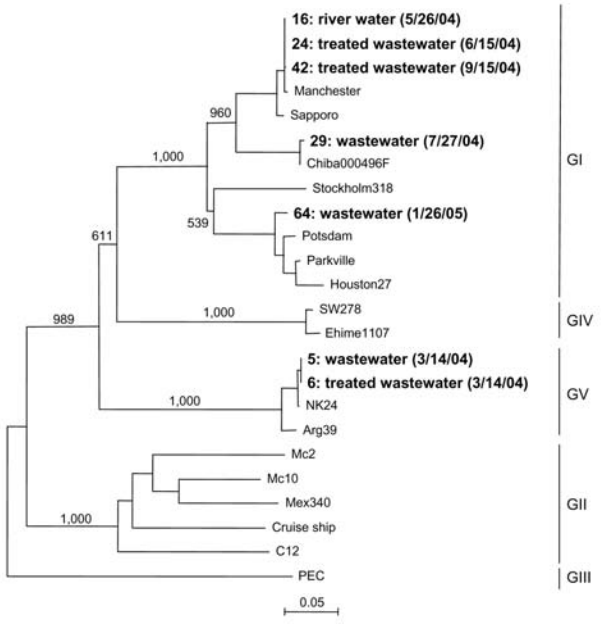

Figure 2. Phylogenetic analysis of sapovirus capsid nucleotide sequence showing different genogroups. Items in **boldface** are sequences isolated in this study and dates of isolation. Numbers on each branch indicate bootstrap values for the genotype. Bootstrap values ≥950 were considered statistically significant for the grouping. The scale bar represents nucleotide substitutions per site. Manchester, X86560; Sapporo, U65427; Chiba000496F, AJ412800; Stockholm318, AF194182; Potsdam, AF294739; Parkville, U73124; Houston27, U95644; SW278; DQ125333; Ehime1107, DQ058829; NK24, AY646856; Arg39, AY289803; Mc2, AY237419; Mc10, AY237420; Mex340, AF435812; cruise ship, AY289804; C12, AY603425; PEC, AF182760.

Conclusions

Human SaVs infections are being detected more often worldwide (*7*,*11*,*12*). These novel results have shown that like NoV (*5*), SaV can also be detected in water samples. Most sequences detected in water samples (5 of 7) belonged to GI. This genogroup likely represents the dominant genogroup worldwide (*7*,*10*,*13*). Two sequences (strains 5 and 6) belonged to GV, which has not yet been reported in Japan.

In a similar study, NoV was detected from water samples from the same research locations (*5*). Detection of SaV in river water samples upstream from the wastewater treatment plant suggests human fecal contamination in the river and that SaVs persist in freshwater. Screening for SaV may be worthwhile in oyster samples because NoVs were detected in oysters from local oyster farms (*5*). However, the failure to detect SaV in seawater samples may indicate that the sampling sites were not affected by human fecal contamination or that SaVs do not survive in marine waters. Nevertheless, further environmental studies are clearly needed to address this issue.

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Rabies Encephalitis in Malaria-Endemic Area, Malawi, Africa

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In a malaria-endemic area of Africa, rabies was an important cause of fatal central nervous system infection, responsible for 14 (10.5%) of 133 cases. Four patients had unusual clinical manifestations, and rabies was only diagnosed postmortem. Three (11.5%) of 26 fatal cases originally attributed to cerebral malaria were due to rabies.

Rabies is a viral infection of the nervous system, caused principally by rabies virus (genus *Lyssavirus*, family *Rhabdoviridae*) and occasionally by other related enzootic viruses (*1*,*2*). After a brief nonspecific febrile prodrome, patients usually manifest either furious (hydrophobic) or paralytic rabies (*3*). The incidence of rabies in many parts of Africa is unknown, but rabies is probably underdiagnosed.

Cerebral malaria is a common cause of death in African children. It is diagnosed clinically in comatose patients with acute *Plasmodium falciparum* infection and no other apparent cause of reduced consciousness (*4*). However, asymptomatic *P. falciparum* infection is common, and other infections must be excluded before coma is attributed to the parasites alone. As part of a prospective clinical study of viral central nervous system (CNS) infections and cerebral malaria in Malawian children, we investigated fatal cases for rabies virus.

The Study

At the Queen Elizabeth Central Hospital, Blantyre, Malawi (an area hyperendemic for *P. falciparum* malaria), for 3 years beginning March 2002, we enrolled children (2 months to 15 years) with suspected CNS infection into a study. Suspected CNS infection was defined as a fever or history of fever and at least 1 of the following (*5*): reduced level of consciousness (Blantyre coma score [BCS] ≤4 [*6*] or for children ≥10 years of age, Glasgow coma score ≤14); neck stiffness; photophobia; Kernig sign; tense fontanelle; focal neurologic signs; convulsions. Children were excluded if they had a simple febrile convulsion (*7*) or acute bacterial meningitis (cerebrospinal fluid [CSF] leukocyte count ≥1,000 cells/μL or a positive Gram stain or bacterial culture).

A full history was obtained and detailed examination performed on admission and at least twice daily until discharge or death. Blood was taken for hematocrit determination, examination for asexual forms of *P. falciparum*, full blood count, blood cultures, biochemical screen, and viral serologic tests. On admission, a lumbar puncture was performed, and CSF was taken for cell count; differential cell count; protein and glucose concentrations; Gram stain; bacterial culture; viral PCR; and viral culture.

Patients with hydrophobia (defined as phobic generalized spasms and inspiratory spasms against a closed glottis in response to the offer of a glass of water) or aerophobia (a similar response to blowing air across the cheek) were defined clinically as having rabies encephalitis. Children with a CSF count of 5–1,000 leukocytes/μL or features of septicemia were treated with broad-spectrum antimicrobial agents. Children with malaria parasitemia were treated with parenteral quinine. For fatal cases, permission was sought for autopsy or supraorbital needle biopsy. Samples of human brain tissue were analyzed for rabies virus by using the fluorescent antibody test (*8*), the rabies tissue culture inoculation test, and the mouse inoculation test (*9*). We used reverse transcriptase–PCR to produce sequence data for phylogenetic analysis (*10*). The study was approved by ethics committees in Blantyre and Liverpool, and signed informed consent was obtained from patients' relatives.

During the 3 years, 1,183 children with suspected CNS infections were assessed; 394 with bacterial meningitis or simple febrile convulsions were excluded, which left 789 children in our study. Rabies encephalitis was diagnosed in 10 children (1.3%) on the basis of history of exposure to a rabid animal and initial clinical manifestations (Table). These included 2 children with *P. falciparum* parasitemia. A total of 779 children had no clinical features of furious rabies at the time of admission; 487 of these children had malaria parasitemia, including 341 with a BCS ≤2/5 who met the case definition of cerebral malaria. In total, 133 (16.9%) of 789 children died, including 58 (17%) of those with a diagnosis of cerebral malaria and all 10 children who had clinical features of rabies. Consent to perform autopsies or postmortem examination of needle-aspirate samples was requested from the relatives of 82 of

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the 133 children who died. Twenty-nine (35.4% of those requested) gave consent; 23 for autopsy and 6 for needle samples. This number included 26 children with a diagnosis of cerebral malaria, 2 with a clinical diagnosis of rabies, and 1 with suspected meningitis. Six of these 29 patients who died were positive for rabies virus, including the 2 with typical rabies manifestations (patients 9 and 10), 1 diagnosed with meningitis (patient 12), and 3 who had had a diagnosis of cerebral malaria (patients 5, 6, and 11). Thus, overall 14 (10.5%) of the 133 fatal cases were rabies: 10 diagnosed clinically and 4 that were not diagnosed until postmortem material was studied virologically. Three (11.5%) of 26 patients who died with a diagnosis of cerebral malaria and for whom postmortem material was

Table. Clinical and diagnostic features of 14 patients for whom the ultimate diagnosis was rabies encephalitis*

Patient no (sex/age,y)	Clinical features	History of animal exposure	Admission coma score†	Malaria slide‡	Clinical diagnosis	Time to death	Postmortem positive results
1 (F/13)	Fever and confusion for 2 d; convulsions, hypersalivation, hydrophobia, aerophobia	Uncertain	13/15	Neg	Rabies	24 h	ND
2 (M/13)	Hallucinations, confusion, for 2 d; thought had "been bewitched"; pyrexia, neck stiffness, drooling hydrophobia, aerophobia	Possible dog bite 6 mo earlier	12/15	Neg	Rabies	12 h	ND
3 (M/6)	Fever for 2 d, convulsions for 1 d; agitated, hydrophobia, aerophobia	Dog bite 3 mo earlier	3/5	Pos (1+)	Rabies	4 d	ND
4 (M/7)	Fever for 2 d, confusion, drooling, hydrophobia, aerophobia	Dog bite 3 mo earlier	4/5	Neg	Rabies	24 h	ND
5 (M/8)	Fever for 2 d, convulsions; confused; rapid deterioration	None§	4/5	Pos (2+)	Cerebral malaria	3 d	FAT, PCR, MIT
6 (M/7)	Headache, fever for 3 d, weak, confused; mild neck stiffness, reduced tone, reflexes; CSF 8 leukocytes /mm^3, protein 40 mg/dL, glucose 4.8 mmol/L; venous glucose 5.5 mmol/L; deteriorated over 10 d	Cat scratch 3 mo earlier	2/5	Pos (1+)	Cerebral malaria	10 d	FAT,¶ PCR, MIT
7 (M/6)	Fever for 1 d, convulsions; neck stiffness, hydrophobia, aerophobia	Dog bite 2 mo earlier	3/5	Pos (2+)	Rabies	<6 h	ND
8 (M/13)	Restlessness, hypersalivation, hematemesis for 1 d; confused, hydrophobia, aerophobia	Dog bite 3 mo earlier	13/15	Neg	Rabies	<6 h	ND
9 (F/11)	Fever, restlessness for 1 d; agitated, hydrophobia, aerophobia	Dog bite, 1 mo earlier	14/15	Neg	Rabies	4 d	FAT, PCR, MIT
10 (M/7)	Fever confusion for 1 d, hallucination, “bewitched”, hypersalivation, confusion, hydrophobia, aerophobia	Dog bite 2 mo earlier	1/5	Neg	Rabies	24 h	FAT, PCR, RTCIT
11 (F/6)	Fever convulsions for 1 d; status epilepticus, hypotonia, areflexia developed; diffuse slow waves on EEG	None§	1/5	Pos (2+)	Cerebral malaria	<6 h	PCR
12 (M/12)	Fell off bike, head injury, no loss of consciousness; ataxia and confusion developed; neck stiffness, fever; CSF 65 leukocytes/mm^3 (70% PMN cells) protein 30 mg/dL, glucose 4.2 mmol/L	None§	14/15	Neg	Meningitis	3 d	FAT, PCR, MIT
13 (M/7)	Fever for 2 d, convulsion, reduced conscious; agitated, convulsions, hydrophobia, aerophobia	Dog bite 6 wk earlier	2/5	Neg	Rabies	24 h	ND
14 (M/6)	Fever for 2 d, vomiting 1 d, no convulsions; confused, hydrophobia, aerophobia	Dog bite 2 mo earlier	4/5	Neg	Rabies	24 h	ND

*Neg, negative; Pos, positive; ND, not done; FAT, fluorescent antibody test; PCR, reverse transcriptase–PCR; MIT, mouse inoculation test; CSF, cerebrospinal fluid; RTCIT, rabies tissue culture inoculation test; EEG, electroencephalogram; PMN, polymorphonuclear.

†Glasgow coma score /15 or Blantyre coma score /5.

‡*Plasmodium falciparum* parasitemia, graded according to the number of parasitized red blood cells per high-powered field (HPF) 1+= 1–100/100 HPF, 2+ = 1–9/10 HPF, 3+ = 1–9 per field, 4+ = >10/HPF) (*11*).

§For patients 5, 11, and 12, a possible exposure history was elicited after the diagnosis of rabies encephalitis became apparent. Patient 5 had been scratched by a dog 6 wks earlier, but it had not appeared rabid; patient 11 had been bitten by a neighbor’s dog 6 mo before admission, although this animal had been vaccinated against rabies, and remained well; patient 12 had been bitten by a neighbor’s dog 4 mo earlier, the dog remained well, although another dog had died after apparently “choking on a rat” (rabid dogs often appear to have something stuck in their throat).

¶Patient 6 CSF was negative for rabies virus by FAT at the Veterinary Laboratories Agency Weybridge but positive when tested in Malawi.

examined actually had rabies encephalitis. Although the initial clinical manifestations of these 3 children were indistinguishable from those of children with cerebral malaria, histologic examination showed that none of these 3 patients had sequestration of parasitized erythrocytes in cerebral tissue (the pathologic hallmark of cerebral malaria). None of these children, nor the 1 with a diagnosis of meningitis, had hydrophobia or aerophobia, which are characteristic of furious rabies; nor did they have limb pain or paresthesia, which are often reported. The disease progression did have unusual features, however. Patient 6 became comatose, flaccid, and areflexic. Patient 11 had periodic episodes of limb shaking; an electroencephalogram during these events showed generalized slow waves but no seizure activity. Patient 12 behaved oddly when a bag of intravenous fluid was set up, but he was not hydrophobic and readily drank water. None of these 4 children had an obvious history of exposure to a rabid animal, although detailed questioning after admission showed possible exposures (Table).

To determine whether the virus isolates in our study were genotype 1 rabies virus, representative of viruses circulating locally, or from other lyssavirus genotypes that also circulate in Africa (e.g., Lagos bat virus genotype 2 or Duvenhage virus genotype 4), we performed a phylogenetic analysis. This analysis included our clinical isolates, rabies viruses we isolated from rabid animals during the course of the study, and other representative strains. We generated consensus trees and bootstrap values as described previously (*12*) and visualized phylogenetic trees with Treeview version 3.2 (Figure). This analysis showed that all of our clinical isolates were African genotype 1 rabies viruses and were closely related to a virus isolated from a rabid dog during the study.

Conclusions

This study showed that rabies encephalitis is an important cause of death among children in Malawi. Overall 14 (10.5%) of 133 children who died from suspected CNS infections had rabies. For 10 of these children with hydrophobia, aerophobia, or both, rabies was diagnosed clinically (2 of the cases were also confirmed virologically), but rabies in 4 children with no hydrophobia or aerophobia was not diagnosed until brain material was examined. In 3 of these 4 children, cerebral malaria had been diagnosed. Hypotonia and areflexia developed in 2 children, which might have been a clue that rabies was involved, but these conditions have also been reported in cerebral malaria (*14*). Whether coinfection with malaria parasites might have affected the initial clinical manifestations of rabies is not certain. Overall ≈11% of deaths initially attributed to cerebral malaria were actually due to rabies virus.

Ours was a hospital-based study. The number of rabies cases in the community is likely to be much higher. In some parts of Africa, up to 100 rabies cases are estimated

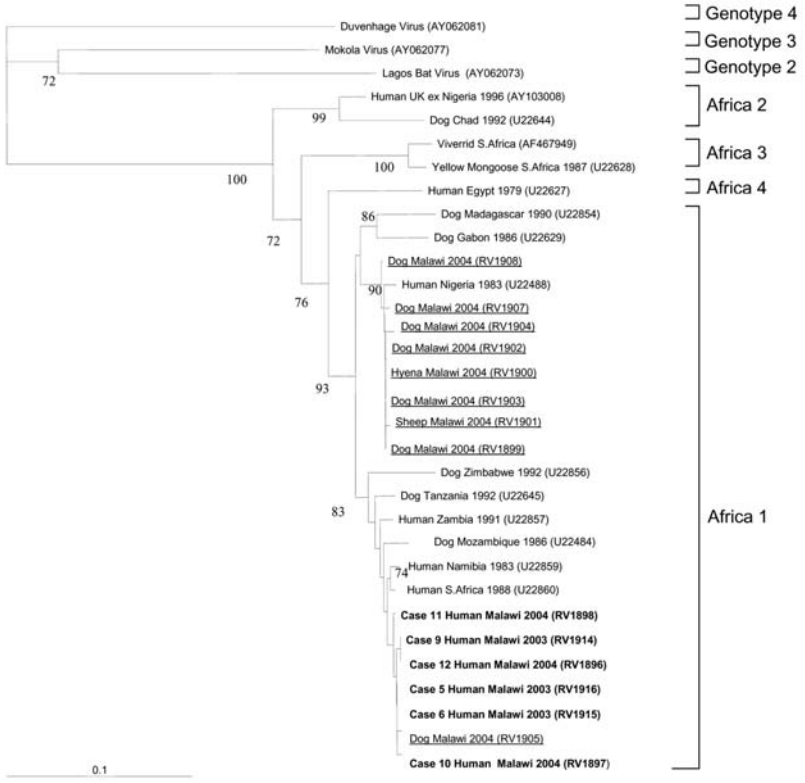

Figure. Phylogenetic tree, based on 400 nucleotides of the nucleoprotein gene (10), showing the relationship between rabies virus isolates in this study from humans (in **boldface**) and animals (underlined), and other representative isolates; GenBank accession nos. are in parentheses. Bootstrap values ≥70% are considered significant and are included within the figure. The Africa 1, 2, 3, and 4 genotypes are within rabies virus genotype 1 (*13*). For a larger representation of this image, see www.cdc.gov/ncidod/EID/13/1/136-G.htm

to occur for each 1 officially reported (*15*). Although rabies is a notifiable disease in Malawi, national reporting is hindered by difficulties with the system and the lack of diagnostic facilities. Rabies is preventable by treatment with rabies vaccine after exposure to the virus, and in Malawi several hundred to several thousand people are treated annually with purified chick embryo vaccine. However, in many areas the vaccine is not available, and it was not given to any of our patients, even though several did seek treatment after a dog bite.

In summary, rabies is an important cause of death in children in Malawi, including some for whom cerebral malaria had been diagnosed. Although these patients had unusual disease progression for cerebral malaria, only by examining postmortem material was the diagnosis of rabies made. Rabies virus should be included in the list of pathogens to consider before diagnosing cerebral malaria, especially in fatal cases with hypotonia and areflexia. Attempts should be made to get tissue for diagnosis, e.g., a nuchal skin biopsy, saliva, or CSF antemortem, or a brain sample postmortem.

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Death Rates from Malaria Epidemics, Burundi and Ethiopia

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Death rates exceeded emergency thresholds at 4 sites during epidemics of *Plasmodium falciparum* malaria in Burundi (2000–2001) and in Ethiopia (2003–2004). Deaths likely from malaria ranged from 1,000 to 8,900, depending on site, and accounted for 52% to 78% of total deaths. Earlier detection of malaria and better case management are needed.

Plasmodium falciparum malaria epidemics are poorly documented, partly because they occur in remote, underresourced areas where proper data collection is difficult. Although the public health problems from these epidemics are well recognized (*1*,*2*), quantitative evidence of their effect on death rates is scarce (*3*). Hospital-based death data, when available, provide a grossly incomplete picture because most malaria patients do not seek healthcare and, thus, these cases are not reported (*4*). Thus, current estimates (*2*) rely on extrapolations of limited site-specific or empirical observations. Accurate information is needed not only to improve our knowledge of malaria epidemics, but also to assess progress of malaria control initiatives that aim to decrease deaths from malaria worldwide by 50% by 2010 (*5*). We report community-based death rates from 2 *P. falciparum* malaria epidemics (Burundi, 2000–2001; Ethiopia, 2003–2004) in which Médecins Sans Frontières intervened.

Detailed information about these epidemics, their determinants, and their evolution is provided elsewhere (*6*). Briefly, the inhabitants of the Kayanza, Karuzi, and Ngozi provinces (population 1,415,900) of Burundi, which borders Rwanda, live in small farming villages, most at an altitude >1,500 m. Before the 2000–2001 epidemic, these areas were considered to have low malaria transmission. Rapid surveys of febrile outpatients confirmed the epidemic (>75% had *P. falciparum* infections; Médecins Sans Frontières, unpub. data). For all 3 provinces, 1,488,519 malaria cases were reported (attack rate 109.0%). Figure 1 shows the number of cases each month. In Kayanza, 462,454 cases were reported from September 2000 through May 2001 (attack rate 95.9%, average cases/month 51,383) (*7*); case counts peaked in January. In Karuzi, 625,751 cases were reported from October 2000 through March 2001 (attack rate 202.8%, average cases/month 10,429); case counts peaked in December (*7*). Ngozi reported 400,314 malaria cases from October 2000 through April 2001 (attack rate 67.7%, average cases/month 57,187); case count peaked in November (*7*).

Damot Gale district (286,600 inhabitants, altitude 1,600–2,100 m), considered a low-transmission area, is located in Wolayita Zone, Southern Nations Nationalities and Peoples Region, central Ethiopia**.** The malaria epidemic was confirmed locally by a sharp increase in *P. falciparum*–positive results among children treated in Médecins Sans Frontières feeding centers; the increase started in July 2003 (*6*). Reported caseload decreased in August and September, probably because of drug shortages and subsequent untreated and unreported patients; caseload rose sharply in October, November, and December (Figure 2). During these 3 months in 2003, 10,308 cases were reported by the 8 district health facilities (attack rate 3.6%, average no. cases/month 3,436), more than 10-fold the corresponding total in 2002 (n = 744) (Médecins Sans Frontières, unpub. data).

The Study

During the epidemics, a retrospective survey of deaths was conducted at each site. Surveys were approved by local authorities, and respondents gave oral consent. Thirty clusters of 30 households were selected by using 2- or 3-stage sampling (*8*). Households were defined as groups of persons who slept under the same roof under 1 family head at the time of the survey; occasional visitors were excluded. Selection within each cluster followed a standard rule of proximity (*9*). Information collected included number, age, and sex of persons living in the household; number of deaths (age, sex, and date of death) since the beginning of the recall period; and cause of death. Malaria was defined as the probable cause if a decedent's household reported "presence of fever" (Burundi) or "fever and shivering without severe diarrhea or severe respiratory infection" (Ethiopia). Recall periods were defined by easily recognizable starting dates (Table 1).

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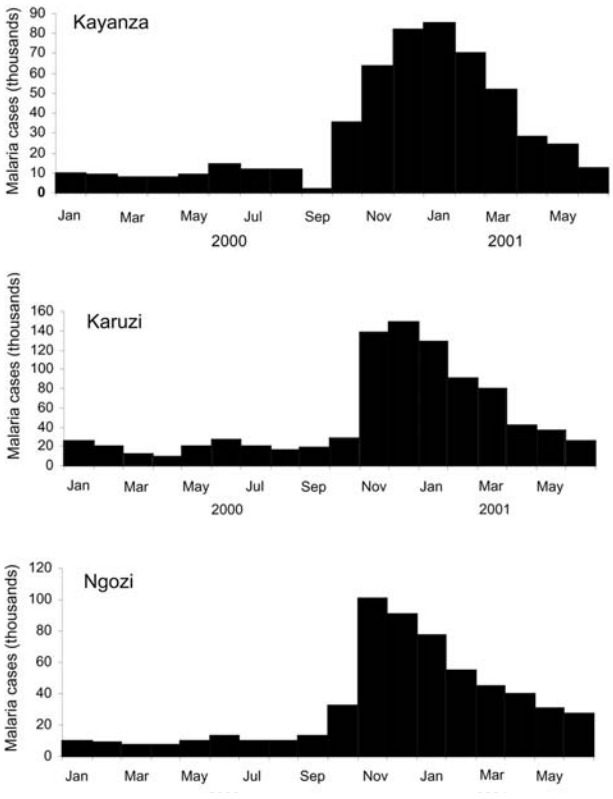

Figure 1. Clinical cases of malaria reported from Kayanza, Karuzi, and Ngozi provinces (Burundi), January 2000–June 2001.

Data were analyzed by using EpiInfo (Centers for Disease Control and Prevention, Atlanta, GA, USA). Death rates were expressed as deaths/10,000 persons/day, and 95% confidence intervals (CIs) were adjusted for design effects. Mortality rates were compared with standard emergency thresholds of 1 death/10,000/day (crude mortality rate [CMR]) and 2 deaths/10,000/day (under 5 mortality rate [U5MR]) (*10*). Excess number of deaths probably due to malaria was estimated by applying the specific death rates due to self-reported malaria to the population and time period covered by each survey.

CMR and U5MR exceeded respective emergency thresholds (Table 1). In the total population, proportion of deaths probably due to malaria varied from 51.7% (Karuzi) to 78.3% (Kayanza) and from 53.0% (Ngozi) to 82.3% (Damot Gale) for children <5 years of age (Table 1).

Deaths probably due to malaria ranged from 1,000 in Kayanza to 8,900 in Ngozi; >50% were among children <5 years (Table 2). Estimates reflect only portions of the epidemic periods (Table 2). When surveys covered most of the epidemic duration (74% in Ngozi, 85% in Karuzi, 83% in Damot Gale), malaria was the probable cause of death for a comparable proportion of the population (1.5% [8,900/574,400] in Ngozi, 0.9% [2,800/308,400] in Karuzi, and 1.9% [5,400/286,600] in Damot Gale).

Conclusions

We provide novel data based on representative population sampling, rather than health facility–based reporting. *P. falciparum* epidemics seem responsible for high death rates: the estimated number of deaths probably due to malaria at our sites (≈18,000) represents about 10% of the worldwide total estimated annual deaths due to epidemic malaria (*2*).

The limitations of retrospective mortality surveys are well known (*11*); hence, results should be interpreted with caution. Reporting bias was minimized by defining a limited recall period and by training interviewers extensively. In Kayanza, the survey was conducted before the epidemic peak; the estimated death rate may have been lower than average for the entire epidemic, which may have led to underestimation of the true death rate. Generally, postmortem diagnosis of malaria at the household level is difficult, and even advanced verbal autopsy techniques (not used in these surveys due to lack of skilled human resources) are of limited accuracy (*12*). Decedents' next of kin may underreport or overreport certain signs and symptoms. Malaria deaths may thus have been overestimated, particularly in Burundi, where fever was the sole criterion of probable malaria; use of this 1 criterion may have masked other causes, such as acute respiratory infection. Furthermore, in 3 of the areas surveyed (Kayanza excepted), the epidemics occurred concurrently with nutritional crises. Malnutrition as a cause of death could not be assessed because of its implication in various infectious diseases, but high prevalence of malnutrition is usually associated with excess U5MR (*13*). Nevertheless, mortality rates among persons ≥5 years of age (CMR – [U5MR × proportion of children <5 years in survey sample]/[1 – proportion of children <5 years in survey sample]) were also elevated. Rates ranged from 0.5 in Kayanza to 1.7 in Damot Gale, higher than the expected rate of 0.27 in sub-Saharan Africa (*14*). In the absence of other specific causes of acute death for adults, we speculate that malaria was largely responsible for these excess deaths.

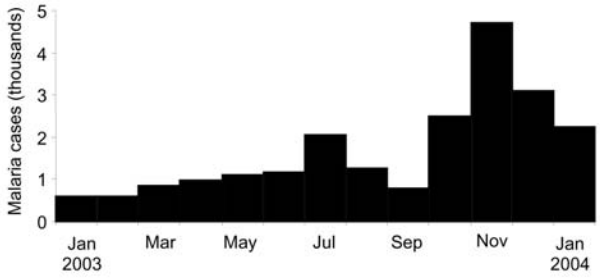

Figure 2. Clinical cases of malaria reported from Damot Gale health facilities (Ethiopia), January 2003–January 2004.

Table 1. Results of retrospective surveys of deaths in Burundi (2000–2001) and Ethiopia (2003–2004)*

	Burundi			Ethiopia
	Kayanza	Ngozi	Karuzi	Damot Gale
Dates of survey	December 2000	February 2001	March 2001	January 2004
Beginning of recall period	Anniversary of Prince Rwagasore murder†	4 wk before anniversary of Prince Rwagasore murder	Anniversary of Prince Rwagasore murder	Last Meskal‡
Recall period, d	53	155	153	125
No. persons in sample	4,308	3,639	4,925	5,619
No. children <5 y in sample (%)	729 (16.9)	639 (17.5)	961 (19.5)	1,167 (20.8)
No. deaths during recall period	23	100	87	148
No. deaths, children <5 y	14	51	45	51
CMR (95% CI)	1.0 (0.6–1.7)	1.8 (1.3–2.3)	1.1 (0.9–1.5)	2.1 (1.5–2.9)
U5MR (95% CI)	3.6 (1.7 - 7.2)	5.0 (3.3–7.4)	3.0 (2.0–4.4)	3.4 (2.3–5.1)
No. deaths probably due to malaria (%)	18 (78.3)	58 (58.0)	45 (51.7)	106 (66.0)
No. deaths probably due to malaria, children <5 y (%)	9 (64.3)	27 (53.0)	24 (53.3)	42 (82.3)
Probable malaria-specific mortality rate (95% CI)	0.8 (0.4–1.5)	1.0 (0.7–1.5)	0.6 (0.4–0.8)	1.5 (1.0–2.1)
Probable malaria-specific mortality rate, children <5 y (95% CI)	2.3 (0.9–5.4)	2.6 (1.5–4.6)	1.6 (0.9–2.8)	2.8 (1.9–4.1)

*CMR, crude mortality rate (deaths/10,000/d); CI, confidence interval; U5MR, mortality rate for children <5 y of age (deaths/10,000/d).
†October 13, 2000.
‡September 28, 2003.

At all sites, early warning systems were not operational and surveillance was ineffective, which led to substantial delays in epidemic detection (*6*). First-line treatment regimens (chloroquine in Burundi, sulfadoxine/pyrimethamine in Ethiopia) were not very effective. In Damot Gale, access to treatment was poor (data not shown), probably due to the dearth of health facilities. All these factors may have exacerbated the epidemics and contributed to excessive death rates.

Early diagnosis and prompt treatment of malaria remain cornerstones of the global malaria control strategy (*15*). The degree to which these interventions will be made available will largely determine the death rates in future epidemics.

Acknowledgments

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Each survey was supervised by an Epicentre epidemiologist. The work was done in collaboration with National Ministries of Health, which authorized inspection of records and provided the necessary information when appropriate.

All surveys, as well as this review, were financed by Médecins Sans Frontières.

Dr Guthmann is a physician and senior epidemiologist who has worked at Epicentre since January 2000. Although his main interest is the epidemiology of malaria, he has also conducted research on other topics such as leishmaniasis and measles.

Table 2. Estimated number of deaths, total population and children <5 years of age, Burundi (2000–2001) and Ethiopia (2003–2004) malaria epidemics*

	Burundi			Ethiopia
	Kayanza	Ngozi	Karuzi	Damot Gale
Estimated source population (total)	246,500	574,400	308,400	286,600
Estimated no. children <5 y†	41,900 (17%)	103,400 (18%)	61,100 (19.8%)	59,900 (20.9%)
Recall period, d (approximate proportion of entire epidemic period)	53 (20%)	155 (74%)	153 (85%)	125 (83%)
Estimated no. deaths, all causes (95% CI)	1,300 (800–2200)	16,000 (11,600–20,500)	5,200 (4,700–7,100)	7,500 (5,400–10,400)
Estimated no. deaths, all causes, children < 5 y (95% CI)	800 (400–1,600)	8,000 (5,300–11,900)	2,800 (1,900–4,100)	2,500 1,700–3,800)
Estimated no. deaths probably due to malaria (95% CI)	1,000 (500–,2,000)	8,900 (6,200–13,400)	2,800 (1,900–3,800)	5,400 (3,600–7,500)
Estimated no. deaths probably due to malaria, children <5 y (95% CI)	500 (200–1,200)	4,200 (2,400–7,400)	1,500 (800–2,600)	2,100 (1,400–3,100)

*CI, confidence interval.
†Using estimates from respective surveys, the mid-period proportion of children <5 years was calculated as follows: (children alive at end of period + [0.5 × deaths for children <5 y during period])/(all persons alive at end of period + [0.5 × all deaths during period]).

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Gastroenteritis Caused by Norovirus GGII.4, the Netherlands, 1994–2005

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From 1994 through 2005, gastroenteritis outbreaks caused by norovirus generally increased in the Netherlands, with 3 epidemic seasons associated with new GGII.4 strains. Increased percentages of GGII.4 strains during these epidemics, followed by a sharp decrease in their absolute and relative numbers, suggest development of immunity.

Noroviruses (NoVs) cause large outbreaks of gastroenteritis in settings of close human contact such as hospitals, institutions, military bases, and cruise ships, as well as sporadic cases. In recent years, human NoVs have increasingly been recognized as a common cause of gastroenteritis. Since the introduction of rapid molecular detection techniques, a high proportion of acute gastroenteritis outbreaks have been attributed to NoVs (*1–3*). We describe trends in occurrence of NoV in the Netherlands, with a focus on the predominant GGII.4 strains.

The Study

Surveillance of viral gastroenteritis outbreaks was initiated just over a decade ago at the National Institute for Public Health and the Environment in the Netherlands (RIVM). Outbreaks were typically reported by the municipal health service or the food inspection services to the RIVM, and samples were collected in close collaboration with these agencies. To determine the role of NoV and possible differences between different NoV strains in gastroenteritis outbreaks, a minimal set of epidemiologic data (setting, date of onset, number of persons affected, most probable mode of transmission, and number of hospitalizations) was collected for reported outbreaks. These data were supplemented with results of molecular biologic detection and typing techniques to enable more in-depth analysis of surveillance data.

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Preliminary typing of strains was performed by sequencing region A (280 nt in the polymerase gene) of the virus. A systematic selection of strains was also typed by sequencing region C (277 nt in the capsid gene) (*1*,*2*). Because NoV activity is much higher in winter months, seasons were analyzed from July through June, rather than per calendar year.

From December 18, 1993 to December 26, 2005, a total of 1,032 gastroenteritis outbreaks were reported to the RIVM. Samples from 942 outbreaks were received and analyzed. Of these, 695 (74%) outbreaks met our inclusion criteria for a NoV outbreak (≥25% of samples positive by reverse transcription–PCR). Overall, we observed an increasing trend in the number of reported outbreaks per year (Figure, panel A). In the 1995–96, 2001–02, 2002–03, and 2004–05 seasons, more NoV outbreaks were reported (66, 90, 154, and 161, respectively). GGII virus strains were predominant in all years and caused 577 (91%) of 631 outbreaks with known genotypes, compared with 36 (6%) of 631 outbreaks caused by GGI or GGIV viruses and 18 (3%) of 631 outbreaks caused by mixed infections with viruses of different genotypes (Table).

GGII.4 strains have been detected since 1995, with the highest proportions observed in years with high numbers of outbreaks. In the epidemic seasons of 1995–96, 2002–03, and 2004–05 the percentages of outbreaks caused by GGII.4 were 82%, 83%, and 89%, respectively, compared with an overall average of 68% (Figure). In seasons after these epidemics, the percentage caused by GGII.4 decreased to 39% in 1996–97, 55% in 2003–04, and 32% in the first half of 2005–06. Multiple NoV genotypes co-circulated throughout the years of the study, but in postepidemic years, outbreaks caused by non-GGII.4 strains were more common (Figure, panel C). The high number of outbreaks in 2001–02 may be partially explained by emergence of a new variant of GGII.4 in the spring of 2002 (*4*), which caused uncharacteristically high numbers of outbreaks between April and June. The epidemic increases in the number of outbreaks and seasonality of outbreaks were mainly attributable to GGII.4. Strains with genotypes other than GGII.4 were found at similar levels throughout the year (data not shown).

A total of 548 (79%) outbreaks were reported in healthcare settings (hospitals and residential institutions) compared with 102 (15%) in nonhealthcare settings; for 45 (6%) outbreaks no data were available (Table). A total of 407 (81%) of 502 outbreaks with genotyping information in healthcare settings were caused by GGII.4 NoV strains (Table). In the nonhealthcare settings, GGII.4 was significantly less prevalent (39 [43%] of 91, $p<0.0001$), resulting in a relative risk at least 2.17× higher for acquiring a GGII.4 infection in a healthcare setting than in other settings.

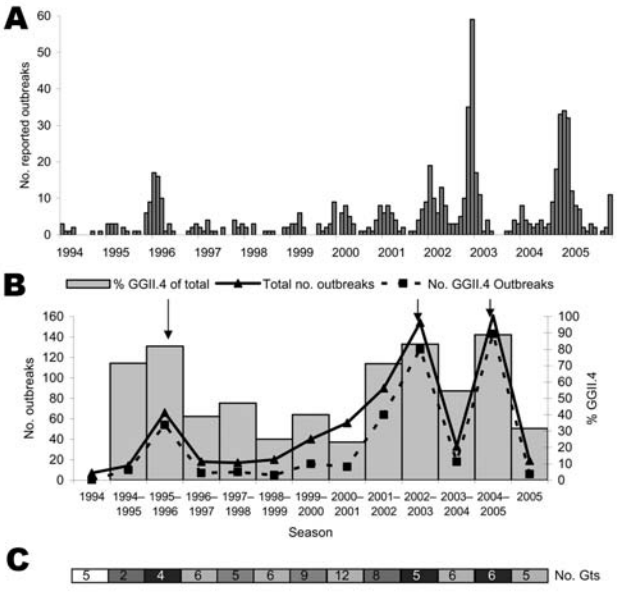

Figure. A) Number of norovirus outbreaks reported per month in the Netherlands, 1994–2005. B) Total no. of outbreaks per season and fraction of GGII.4 outbreaks reported in the Netherlands. Total no. is indicated by the solid line, no. of GGII.4 outbreaks by the dotted line (values on left y-axis), bars indicate percentage of GGII.4 outbreaks of the total no. (values on right y-axis), and arrows indicate epidemic seasons. Seasons run from July through June. C) Total no. of genotypes (Gts) circulating per season. Shading of the bar indicates the percentage of GGII.4, ranging from white (0%–20%), in steps of 20%, to black (80%–100%).

A mode of transmission was reported for 272 (39%) outbreaks. Data confirmed that the main transmission route was person to person (60%, 163/272) (Table) (*5*,*6*). GGII.4 strains were found in 73% of person-to-person outbreaks compared with 44% of food-related outbreaks ($p<0.0001$). When outbreaks for which no genotype was known were counted as non-GGII.4 strains, the relative risk of finding GGII.4 in an outbreak caused by person-to-person transmission was 2.3× greater than finding it in a foodborne outbreak or relative to other genotypes. Multiple GGII NoV strains were found in 18 outbreaks (3 foodborne, 1 waterborne, 4 person-to-person, and 10 with unknown modes of transmission).

Conclusions

Detailed molecular epidemiologic data from long-term surveillance on NoV outbreaks are rare because NoV molecular detection techniques became available only in the mid-1990s. In our 12-year surveillance study, we observed large differences in the magnitude of the annual winter peak of NoV infection. All epidemic peaks were associated with predominance of GGII.4 strains. Although this finding has been observed in studies covering a shorter period (*3*,*5*–*7*), our data suggest an increase in infections with GGII.4 in recent years, particularly in healthcare settings. However, the number of outbreaks reported in healthcare settings is likely overrepresented in our study because of mandatory reporting of illness in such settings. An actual increase in the number of outbreaks cannot be proven based on passive surveillance data alone, but an increase is strongly suggested by increased prevalence of GGII.4 in recent years and supported by reported shifts in the predominant GGII.4 variant associated with large numbers of outbreaks (*8*).

The overall dominance of GGII.4 suggests that this genotype is more transmissible than other genotypes in healthcare settings, where close contact of many persons favors person-to-person transmission. Transmission may also be affected by poorer hygiene or greater susceptibility to infection. Increased transmissibility could result from increased levels of shedding of GGII.4 or altered stability of virus particles outside the host compared with other genotypes. Alternatively, changes in circulating viruses may lead to differences in host cell binding or immune recognition, thereby changing the dynamics of infection or size of the population at risk.

Our group and other researchers have reported the emergence of distinct GGII.4 lineages in 1995–96, 2002, and 2004 (*2*,*4*,*9*,*10*). This suggests that the changing phenotype of GGII.4 strains results in increased numbers of outbreaks (*10*). A detailed characterization of GGII.4 strains is ongoing to determine the molecular mechanisms

Table. Outbreaks of gastroenteritis caused by noroviruses per mode of transmission and setting, the Netherlands, 1994–2005*

Genotype	Mode of transmission per genotype, no. (%)					Setting per genotype, no. (%)							
	MW	F	PTP	U/O	Total	T	S/D	R/C/C	H	PH	RI	U/O	Total
GGI + IV	1 (50)	5 (11)	7 (3)	23 (5)	36	3 (25)	3 (9)	9 (16)	4 (3)	2 (67)	10 (2)	5 (11)	36
GGII.4	0	18 (39)	163 (73)	291 (69)	472	7 (58)	11 (34)	20 (36)	91 (74)	1 (33)	316 (74)	26 (58)	472
GGIInon4	0	14 (30)	33 (15)	58 (14)	105	2 (2)	10 (31)	16 (29)	17 (14)	0	55 (13)	5 (11)	105
Mixed	1 (50)	3 (7)	4 (2)	10 (3)	18	0	4 (13)	2 (4)	3 (2)	0	6 (1)	3 (7)	18
Unknown	0	6 (13)	17 (8)	41 (10)	64	0	4 (13)	8 (15)	8 (7)	0	38 (9)	6 (13)	64
Total	2 (100)	46 (100)	224 (100)	423 (100)	695	12 (100)	32 (100)	55 (100)	123 (100)	3 (100)	425 (100)	45 (100)	695

*MW, mainly waterborne; F, foodborne; PTP, person-to-person; U/O, unknown or other; T, travel associated; S/D, school or daycare center; R/C/C, restaurant, canteen, or caterer; H, hospital; PH, private house; RI, residential institution.

involved in observed epidemiologic patterns. The marked decrease in the percentage of GGII.4 strains during seasons after epidemic seasons caused by variant strains suggests that populations may acquire immunity against these predominant strains.

The value of this surveillance dataset will increase with its continuation, as well as with its expansion as part of a European surveillance network (www.eufoodborne viruses.co.uk). Future research will be directed at understanding the molecular basis for observed changes in the epidemiology of NoV and control of its spread.

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Reemergence of Endemic Chikungunya, Malaysia

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Chikungunya virus infection recently reemerged in Malaysia after 7 years of nondetection. Genomic sequences of recovered isolates were highly similar to those of Malaysian isolates from the 1998 outbreak. The reemergence of the infection is not part of the epidemics in other Indian Ocean countries but raises the possibility that chikungunya virus is endemic in Malaysia.

Chikungunya, a mosquitoborne disease first described in Tanzania (formerly Tanganyika) in eastern Africa in 1952, is caused by chikungunya virus (CHIKV), an alphavirus belonging to the *Togaviridae* family. The disease occurs in Africa and various parts of Asia and is endemic in several southeast Asian countries, including Thailand, Indonesia, and the Philippines. Only 1 known outbreak has occurred in Malaysia, in 1998–1999 when ≥51 persons in Port Klang were infected (*1*).

From March through April 2006, an outbreak of CHIKV infection was reported in Bagan Panchor (4°31′N, 100°37′E), an isolated coastal town 50 km west of Ipoh, the state capital of Perak, in northwest Malaysia. At least 200 villagers were infected, with no deaths reported. This was the second known outbreak in Malaysia, 7 years after the previous one. This reemergence coincided with reports of ongoing epidemics of CHIKV infection in India and almost all the island nations of the Indian Ocean, with >200,000 cases in the French island of Reunion alone since February 2005 (*2*).

Why and how the recent infection reappeared in Malaysia remains unknown. The apparent absence of CHIKV for 7 years may be due to failure to detect low-level, continued transmission in humans, particularly because the symptoms may be mistaken for dengue fever. Alternatively, this outbreak could have originated from a viremic traveler from an endemic country (such as neighboring Thailand or Indonesia), but proximity of Malaysia to the Indian Ocean raises the possibility of an extension of the epidemic, with Malaysia being the furthest point yet of the expanding epidemic frontline.

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The Study

We received serum samples from 11 patients who had symptoms typical of CHIKV infection (Table). Samples were injected into Vero and C6/36 mosquito cells. Indirect immunofluorescence assays for immunoglobulin M (IgM) and IgG were performed using the patients' sera and CHIKV-infected cells fixed onto glass slides, as previously described (*1*). A CHIKV isolate (SM287) reported previously (*3*) was used to prepare the slides as a positive control for subsequent studies. Serum samples from patients who did not have symptoms of chikungunya, including patients with dengue fever, were used as negative controls. Nucleic acid amplification was performed using RNA extracted directly from the patients' sera or from cell cultures (Table). At least 3 different primer pairs specific for envelope glycoprotein E1 (E1), glycoprotein E2 (E2), and nonstructural protein 1 (nsP1) genes of CHIKV were used (*4*,*5*). Confirmation of the amplified DNA fragments was done by DNA sequencing. Phylogenetic relationships were examined using the E1, E2, and nsP1 gene sequences of the isolates and all other available CHIKV sequences obtained from GenBank or the previous studies (online Appendix Table, available from www.cdc.gov/ncidod/EID/13/1/147-appT.htm). Sequences were aligned and phylogenetic trees were drawn as previously described (*6*).

CHIKV infection was confirmed in 8 of 11 patients. CHIKV sequences were amplified directly from serum samples from 5 patients in the acute phase of disease. Of these, 4 CHIKV isolates were eventually cultured. IgM and IgG were detected in serum samples from 3 other patients in the convalescent phase (data not shown). In 1 patient, CHIKV sequences were amplified from serum samples obtained as late as 9 days after onset of symptoms (data not shown). The PCR amplification method, thus, could be useful for early detection of CHIKV infection in suspected outbreak situations.

The genomic sequence of the E1, E2, and nsP1 genes in the CHIKV isolates shared high similarity (>90%) to all the known CHIKV except West African CHIKV (≈86% similarity). The sequences were only ≈70% related to o'nyong-nyong virus, the most closely related alphavirus, which is present only in certain parts of Africa. Previous phylogenetic studies showed that CHIKV strains were clustered into 3 distinct groups based on origin from West Africa, Central/East Africa, or Asia (*7*–*13*). Phylogenetic trees drawn using E1 (Figure), E2, and nsP1 (data not shown) gene sequences clustered the recent Malaysian isolates into a group with other known CHIKV Asian isolates. The cluster, however, was distinctly separated (100% bootstrap support) from the African isolates and all the known isolates of the ongoing CHIKV epidemics of the Indian Ocean islands (*7*–*9*,*11*,*13*). This makes it unlikely that the outbreak in Malaysia is part of the ongoing epidemics,

Table. Identification of virus by PCR amplification and serologic analysis*

Patient		Chikungunya						Dengue fever	
		PCR†			Serology				Serology
Age (y)	Sex	E1	E2	nsP1	IgM	IgG	Culture	PCR‡	IgM
6	M	+	+	+	–	–	+§	–	–
34	M	+	+	+	–	–	+¶	–	–
40	M	+	+	+	–	–	+#	–	–
26	F	+	+	+	–	–	+**	ND	–
62	M	+	+	+	–	–	–††	ND	–
		(day 5 after onset)							
		–	–	–	+	+	ND	ND	ND
		(day 15 after onset)							

*IgM, immunoglobulin M; IgG, immunoglobulin G; +, positive; –, negative; ND, not determined.
†PCR amplifications were performed for detection of envelope glycoprotein E1 (E1), glycoprotein E2 (E2), and nonstructural protein 1 (nsP1) genes of chikungunya virus.
‡Multiplex PCR amplifications were performed for detection of dengue virus type 1–4.
§Isolate MY/0306/BP37348.
¶Isolate MY/0306/BP37350.
#Isolate MY/0306/BP37352.
**Isolate MY/0406/BP37437.
††Isolate MY/0306/BP34198.

despite its proximity to the region and timing of the outbreak. The phylogenetic tree, on the other hand, suggests that the isolates from the current Malaysia outbreak share a common ancestral lineage to the 2 Malaysian isolates recovered in 1998 (*4*; GenBank accession nos. AF394210 and AF394211) but have a slight genetic distance from all other Asian isolates.

Conclusions

On the basis of all available sequences of isolates from the neighboring countries where CHIKV is endemic, Thailand and Indonesia, the outbreak in Malaysia likely did not originate from either of these countries, which means the outbreak could have originated from an endemic CHIKV cycle not previously identified in Malaysia. A serologic survey of human serum samples collected during 1965–1969 in west Malaysia showed neutralizing antibodies to CHIKV among adults, especially those inhabiting the rural northern and eastern states bordering Thailand (*14*). The same authors also reported in an earlier study evidence of CHIKV-neutralizing antibodies in wild monkeys, a pig, and a chicken and suggested that a CHIKV sylvatic transmission cycle involving primates and possibly nonprimates exists in Malaysia. A sylvatic transmission cycle of the virus has been described in Africa and may play a role in the episodic emergence and reemergence of CHIKV infection (*15*). Before 1998, CHIKV had not been isolated from humans or animals in Malaysia, and no clinical disease caused by CHIKV had been reported. However, in the absence of active surveillance since the 1965 study, whether the apparent absence of CHIKV over the years and between the 2 recent outbreaks in Malaysia is due to an unidentified sylvatic transmission cycle or silent transmission among humans cannot be determined. Further investigation is required to examine these possibilities. Understanding this disease in Southeast Asia is critical because CHIKV shares the same mosquito vectors as dengue virus, which is endemic to the region.

Phylogenetic analysis showed that CHIKV from the recent 2006 outbreak in Malaysia is highly similar to isolates from the 1998 outbreak. At the 3 genes examined,

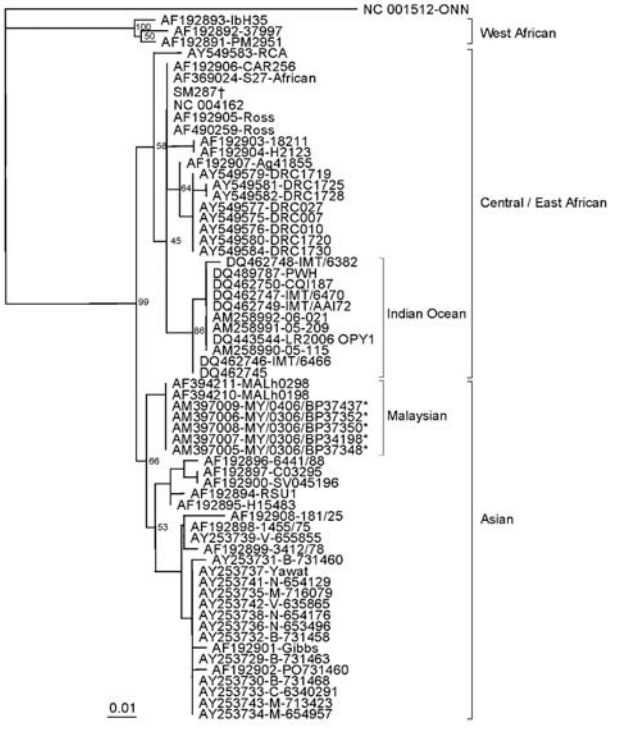

Figure. Phylogenetic relationships of chikungunya virus isolates from the 2006 Malaysia outbreak. The neighbor-joining tree was constructed using nucleic acid sequences of the envelope glycoprotein E1 gene, with o'nyong nyong virus (GenBank accession no. NC_001512) as the outgroup virus. * indicates isolates from the Malaysia 2006 outbreak; † indicates Australia SM287. Bootstrap values are shown as percentages derived from 1,000 samplings. The scale reflects the number of nucleotide substitutions per site along the branches.

the isolates differ from the ongoing Indian Ocean epidemic isolates and known isolates from Thailand and Indonesia. These findings support the possibility that the outbreak originated from an endemic infection in Malaysia.

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Human Subcutaneous Dirofilariasis, Russia

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We report 14 cases of human subcutaneous dirofilariasis caused by *Dirofilaria repens*, diagnosed from February 2003 through July 2004, in patients from Rostov-on-Don, Russia. Serologic analysis showed evidence of high risk of exposure to *D. repens*. Surveillance studies on prevalence and prevention effectiveness of canine infection are needed to control this emerging zoonosis.

Human subcutaneous dirofilariasis (HSD) is a zoonotic filariasis caused by infection with several species of worms belonging to the genus *Dirofilaria*; most documented cases are attributed to *Dirofilaria repens* (*1*). Dirofilarias are natural parasites of a great variety of animals and, with the exception of *D. immitis*, live in the subcutaneous tissue of their hosts, produce circulating microfilariae, and are transmitted by mosquitoes (*2*). The principal reservoir of *D. repens* is the dog. Humans are accidental hosts with patent infections being extremely rare. Differential diagnoses of HSD include neoplasia and other granulomatous diseases, and a definitive diagnosis usually requires surgical removal and examination of a granuloma.

Current epidemiologic studies indicate that human dirofilariasis is increasing in prevalence, and several authors have recently described it as an emerging disease in different areas of the world. Pampiglione et al. (*3*) reported 60 new cases in Italy. Eleven cases of subcutaneous dirofilariasis have been diagnosed near Moscow, Russia (*4*). Cases have been reported in Taiwan (*5*). A total of 48% of human dirofilariasis cases reported in France have been diagnosed in the previous 10 years (*6*). We report 14 confirmed cases of HSD diagnosed from February 2003 through July 2004 in patients from Rostov-on-Don in southeastern Russia, and serologic evidence of high risk of exposure to *D. repens* infection in the local population.

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The Study

Skin nodules were removed from 14 patients from February 2003 through July 2004. Eleven patients were female, and 3 were male (age range 23–66 years). Nodule localization included the head, trunk, inguinal area, and feet (Table). Nodules ranged from ≈4 mm to ≈2 cm and were examined by routine histologic analysis. In 1 case, genomic DNA was extracted from an intact worm excised from a nodule by using the NucleoSpin Tissue procedure (Macherey-Nagel, Düren, Germany). PCR was conducted according to the procedure of Favia et al. (*7*). Amplicons were visualized under a UV transilluminator after electrophoresis on 1.5% agarose gels and staining with ethidium bromide (0.5 μg/mL). Gels were scanned by using a digital photograph system (Gel Logic 100, Eastman Kodak, Rochester, NY, USA).

Serum samples were taken at the time of nodule excision surgery for 9 of the 14 patients. These samples were analyzed with an ELISA for antibody response to *D. repens* somatic antigen, as described (*8*). Briefly, 96-well microplates were coated with 0.6 μg of *D. repens* somatic antigen prepared according to the procedure of Prieto et al. (*9*). All serum samples were analyzed at a dilution of 1:30, and anti-human peroxidase-conjugated immunoglobulin G was used at a dilution of 1:4,000. Optical densities (ODs) were measured at 492 nm in an Easy Reader (Bio-Rad Laboratories, Hercules, CA, USA). Positive antibody response was defined as an OD value greater than the mean value ± 3 standard deviations for 14 serum samples from clinically healthy blood donors living in a *D. repens*–free area. A total of 317 serum samples from a random hospital population in Rostov were divided into categories on the basis of sex and age and analyzed by ELISA as described above.

Routine histologic analysis of all nodules showed an intense inflammatory granuloma around several cross-

Table. Sex, age, and location of nodules of 14 patients with subcutaneous dirofilariasis diagnosed in Rostov-on-Don, Russia, February 2003–July 2004

Patient	Sex	Age, y	Location of nodule
1	F	29	Temporal area
2	M	40	Left upper eyelid
3	F	61	Left groin
4	F	54	Left shoulder
5	F	43	Left shoulder
6	F	23	Oral cavity
7	M	38	Forehead
8	F	39	Left shoulder
9	F	43	Back
10	F	66	Left breast
11	F	43	Cheek
12	F	28	Temporal area
13	M	55	Left foot
14	F	35	Right foot

sections of filarial nematodes (Figure 1A). The average diameter of adult worms was ≈450 μm. These worms had 95 longitudinal ridges on the external cuticle, 2–5 chord nuclei per section, and robust muscle cells, all of which are characteristic features of *D. repens* (*10*). Results of PCR analysis of a worm excised from 1 of the nodules was specific for *D. repens* (Figure 1B). All 9 patients with HSD had significantly higher OD values for total serum immunoglobulin G against *D. repens* somatic antigen (p=0.001) than controls (Figure 2). Thirty-three (10.4%) serum samples from a random hospital population from the area had positive OD values for antibodies against *D. repens* somatic antigen. Prevalence of infection was higher in males (28/235, 12%) than in females (5/81, 6%) and in persons >60 years of age (25%) (data not shown).

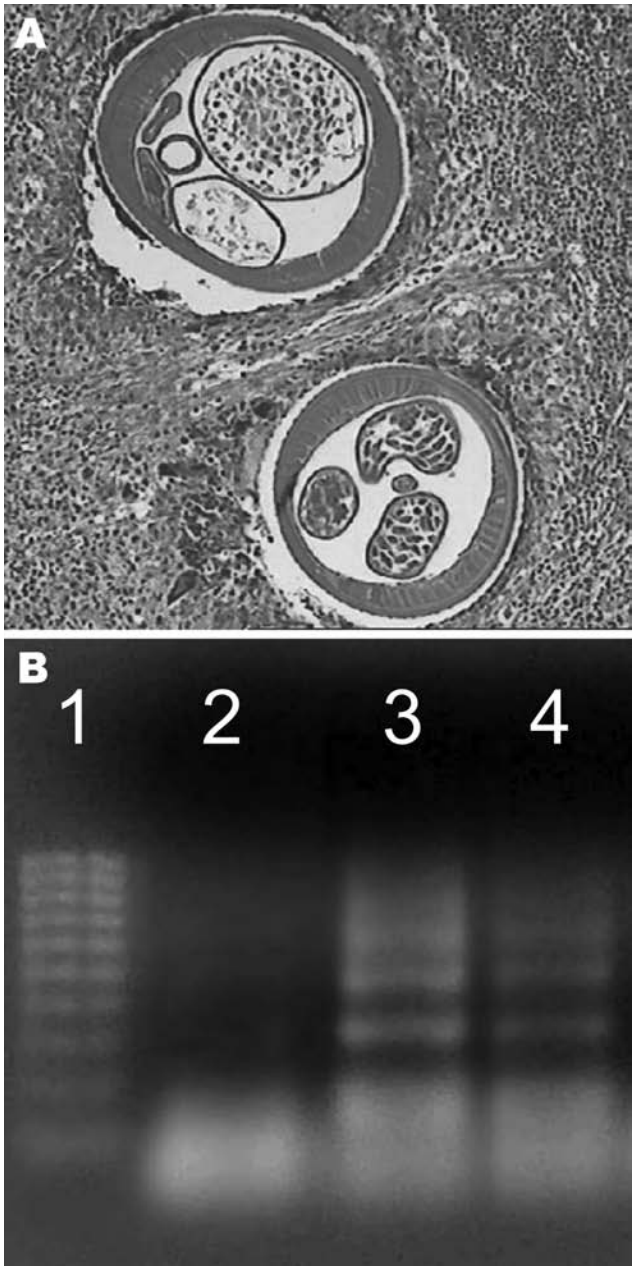

Figure 1. A) Histologic analysis of skin nodules caused by human subcutaneous dirofilariasis. Cross-sections of *Dirofilaria repens* surrounded by an inflammatory granuloma. Note the uteri with developing embryos (hematoxylin and eosin stain, original magnification 10×). B) Analysis of patient samples by agarose gel electrophoresis. Lane 1, 100-bp DNA molecular mass weight marker; lane 2, negative control; lane 3, positive control; lane 4, patient sample showing banding pattern typical of the positive control and similar to the banding pattern described in the original protocol (*7*).

Conclusions

The cases of HSD described were all diagnosed in patients who had never traveled outside the Rostov area. This is the highest number of cases of HSD diagnosed worldwide in such a short period. Histologic analysis and PCR indicate that *D. repens* is the causative agent of HSD in this area, and serologic analysis suggests that the risk for exposure is high.

Domestic and wild canids are definitive hosts of *D. repens*; the dog is the principal reservoir. No epidemiologic data are available on infection prevalence in dogs in southern Russia. In Piedmont, Italy, a region with a high incidence of human dirofilariasis, a survey of dogs conducted in 1966–1967 and repeated in 1991–1992 (*11*) showed a marked increase in the number of infected animals and size of the endemic area. Any increase in the population of vectors and infection of the reservoir may likely be associated with an increase in human dirofilariasis.

Information is also lacking on which mosquito vectors are involved in transmission of *D. repens* in the study area. In other geographic areas where human dirofilariasis is endemic, changes in climatic conditions (temperature, relative humidity, rainfall, rate of evaporation) favor the

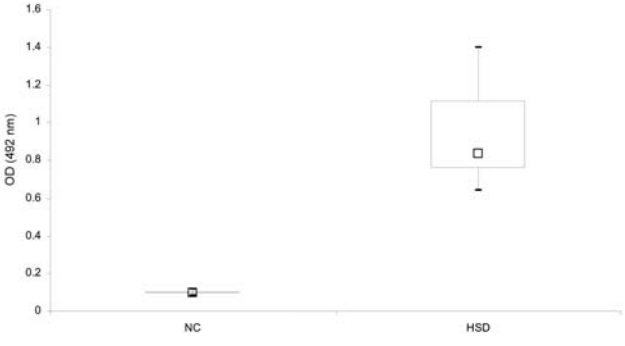

Figure 2. Serologic analysis (box and whisker plot) for antibodies to *Dirofilaria repens* somatic antigen from normal controls (NC) and patients with human subcutaneous dirofilariasis (HSD). The horizontal line shows the optical density (OD) values of 25%–75% of the examined sera. The large box shows OD values between the first and third quartiles, the small box shows the median, and error bars show maximum and minimum OD values. A positive antibody response was defined as an OD value greater than the mean value ±3 standard deviations from 14 normal controls.

development of vector mosquitoes (*12*) and of the larval phase of the nematode in the vector.

Medical awareness of infection risk is essential for a correct diagnosis, and the use of serologic analysis for *D. repens* somatic antigen merits further study as a diagnostic aid. Further monitoring of the HSD situation in this area is needed to establish guidelines for preventive measures, including effective chemoprophylaxis in animals.

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Alkhurma Hemorrhagic Fever Virus in *Ornithodoros savignyi* Ticks

Rémi N. Charrel,* Shamsudeen Fagbo,† Gregory Moureau,* Mohammad Hussain Alqahtani,† Sarah Temmam,* and Xavier de Lamballerie*

Evidence for the tickborne nature of Alkhurma hemorrhagic fever virus (AHFV) is indirect because AHFV has not been detected in arthropods. One *Ornithodoros savignyi* tick from Saudi Arabia contained AHFV RNA. This is the first direct evidence that AHFV is a tickborne flavivirus and confirms the association between human AHFV cases and tickbite history.

Alkhurma hemorrhagic fever virus (AHFV) is a recently described virus within the genus *Flavivirus*. AHFV was discovered in 1995 in a patient with hemorrhagic manifestations and fever in Saudi Arabia (*1*). Subsequently, ≈20 symptomatic patients infected with this virus have been documented by virus isolation in this country. The clinical picture is extremely severe and the case-fatality rate is >30%, which makes AHFV one of the most deadly flaviviruses (*2*). Previous studies have determined that AHFV is a variant genotype of *Kyasanur Forest disease virus*, another biosafety level (BSL) 4 virus that causes viral hemorrhagic fever in certain regions of India (*3*). Accordingly, AHFV is classified as a BSL-3 or BSL-4 agent, depending on country regulations.

To date, AHFV has been isolated only from human samples. Genetic and serologic characterization has grouped AHFV with tickborne flaviviruses (*1*,*3*). A previous study associated AHFV transmission to humans with butchering of sheep and camels (*1*). However, no direct evidence for its association with ticks, such as viral detection in or isolation from ticks, has been documented. To investigate the tickborne nature of AHFV, ticks were collected in western Saudi Arabia and tested by reverse transcription–PCR (RT-PCR) for AHFV.

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The Study

A total of 124 ticks were collected from camels and camel resting places in 3 different locations in western Saudi Arabia. The epidemiologic characteristics of the 124 ticks are shown in Table 1. Ticks were stored in individual containers at room temperature in Saudi Arabia and killed by overnight freezing at –80°C the day before shipment to France, according to the French regulations for importation. Samples were treated as previously described (*4*), and 200 μL of clarified, crushed material was used for purification of total nucleic acid with the MagNA Pure LC system (Roche Diagnostics, Meylan, France). Pools of 10 RNA samples (5 μL each) were prepared and tested by 1-step RT-PCR assay with the Access RT-PCR system (Promega. Madison, WI, USA) by using primers ALK-ES1 and ALK-ER (*2*). We used a cycling profile of 48°C for 45 min, 95°C for 5 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 30 sec, and a final elongation step at 68°C for 7 min. The 10 specimens in the RT-PCR–positive pool were tested individually for confirmation and sample identification. PCR products were then sequenced.

The tick JE7, which was collected southeast of Jeddah, Saudi Arabia, was positive for AHFV, and sequence analysis showed 99.7% homology at the nucleotide level with AHFV strain 1176 (GenBank accession no. AF331718) in the homologous region of the envelope gene. For safety reasons, virus isolation was not attempted in our BSL-3 laboratory. To identify the tick species, morphologic studies and molecular identification were conducted. All 124 ticks were identified by using morphologic keys (*5*).

The complete coding sequence of AHFV-JE7 was determined by amplification and sequencing of overlapping PCR products by using the long PCR product sequencing strategy (*6*) (primers and detailed protocol available on request). The open reading frame sequence was 10,248 nt (GenBank accession no. DQ154114). Pairwise distance comparison with AHFV prototype strain 1176 sequence showed 0.80% and 0.70% divergence at the nucleotide and amino acid level, respectively. A total of 79 nt substitutions were observed, of which 54 mutations were synonymous and 25 mutations were nonsynonymous (Ka:Ks ratio 0.46) (Table 2). The AHFV-JE7 complete sequence has been used with other full-length amino acid sequences of other mammalian tickborne flaviviruses to reconstruct phylogenetic relationships.

No nonsynonymous mutations were observed in the envelope gene of the 11 sequences determined for human AHFV cases or in tick and human AHFV sequences. Ka:Ks ratios within human sequences (n = 11) and between tick and human sequences were 0.33 and 0.50 in the nonstructural protein 3 (NS3) gene and 0.46 and 0.33

Table 1. Epidemiology of ticks collected in Saudi Arabia

Location	Type of location	Ticks isolated	No. ticks	Date of collection
Northeast Jeddah	Camels and camel resting place	*Ornithodoros savignyi* nymphs and adults and *Hyalomma* spp.	64	Jun 2004
Southeast Jeddah	Camels and camel resting place	*O. savignyi* nymphs and adults and *Hyalomma* spp.	32	Jun 2004
Kilaakh (50 km from Taif)	Camels	*H. dromedarii*	28	Jan 2005

in the NS5 gene, respectively. However, these values were not significantly different when sequences were analyzed by χ^2 test. Because of the low genetic heterogeneity observed between AHFV sequences, envelope, NS3, and NS5 sequences of the 12 AHFV isolates were colinearized and aligned with homologous sequences of AHFV and related tickborne flaviviruses to infer phylogenetic relationships, as previously reported (Figure) (*2*).

As shown in the Figure, the AHFV-JE7 sequence was closely related to but unambiguously distinct from all other AHFV sequences from human isolates. AHFV-JE7 constituted a phylogenetic group distinct from human isolates and was supported by a 100% bootstrap value. The genetic distance of AHFV-JE7 from the common ancestor of AHFV isolates is less than that of any of the human isolates. This topology strongly suggests that human isolates are derived from the group including AHFV-JE7. This constitutes a strong argument for the tickborne nature of AHFV.

It could be argued that several mosquitoborne flaviviruses have been isolated from ticks, e.g., Saint Louis encephalitis virus and West Nile virus (*7*,*8*). Phylogenetic analysis showed that these viruses are closely related to other *Culex*-associated viruses and that their evolution is determined by mosquitoes, not by ticks. Thus, the evolution of AHFV is clearly determined by ticks because all the most closely related viruses are also known to be associated with ticks, although in at least 1 case (Powassan virus) there is some evidence that the virus is also found in mosquitoes (*9*). Because ticks blood-feed on vertebrates, detection of AHFV RNA could be due to AHFV in the blood of the camel; however, this virus may not replicate in the tick. Because no obvious sign of blood was noted in the JE7 tick, detection of a full-length open reading frame sequence is convincing evidence that the virus had replicated in the tick, thus increasing the likelihood that the tick is a vector for the virus.

Table 2. Genetic differences between Alkhurma hemorrhagic fever virus coding sequences of human (strain 1176 AF331718) and tick (strain JE7, DQ154114) origin*

	No. mutated sites/no. sites in gene (%)		
Gene	Synonymous mutations	Nonsynonymous mutations	Ka:Ks
VirC	2/291 (0.69)	–	–
CTHD	–	1/60 (1.67)	–
prM	2/267 (0.75)	–	–
M	4/225 (1.78)	1/225 (0.45)	0.20
E	6/1,488 (0.41)	2/1488 (0.13)	0.25
NS1	4/1,062 (0.32)	3/1,062 (0.28)	0.43
NS2a	8/687 (1.16)	1/687 (0.15)	0.11
NS2b	2/393 (0.51)	3/393 (0.76)	0.60
NS3	9/1,863 (0.48)	5/1,863 (0.27)	0.36
NS4a	2/378 (0.53)	2/378 (0.53)	0.50
2K	1/69 (1.45)	–	–
NS4b	4/756 (0.53)	4/756 (0.53)	0.50
NS5	10/2,709 (0.37)	3/2,709 (0.15)	0.11

*Ka:Ks, ratio of nonsynonymous-to-synonymous nucleotides; VirC; mature virion C protein; CTHD, C-terminal hydrophobic domain; prM, premembrane; M, membrane; E, envelope; NS, nonstructural; 2K, transmembrane domain.

Conclusions

In the arid ecosystems of Saudi Arabia and other parts of the Persian Gulf, *Ornithodoros savignyi*, the sand tampan, has been associated with camels, their resting places, and to a lesser extent, other domestic and wild animals (*5*) found in camel resting places. *O. savignyi* is a multiple-host–seeking, nocturnally active, cryptic tick that commonly attacks humans and other animals resting under trees (*10*), which supports its role as a vector and transmitter of AHFV. The closely related *Kyasanur Forest disease virus*, which is endemic in certain regions of India, is the only tickborne hemorrhagic fever virus that has been isolated from *Ornithodoros* spp. (*11*). AHFV is the first human pathogenic RNA virus to be detected in *O. savignyi*. Recent reports of mosquito transmission of AHFV (*12*) remain unverified (*13*) but merit investigation by virologic analyses of field-collected mosquito pools. Our study provides the first unequivocal evidence that AHFV is a tickborne flavivirus, confirms previous phylogenetic analysis linking human AHFV isolates to a tick source, and shows the value of molecular techniques in rapidly and safely detecting arboviral activity in local arthropod fauna.

Wide-ranging implications arise from the identification of *Ornithodoros* ticks, a well-established genus in the Persian Gulf region in the transmission cycle of AHFV. First, the ability of the unfed tick to remain dormant in harsh conditions for long periods may give it an extended reservoir role for AHFV. Second, the wide distribution of *Ornithodoros* ticks in the region suggests that the geographic limits of AHFV may be larger than presently assumed. A recent clinical case from Najran, which is >600 km from the Jeddah–Makkah area, supports this view (*14*). This finding reinforces the need for physicians in the region and elsewhere to consider AHFV in the etiology for undifferentiated fever.

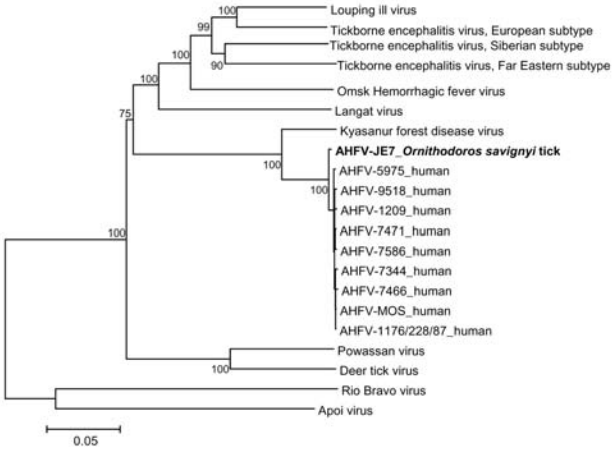

Figure. Phylogenetic analysis of AHFV-JE7 (shown in **boldface**) detected in an *Ornithodoros savignyi* tick and homologous sequences of related mammalian tickborne flaviviruses based on colinearized nucleotide sequences. Distances and groupings were determined by the p-distance algorithm and neighbor-joining method. Bootstrap values are indicated and correspond to 500 replications. Rio Bravo and Apoi viruses were used to root the tree. The scale bar at the lower left indicates a genetic distance of 0.05-nt substitutions per position.

The relationship between *Ornithodoros* ticks and military activity–related tickborne disease incidence has been recently documented in the Middle East (*15*). A large number of foreign military and civilian personnel are presently based in the region, thus expanding the opportunity for exporting clinical disease or infected ticks. The association of JE7 with camels further supports the role of camels in AHFV transmission cycle as well as the zoonotic nature of the disease. Larger studies involving more tick species are needed to better understand AHFV ecology and transmission dynamics. Investigations to obtain better knowledge of the geographic distribution of AHFV are necessary in countries near Saudi Arabia.

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Major Outbreak of Hepatitis A Associated with Orange Juice among Tourists, Egypt, 2004

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In 2004, a major outbreak of hepatitis A among tourists returning from Egypt involved 351 case-patients from 9 European countries who were infected with a single strain (genotype 1b). The case-control study identified orange juice as the most likely infection vehicle. Vaccination against hepatitis A virus is strongly recommended before travel to disease-endemic areas.

Nonimmune travelers to hepatitis A (HA)–endemic countries carry a substantial risk of acquiring the disease, yet little is known about the epidemiology of HA virus (HAV) infection in travel-associated outbreaks. In Germany, approximately half of the 1,400–2,300 cases of laboratory-confirmed HA reported annually since 2001 were acquired abroad. In mid-August 2004, infectious disease surveillance in Germany showed a strong increase of HAV infections in tourists returning from Egypt, where HA is highly endemic (*1*,*2*). The overwhelming majority had stayed at hotel X in the Red Sea resort of Hurghada. Prevention measures were implemented at the hotel (e.g., HA vaccination of guests). An outbreak investigation was carried out.

The Study

The line listing of HA patients included persons with laboratory evidence of recent HAV infection (anti-HAV immunoglobulin M [IgM]) who stayed at hotel X after June 1, 2004. Also listed were hotel guests with HA disease (jaundice, elevated liver enzyme levels), without laboratory confirmation, who had traveled with persons with laboratory-confirmed acute HAV infection.

A case-control study was performed among hotel X guests >17 years of age residing in 3 German states. The time span between the earliest arriving case-patient's last day at hotel X and the latest arriving case-patient's first day there was defined as the "minimum period of transmission" (MPT). Case-patients came from the line listing. Healthy hotel guests who stayed at the hotel during the MPT who had neither been vaccinated against HAV nor previously infected with HAV were eligible as controls. Telephone interviews were conducted with a standardized questionnaire that elicited information on demographic factors, foods and drink consumption, and participation in recreational activities such as swimming, day trips, etc.

For statistical analysis, exposures were dichotomized into "ever" versus "never," and the "number of days exposed" was calculated. Univariate analysis (χ^2 tests) and multiple logistic regression were performed (SPSS version 12.0.2, SPSS Inc., Chicago, IL, USA); $p<0.05$ was considered statistically significant. The Egyptian authorities' investigation included testing all hotel employees for HAV antibodies (IgM, IgG), and scrutiny of food suppliers. Serum samples were obtained from German case-patients for testing by reverse transcription–PCR (RT-PCR) in the VP1–2A junction and sequencing of a 160-bp-long PCR fragment of the VP1 region.

The outbreak lasted from July 10 to September 8, 2004 (Figure 1). A total of 351 case-patients made up this outbreak: 271 primary and 7 secondary infections in Germany, plus 60 primary infections reported to the national public health institutes in 8 other European countries. Austria recorded a secondary outbreak with 13 cases caused by an infected food handler who had stayed at hotel X (*3*). In preceding years, in Germany only 2–8 HA cases were reported after travel to Egypt with disease onset between July 10 and September 8.

Of the German reported primary cases, overt clinical HA developed in 263 (97%) persons. Case-patients were 2 to 67 years of age (median 34 years) and 54% were male. Overall, 47% of case-patients were hospitalized. Risk for hospitalization rose with increasing age (p for trend = 0.001). The MPT lasted from June 24 to July 23 (Figure 1). No more than 52% of the case-patients had stayed together at the hotel on any single day. Case-patients had stayed at hotel X from 6 to 21 days, and 70% had stayed 13 days or longer.

Sixty-nine HA case-patients (60% response among the 115 case-patients in the 3 states) and 36 controls were included in the statistical analysis. Eighty-seven percent of the case-patients reported absence from work for 3 to 56 days (median 26 days), and 54% of the case-patients were hospitalized for 2 to 25 days (median 9 days).

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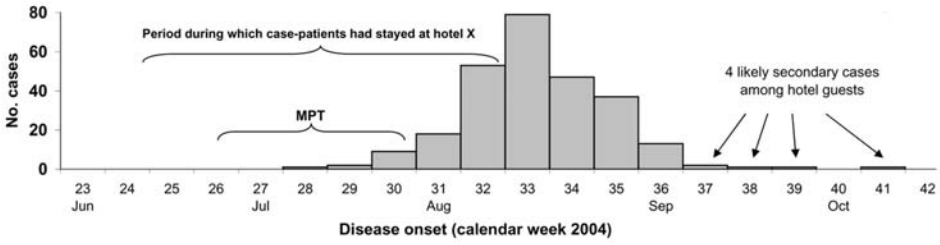

Figure 1. Epidemic curve: distribution of dates of disease onset for outbreak-associated hepatitis A case-patients from Germany (n = 264), and minimum period during which hepatitis A virus transmission occurred (MPT).

Case-patients and controls did not differ significantly by age, sex, recreational activities, consumption of ice cream or salads, or other foods consumed, or behavioral characteristics. Case-patients were significantly more likely to have drunk orange juice served at the breakfast buffet (82%) than were controls (64%) (odds ratio 2.6; 95% confidence interval 1.1–6.6). In multivariate analysis, no other exposures were retained. A dose-response relationship became apparent between number of days of orange juice consumption and HA (Figure 2). Case-patients consumed orange juice for a median of 11 days, and controls consumed it for a median of 5 days. In 22 (52%) of the 42 serum samples available for testing, HAV RNA was identified. All samples compared by sequencing were identical and belonged to genotype 1b (Figure 3).

The on-site investigations in Egypt did not identify hotel staff positive for HAV IgM. Minimal fluctuation among hotel staff renders it unlikely that an HAV-positive employee was missed. Investigations at the orange juice producing plant found significant hygiene problems. In addition, the finished product did not undergo heat treatment. This producer did not cater to other hotel chains in Hurghada.

Conclusions

This large outbreak demonstrates risk and clinical impact of HA for nonimmune travelers to HA-endemic countries. In Germany, the outbreak accounted for 12% of all HA case-patients notified in the year 2004.

The results of the outbreak investigation strongly point to orange juice as the infection vehicle. In the case-control study, among a broad range of foodstuff, beverages, and recreational activities queried, the consumption of orange juice was the only exposure significantly associated with HA, with higher doses of juice significantly increasing HA risk. These findings are corroborated by the inspection of the hygienic conditions under which the juice was produced in Egypt. The juice was most likely contaminated during the manufacturing process, e.g., by an infected worker with imperfect hand hygiene or by contact of fruit or machinery with sewage-contaminated water.

Citrus fruit and citrus juices have only rarely been implicated as vehicles of HA outbreaks, with contamination typically described during preparation just before consumption (*4*,*5*). Salmonella outbreaks caused by orange juice contaminated at the production site have been identified (*6*–*8*). As HAV is quite resistant to acid (*9*), it likely survives for prolonged periods in orange juice. Less stable pathogens such as *Escherichia coli* have been shown to survive in orange juice for ≥15 days (*10*).

The fact that juice was consumed by 60% of healthy controls may be explained in part by fluctuating virus concentration within the juice, which resulted in varying degrees of infectiousness during the 4-week period. A contaminated lot may have been phased-in and out slowly by gradual mixture with other lots. Also, the study design did not allow the exclusion of controls who did not know they were immune.

The Hurghada outbreak-strain clearly differs from strains that have caused nontravel-associated outbreaks in Germany in recent years. Two large autochthonous outbreaks were caused by HAV type 1a strains (*11*). In the Netherlands, HAV strains in autochthonous cases mostly also belonged to HAV 1a, whereas 1b strains were found more often in children of Moroccan origin (*12*). Extended monitoring of HAV strains, for example, as performed in the United States (*13*), could find hidden clusters and demonstrate links between imported and autochthonous cases. Similar monitoring should be introduced in Germany.

Vaccination against HA is recommended for all nonimmune travelers to HA-endemic areas (*14*). This outbreak showed that a high proportion of German tourists in Egypt were either not adequately informed about HA risk and the benefits of vaccination or were informed yet still decided

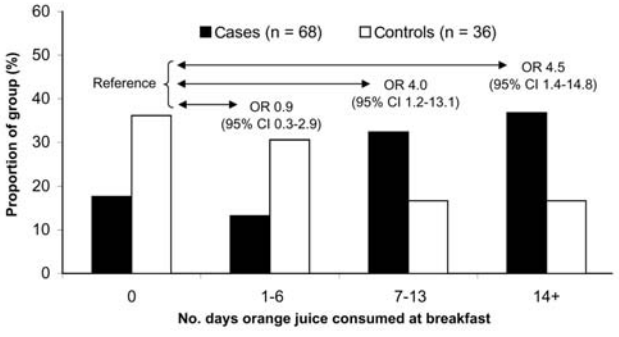

Figure 2. Days of orange juice consumption among hepatitis A patients and controls. OR, odds ratio; CI, confidence interval.

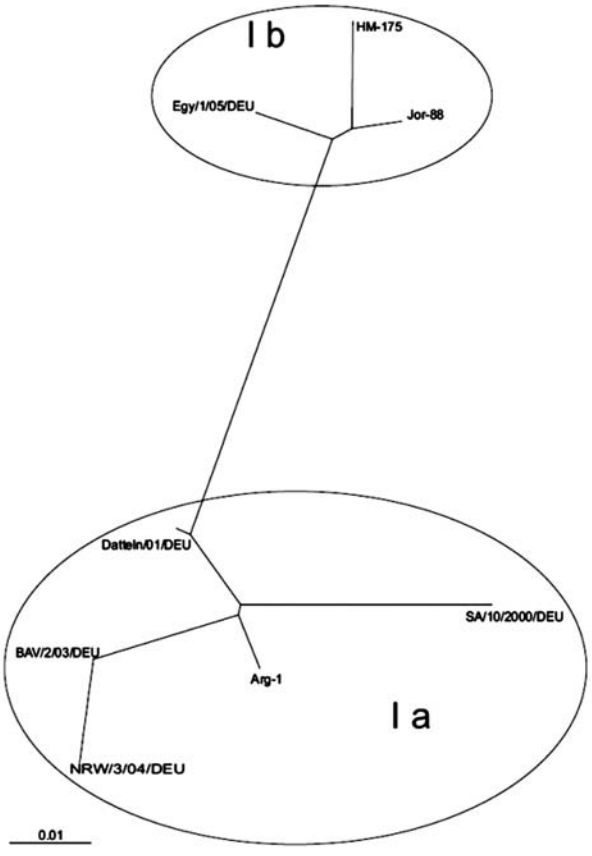

Figure 3. Phylogenetic analysis of the VP1-2A junction region. The sequence of a 160-bp-long PCR fragment (isolate Egy/1/05/DEU, GenBank accession no. AY741663) of the hepatitis A VP1 region was compared to published reference sequences of hepatis A virus. Genotype Ia: GenBank accession nos. AJ306374(Arg-1), AY656712 (BAV/2/03/DEU*), AY028976 (SA/10/2000/DEU), AY046073 (Datteln/01/DEU), and AY747173 (NRW/3/04/DEU*). Genotype Ib: L07728 (Jor 88), M14707 (HM-175). The scale represents nucleotide substitutions per position; * denotes previous German outbreak-causing strains.

against vaccination. The outbreak emphasizes the importance of adequate pretravel advice, preferably from an institution specialized in travel medicine. Travel agencies should incorporate adequate immunization advice into their catalogs.

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Henipavirus and Tioman Virus Antibodies in Pteropodid Bats, Madagascar

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Specimens were obtained from the 3 Malagasy fruit bats, *Pteropus rufus*, *Eidolon dupreanum*, and *Rousettus madagascariensis*. Antibodies against Nipah, Hendra, and Tioman viruses were detected by immunoassay in 23 and by serum neutralization tests in 3 of 427 serum samples, which suggests that related viruses have circulated in Madagascar.

The Old World fruit bats of the family *Pteropodidae*, particularly species belonging to the genus *Pteropus*, have been considered natural hosts for viruses emerging in Australia (Hendra virus [HeV], Australian bat lyssavirus [ABLV], and Menangle virus), Malaysia, Singapore, and Bangladesh (Nipah virus [NiV]) (*1*,*2*). The geographic distribution of the henipaviruses NiV and HeV or related unrecognized viruses may overlap with that of *Pteropus* spp. outside Australia, Malaysia, and Bangladesh. This hypothesis was confirmed with the evidence of NiV in *P. lylei* from Cambodia and Thailand (*3*,*4*). NiV emergence represents a human and animal health problem because the virus causes severe febrile encephalitis associated with death in humans and respiratory illness in domestic pigs. Isolated human cases have been reported for the 3 Australian viruses (*1*). Another paramyxovirus, the rubulavirus Tioman (TiV), was isolated from the urine of a *P. hypomelanus* bat, collected on Tioman Island, Malaysia (*5*). Although closely related to Menangle virus and associated with *Pteropus* spp., TiV has not yet been associated with any human disease.

Pteropodids have less species diversity in Madagascar than in other Old World tropical regions. Three species, all endemic, are found on the island: *P. rufus*, *Eidolon dupreanum*, and *Rousettus madagascariensis*. These species are distributed across much of the island and are more common in lowland areas than in the highlands (*6*). We report on a survey of Malagasy fruit bats to assess the presence and distribution of Hendra, Nipah, and Tioman (like) viruses on this island.

The Study

The samples were collected May 2003–July 2005 at different locations (Figure), most with multiple sampling sites. Most fruit bats were captured during the dry season by using mist nets set near roosting places (trees or caves). Bats were immobilized face up, and a blood sample was taken using sterile procedures. Urine was collected directly from the bat's urogenital opening, before the blood sampling, with a cotton swab that was immediately placed in a cryotube containing 1 mL viral transport medium. When a urine sample could not be obtained from a given animal, a pharyngeal sample was collected with a cotton swab that was then directly placed in the viral transport medium. Most animals were subsequently released. Urine samples were also collected under 1 *P. rufus* tree roost, as described previously (*7*).

Figure. Madagascar, showing bat collection sites by species. For reference, the X indicates the location of the capital city, Antananarivo, where no samples were collected.

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Blood was centrifuged in the field at ambient temperature at 3,000 rpm for 15 min. Serum, blood pellets, urine, and pharyngeal samples were placed in a container of charged nitrogen until their arrival at the laboratory, where they were stored at –80°C.

Bat sera were screened for antibodies against NiV, HeV, and TiV by enzyme-linked immunosorbent assay (ELISA), as described previously (*3*). In total, 427 serum specimens were tested (Table); 23 (5.4%) were positive for 1 of the viruses. Antibodies to NiV and HeV were mainly detected in *E. dupreanum* sera (14/73) but also in *P. rufus* sera (8/349), whereas antibodies to TiV were found in 2 *P. rufus* serum specimens and 1 *R. madagascariensis* specimen. All samples positive for antibodies to HeV were also positive for antibodies to NiV. ELISA-seropositive bats were detected in 5 of 7 locations investigated (Table, Figure).

Serum neutralization tests were carried out by using NiV, HeV, or TiV. Serum samples were heated for 30 min at 56°C and then titrated with 2-fold dilutions (1:10 to 1:640) as previously described (*3*). Positive control for the NiV and HeV tests was an anti-NiV serum sample obtained from a convalescent-phase patient. Positive control for the TiV test was serum collected from a hamster experimentally infected with TiV. Of the 23 ELISA-positive serum samples, 21 could be tested by neutralization test, using NiV, HeV, and TiV. Neutralizing antibodies to NiV and HeV were detected in 2 of the 13 *E. dupreanum* serum samples that were ELISA positive for HeV, NiV, or both (titers 1:40 and 1:80 for NiV, 1:20 and 1:10 for HeV, respectively). Another *E. dupreanum* bat was also confirmed positive only for antibodies to NiV (1:40); the 10 other *Eidolon* bats were negative. One bat that was ELISA seropositive for NiV and HeV *P. rufus* was confirmed positive for neutralizing antibodies to HeV (1:160) and was found to be positive for neutralizing antibodies to TiV (1:80); the 7 other *Pteropus* bats were negative. All ELISA-positive sera confirmed by the neutralization test were obtained in the same geographic area, Moramanga District (Figure).

Virus isolation experiments were performed on 118 urine and 285 pharyngeal specimens. Thus, subconfluent Vero E6 cells (ATCC CRL-1586) were inoculated with 500 µL of viral transport medium containing a cotton swab impregnated with urine or pharyngeal epithelial cells, as previously described (*3*). Results were negative, but 22 were inconclusive because of bacterial and fungal contamination.

Conclusions

Our study provides the first evidence that the 3 pteropodid bats on Madagascar (*P. rufus*, *E. dupreanum*, and *R. madagascariensis*) have been in contact with viruses of the *Paramyxoviridae* family, and especially of the genus *Henipavirus*. The distribution of *Pteropus* is limited to islands of the Pacific and Indian Oceans and continental areas from Pakistan east across Southeast Asia to Australasia. The genus *Rousettus* is found in both Africa and Asia; the genus *Eidolon*, only in Africa and Madagascar. The presence of antibodies to henipaviruses (detected by ELISA or serum neutralization test) in *Eidolon* could suggest the possible presence of these viruses in Africa, where the only other species in this genus, *E. helvum*, is found. The distributional pattern of *Eidolon* indicates that a dispersal event occurred between Africa and Madagascar (*8*). A lateral transfer on Madagascar between *E. dupreanum* and the other pteropodids could have occurred on that island. *E. dupreanum* and *R. madagascariensis* are known to share cave roost sites during the day (*9*) and, with *P. rufus*, can be found feeding at night in the same fruit trees (*10*). A survey for henipaviruses in Africa should be considered to confirm the hypothesis of a wider distribution of these pathogens.

In Cambodia, the circulating NiV isolated strain was very similar to the Malaysian strain used in ELISA and serum neutralization tests. Therefore, most of the ELISA-

Table. Serum samples ELISA-reactive to Nipah, Hendra, and Tioman viruses collected from fruit bats, Madagascar, 2003–2005

Species and location	No. tested	Seropositive samples			
		Nipah virus	Hendra virus	Tioman virus	Total (%)
Eidolon dupreanum					14/73 (19.2)
Marozevo (Moramanga)	53	10	10	0	10
Miandrivazo	2	0	0	0	0
Ankarana	18	4	1	0	4
Pteropus rufus					8/349 (2.3)
Marovitsika (Moramanga)	33	2	2	0	2
Miandrivazo	112	4	0	1	5
Marovoay	140	0	0	1	1
Beroboka	27	0	0	0	0
Vangaindrano	37	0	0	0	0
Rousettus madagascariensis					1/5 (20.0)
Ankarana	5	0	0	1	1
Total	427	20	13	3	23/427 (5.4)

positive bat serum specimens (95%) were confirmed by the serum neutralization test (*3*). In our Madagascar study, in which the Malaysian strain was also used, few ELISA-positive specimens (16%, n = 19) could be confirmed by the serum neutralization test. We hypothesize that henipaviruses circulate in Madagascar and are sufficiently divergent from the NiV and HeV strains used in this study, which explains these discordant results. To attempt to isolate and characterize the circulating viruses, a long-term survey among pteropodids is being conducted in the Moramanga District, where positive neutralization test serum specimens were obtained.

Day roost sites of Malagasy pteropodids are extremely rare in proximity to human settlements. However, these animals are hunted for food and can be found alive or dead in local markets and in restaurants. Further, humans eat fruits from trees where pteropodids have fed. More research is needed to clarify the possible risk of pathogen transmission to humans.

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Ophthalmic Surgery in Prion Diseases

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Eleven (1.8%) of 597 patients underwent ophthalmic surgery within 1 month before the onset of prion disease or after the onset. All ophthalmologists reused surgical instruments that had been incompletely sterilized to eliminate infectious prion protein. Ophthalmologists should be aware of prion diseases as a possible cause of visual symptoms and use disposable instruments whenever possible.

Visual impairment occurs in 10% to 20% of patients with sporadic Creutzfeldt-Jakob disease (sCJD) during an early stage of the disease (Heidenhain variant) (*1*,*2*). Some patients with prion diseases may visit ophthalmologists with visual impairment due to prion diseases or with coexisting age-related eye diseases (*3*,*4*).

Infectious prion protein (PrP^{Sc}) was identified in the retina and optic nerve in patients with variant CJD (vCJD) and sCJD (*5*,*6*), and CJD has been transmitted by corneal transplantation (*7*,*8*). In the World Health Organization (WHO) guidelines, eyes were classified as highly infectious tissues (*9*).

Secondary transmission of PrP^{Sc} through ophthalmic surgery could possibly be prevented around the onset of prion diseases, although surgery that is performed long before the onset of prion diseases would not have that potential. It is important to understand the current status of ophthalmic surgery for patients with prion diseases and to clarify the clinical features of the patients with prion diseases who undergo ophthalmic surgery. Here, we describe the relevant data from CJD surveillance in Japan.

The Study

We analyzed the patients with prion diseases who had been registered by the CJD Surveillance Committee in Japan from April 1999 through March 2005. We prospectively investigated each patient with a surveillance protocol that assembled information about life history, previous medical history, clinical history, laboratory data, and results of molecular genetic and pathologic analyses. Written consent, approved by the Institutional Ethics Committee, was obtained from all the patients' families; members of the Surveillance Committee examined the patients and collected the data.

We classified the patients into 4 categories: sCJD, infectious prion diseases, inherited prion diseases, and unclassified prion diseases. sCJD was diagnosed according to the classical criteria established by Masters et al. (*10*). Infectious prion diseases included CJD associated with cadaveric dura mater graft (dCJD) or other iatrogenic opportunities for prion infection, in which the criteria for sCJD were applied for the diagnosis, and vCJD, in which the diagnosis was based on WHO criteria (2001) (*11*). Regarding the accuracy of the diagnosis of inherited prion diseases, cases verified by pathology report were defined as definite, and cases with mutations in the prion protein gene and neuropsychiatric manifestations compatible with prion diseases were defined as probable.

Among patients with a history of ophthalmic surgery, we directed special attention to the patients who had a history of eye surgery within 1 month before the obvious onset of prion disease or after the onset. Because the onset of prion diseases often overlaps with various kinds of prodromal symptoms, determining the precise time point of onset is difficult; therefore, we included the period of 1 month before the obvious onset. To gather information about the ophthalmic surgery, we mailed questionnaires to the ophthalmologists who operated on these patients, requesting the following information: diagnosis of ophthalmologic diseases, surgical procedures performed, changes in the symptoms after the surgery, whether the instruments were reused, and methods of cleaning reused instruments.

To ascertain the clinical features of prion diseases, we analyzed the patient's age at onset and duration of disease course, which was calculated as the interval between the onset and the appearance of the akinetic mutism state or death in the patients who died without akinetic mutism. Among early clinical manifestations of prion diseases, dementia and visual disturbance are major determinants that would influence the indication for ophthalmic surgery, so we grouped the patients according to whether they had dementia or visual impairment within 2 months after onset of symptoms.

The sex distribution of the patients who had ophthalmic surgery around the time of onset of clinical symp-

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toms and those who did not was compared by Fisher exact tests, and differences in age at onset and disease duration were compared by Mann-Whitney U tests. We used χ^2 tests to compare the distribution of the patients with or without dementia or visual impairment within 2 months of onset. Statistical significance was defined as $p<0.05$.

We found 597 patients with definite or probable diagnosis of prion diseases: 468 (78.4%) with sCJD; 78 (13.1%) with inherited prion diseases; 48 (8.0%) with infectious prion diseases, including 47 cases of dCJD; and 1 patient with vCJD and 3 patients with unclassified CJD.

Thirty-seven patients (6.2%) had a history of ophthalmic surgery at some time in their lives. Among them, 11 patients (1.8%) underwent ophthalmic surgery within 1 month before the obvious onset of prion disease or after the onset. Except for 1 patient with Gerstmann-Sträussler-Scheinker disease, all of these patients had sCJD. There have been no reports of the development of prion diseases in patients who underwent ophthalmic surgery after the ophthalmic surgery of patients with prion diseases.

Ten patients with sCJD underwent ophthalmic surgery within 14 months of symptom onset, and 8 of them had ophthalmic surgery within 4 months of symptom onset (Table 1). At clinical onset, 4 patients exhibited visual symptoms, 5 had dementia, and 1 patient had a gait disturbance. All patients underwent surgery for cataracts, except for 1 patient who underwent surgery for a detached retina. According to the reports on the surgical outcome by the ophthalmologists of 7 patients, visual disturbance was unchanged in 2 patients, deteriorated in 1, and improved to some extent in 4 after surgery. All ophthalmologists reused some surgical instruments and cleaned instruments by either autoclaving or the ethylene oxide gas method, which have been reported to incompletely sterilize PrP^{Sc} (*9*,*12*).

Clinical features were compared between sCJD patients who did and did not have ophthalmic surgery (Table 2). The patients who had ophthalmic surgery had a significantly longer disease duration than those without ($p=0.0004$). Regarding early clinical symptoms within 2 months after onset, the subgroup with visual symptoms without dementia was significantly overrepresented among the patients who had ophthalmic surgery compared with those who did not have surgery ($p= 0.0004$).

Conclusions

Our study showed that, in 1.8% of the patients with prion diseases, eye tissues were operated on within 1 month before the obvious onset of prion disease or after the onset. In addition, the sCJD patients who underwent surgery had a significantly longer duration of the disease course as well as significant overrepresentation of visual symptoms without dementia in the early phase, compared with patients who did not have ophthalmic surgery.

The prevalence of ophthalmic surgery around the time of clinical onset of prion diseases in our study is similar to that (2.0%) in a report from the United Kingdom (*13*). In the UK study (*13*), patients with Heidenhain variant cases constituted 40% of sCJD patients who had ophthalmic surgery. Early visual impairment (due to prion diseases) would prompt ophthalmologists to perform surgery.

Currently, cataract surgery is recommended to improve physical or cognitive function in elderly patients (*14*,*15*). It should be noted that, after performing eye surgery on patients with prion disease, all ophthalmologists reused surgical instruments that were sterilized with procedures that are incomplete for the sterilization of PrP^{Sc}, although the WHO infection control guidelines for prion diseases (*9*) strongly recommend single-use surgical

Table 1. Characteristics of sCJD patients and ophthalmic surgery*

Patient no.	Sex/age, y†	Disease duration, mo‡	Symptom at sCJD onset	Ophthalmic disease	Interval, mo§	Visual symptoms after surgery	Reused instruments	Cleaning method
1	M/81	8	Visual	Cataract	4	NA	NA	NA
2	M/61	15	Dementia	Cataract	0	Improved	Yes	Autoclave (135°C for 9 min)
3	F/64	20	Visual	Cataract	14	Not changed	Yes	EOG
4	F/59	3	Dementia	Detached retina	–1	Improved	Yes	EOG
5	F/57	10	Dementia	Cataract	10	NA	NA	NA
6	F/79	5	Dementia	Cataract	–1	Improved	Yes	EOG
7	M/74	16	Visual	Cataract	3	Improved	Yes	Autoclave (132°C for 10 min), EOG
8	F/63	5	Visual	Cataract	1	Deteriorated	Yes	Autoclave (132°C for 10 min)
9	M/79	6	Gait disturbance	Cataract	2	Not changed	Yes	Autoclave (121°C for 60 min)
10	F/66	3	Dementia	Cataract	1	NA	NA	NA

*sCJD, sporadic Creutzfeldt-Jakob disease; visual, visual impairment; NA, not available; EOG, ethylene oxide gas.
†At sCJD onset.
‡Disease duration, the duration from onset to akinetic mutism state or death if the patients never displayed akinetic mutism.
§Between surgery and sCJD symptoms.

Table 2. Clinical symptoms of sCJD within 2 mo after disease onset*

Characteristic	Ophthalmic surgery		Total	p value
	No, n = 458	Yes, n = 10		
Female/male	263/195	6/4	269/199	0.57
Age at onset, y; mean ± SD	66.8 ± 9.9	68.3 ± 9.1	66.8 ± 9.9	0.74
Disease duration,† mean ± SD	4.2 ± 4.8	9.1 ± 6.0	4.3 ± 4.9	0.0004
Clinical symptoms (%)				
Dementia (+)/visual impairment (+)	153 (34.2)	4 (40.0)	157 (34.3)	
Dementia (+)/visual impairment (–)	239 (53.3)	3 (30.0)	242 (52.8)	0.0004
Dementia (–)/visual impairment (+)	16 (3.6)	3 (30.0)	19 (4.1)	
Dementia (–)/visual impairment (–)	40 (8.9)	0	40 (8.7)	

*sCJD, sporadic Creutzfeldt-Jakob disease; SD, standard deviation; +, with; –, without.
†Disease duration, the duration from onset to akinetic mutism or death if patients never displayed akinetic mutism.

instruments for procedures involving highly infective tissues. The fact that no secondary iatrogenic cases that could be attributed to surgical procedures were found during our investigation does not diminish the need for ophthalmologists to be aware of CJD as a cause of visual symptoms (including symptoms mimicking those of cataracts) and highlight the importance of using disposable instruments whenever possible to avoid cross-contamination.

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Human Bocavirus Infection, People's Republic of China

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A newly identified parvovirus, human bocavirus (HBoV), was found in 21 (8.3%) of 252 nasopharyngeal aspirates from hospitalized children with lower respiratory tract infection in Hunan Province, People's Republic of China. Viral loads were 10^4 to 10^{10} copies/mL. Phylogenetic analysis of the VP1 gene showed a single genetic lineage of HBoV worldwide.

Acute respiratory tract infections (ARTIs) are a leading cause of hospitalization, illness, and death in infants and young children (*1–4*). Respiratory syncytial virus (RSV), human metapneumovirus (HMPV), influenza viruses, human coronaviruses, rhinoviruses, and adenoviruses are some of the most important viral agents for this group of patients. However, in a substantial proportion of children with respiratory tract diseases, no pathogen can be identified (*1*).

Until recently, the only parvovirus known to be pathogenic for humans is B19 (*5*). In 2005, a new human virus of the genus *Bocavirus* considered to be pathogenic for humans, provisionally named human bocavirus (HBoV), was described in Sweden (*1*). Subsequently, HBoV infection was reported in children with ARTIs in Australia (*6*), Japan (*7*), Canada (*8*), France (*9*), and the United States (*10*). In our study, 252 nasopharyngeal aspirates (NPA) obtained from November 3, 2005, to April 3, 2006, from hospitalized children with lower respiratory tract infections were analyzed for the presence of HBoV because of associated clinical manifestations.

The Study

Participants in the study were children ≤10 years of age who were hospitalized from November 3, 2005, to April 3, 2006, in Hunan Province, People's Republic of China. They were admitted mostly for bronchitis, pneumonia, and bronchopneumonia; their NPA were collected for investigation of the cause. All children were admitted 2–6 days after the onset of ARTI. All specimens were collected after the parents of the enrolled children had given informed consent.

DNA was extracted from NPA specimens by using the QIAamp Viral DNA Mini Kit (QIAGEN, Beijing, China). HBoV in extracted DNA was detected by PCR amplification of a 291-bp fragment of the NS1 gene as described previously (*6*). To acquire the complete sequence of the VP1 gene, we used primers 5′-GATAACTGACGAGGAAATG-3′ and 5′-GAGACGGTAACACCACTA-3′ based on the published genomic sequence of HBoV (GenBank accession no. NC_007455). The PCR cycle included an initial heating at 95°C for 15 min; 40 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 2 min; and a final extension at 72°C for 10 min. Both short and long PCR products were sequenced. Sequencing was performed on an Applied Biosystems 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) by using both the forward and reverse primers. The complete sequences of the VP1 gene obtained were aligned with sequences available in GenBank by using Clustal X (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). A neighbor-joining tree was constructed by the neighbor-joining method using the MEGA 3.1 program (www.megasoftware.net) and sequences of canine minute virus (MVC) and bovine parvovirus (BPV). Human parvovirus B19 (B19) was used as the outgroup.

A TaqMan real-time PCR targeting the NS1 region of HBoV was conducted to quantify the viral load. In brief, 2 μL genomic DNA was amplified in a 25-μL PCR mixture containing 5 μL ABI TaqMan 2× PCR Master mix, 20 μM of each primer, and 20 μM of the probe. The primer sequences used were 5′-TAATGACTGCAGACAACGCCTAG-3′ and 5′-TGTCCCGCCCAAGATACACT-3′, and the probe was 5′-FAM-TTCCACCCAATCCTGGT-MGB-3′. The cycling conditions included initial incubations at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Plasmid pGEM-T-NS1 containing the target sequences was constructed and used as a positive control for copy number calculation. Sensitivity of the PCR assay was 100 copies per reaction, as determined by dilutions of the plasmid.

RNA was also extracted from each NPA specimen by using the QIAamp Viral RNA Mini Kit (QIAGEN) to screen for HMPV (*11*), RSV (*12*), influenza (A, B, and C) (*13*), parainfluenza (types 1–4) (*13*), and human coronaviruses (229E, OC43, NL63, and HKU1) by standard reverse transcription–PCR technique (*13–15*).

HBoV was detected by the diagnostic PCR in 21 (8.3%) of NPA specimens collected from 252 hospitalized children with ARTI. Serum samples available from 2 of the

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HBoV-positive patients were also positive. Two HBoV-positive patients (patient 7 and patient 10) had coinfection with human coronavirus 229E. Among the HBoV-positive patients, 17 (81%) were male, and 4 (19%) were female (Table). The ages of the infected patients were 2 months to 3 years (median age 10.5 months), with the exception of a 10-year-old boy (patient 8). The most common clinical signs and symptoms were cough (86%), fever (33%), wheezing (33%), and diarrhea (29%) (Table). The 3 main admission diagnoses were pneumonia (6 patients), bronchitis (6 patients), and bronchopneumonia (7 patients). These patients had been admitted to hospital for 2 to 28 days. Chest radiographs were obtained from 12 patients; all showed abnormal findings (6 had airspace shadows, and 6 displayed coarse lung markings). Most HBoV-positive patients had no other underlying illness, with the exception of 1 (patient 3) who had intracranial infection. Although cough and diarrhea were more frequently found in HBoV-infected children (86% and 29%, respectively) than in HBoV-uninfected children (60% and 7.8%, respectively), confirmation of the disease association of HBoV infection requires the analysis of HBoV in a negative control group of healthy children.

HBoV viral loads in NPA specimens ranged from 2.4×10^4 to 2.5×10^{10} copies/mL (Table). The 2 positive serum specimens (from patients 7 and 10) had 1.2×10^5 and 4.1×10^4 copies/mL, respectively, which were almost equal to those found in their corresponding NPA specimens. Most specimens had HBoV viral loads close to10^4 copies/mL. However, 5 (24%) NPA specimens had viral loads >10^9 copies/mL.

The entire VP1 gene of HBoV was sequenced for 5 specimens that had adequate amounts of genomic DNA. An alignment of VP1 sequences obtained from children in China with those previously reported for the prototype strains (ST1 and ST2 strains, GenBank accession nos.DQ000495–DQ000496) showed only minor sequence differences, with a nucleotide identity of 97.7% and an amino acid identity of 98.1%. Thus, HBoV is a highly conserved virus. Phylogenetic analysis of these sequences and those from BPV, MVC, and B19 indicated that HBoV was more related to BPV and MVC (Figure).

Conclusions

The prevalence of HBoV in children and the associated illness have not been well characterized. In this study, we found that HBoV was prevalent in infants and young children in China. The 8.3% prevalence rate is higher than rates (3.1%–5.7%) previously reported for children in Sweden, Australia, Japan, Canada, France, and the United States (*1,6–10*). This difference could be due to the fact that we screened specimens collected during the peak ARTI season. Because this is the first finding of HBoV in children in developing countries, whether the difference

Table Clinical data for children with human bocavirus DNA detected in nasopharyngeal aspirate (NPA) specimens*

Patient no.(sex)	Age, mo	Days in hospital	Diagnosis	Signs/symptoms	Chest radiograph	Copies/mL specimen (NPA)
1 (F)	12	5	P	Fever, vomiting, diarrhea	NR	1.2×10^{10}
2 (M)	18	15	P	Cough, rash	BA-ILM	3.1×10^4
3 (M)	12	28	II	Cough, hypodynamia	RLZ-AS	5.1×10^9
4 (M)	8	3	B	Fever, clonism	NR	4.3×10^4
5 (M)	10	15	BP	Cough	BA-ILM	2.5×10^{10}
6 (F)	2	2	BP	Dyspnea, cyanosis	RLZ-AS	4.7×10^4
7 (M)	4	11	BP	Cough, diarrhea	BA-ILM	1.6×10^5 (1.2×10^5)†
8 (M)	120	10	P	Fever, cough	BA-ILM	4.4×10^4
9 (M)	9	19	B	Cough, wheeze	NR	1.8×10^9
10 (M)	2	16	SP	Cough, foaming, ALTE	BAS	6.7×10^4 (4.4×10^4)†
11 (M)	24	7	P	Cough, wheeze	RUZ-AS	1.8×10^9
12 (M)	14	6	B	Cough, wheeze, polypnea	NR	4.2×10^4
13 (M)	36	14	B	Fever, cough, wheeze	NR	4.8×10^4
14 (F)	8	17	B	Fever, cough, wheeze	NR	5.5×10^4
15 (M)	6	8	BP	Cough, polypnea, diarrhea	BA-ILM	2.4×10^4
16 (F)	6	8	FOU	Fever, cough, wheeze	BA-ILM	7.3×10^4
17 (M)	6	5	BP	Cough, diarrhea	BAS	4.5×10^4
18 (M)	15	9	B	Cough, wheeze, diarrhea	NR	6.1×10^4
19 (M)	7	9	BP	Cough, polypnea	NR	3.8×10^5
20 (M)	2	15	BP	Cough, polypnea	BAS	7.6×10^4
21 (M)	9	6	P	Fever, cough, diarrhea	NR	8.3×10^4

*M, male; F, female; P, pneumonia; NR, none reported; BA, bilateral airspace; ILM, increased lung marking; II, intracranial infection; RLZ, right lower zone; B, bronchitis; BP, bronchopneumonia; SP, severe pneumonia; ALTE, apparent life-threatening event; RUZ, right upper zone; AS, airspace shadows; FOU, fever of unknown origin; BAS, bilateral airspace shadows.
†Parentheses show virus titer in serum sample.

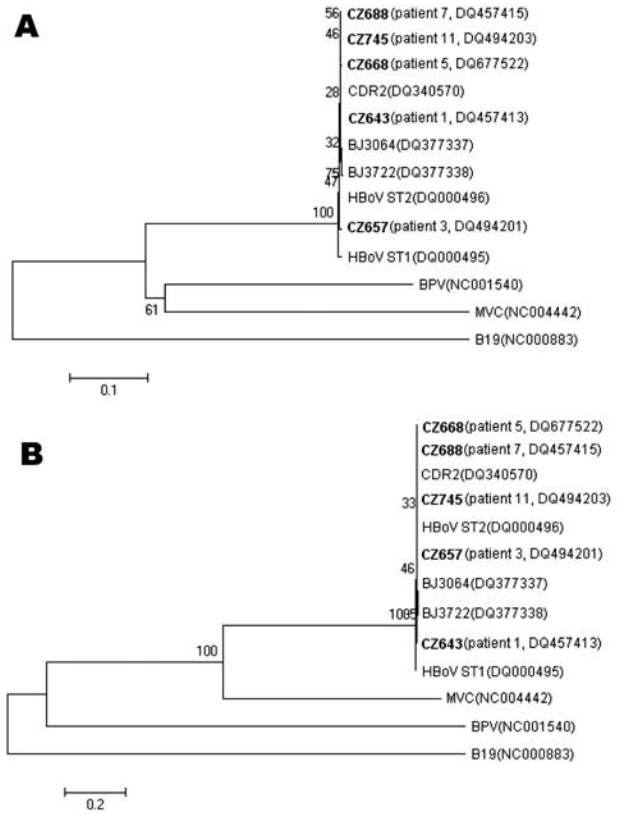

Figure. Phylogenetic analysis of the complete VP1 nucleotide (A) and amino acid (B) sequences of human bocavirus (HBoV). Phylogenetic trees were constructed by the neighbor-joining method by using MEGA 3.1 (www.megasoftware.net), and bootstrap values were determined by 1,000 replicates. Viral sequences in **boldface** were generated from the present study, and other reference sequences were obtained from GenBank. Bootstrap values are shown at each branching point. The sequences generated from the present study were deposited in GenBank under accession nos. DQ457413, DQ457415, DQ494201, DQ494203, and DQ677522.

also reflects a higher prevalence of the infection in developing countries is unclear.

The symptoms associated with HBoV infection in Chinese children are similar to those reported for children from other countries (*6–10*) and are comparable to those observed in children infected with other respiratory viruses, with a predominance of bronchitis or pneumonia (*11*,*13*,*15*). Our results indicate several risk factors for HBoV infection. Consistent with cases reported in the United States (*10*), 57% of our HBoV-positive patients were ≤12 months of age. Chest radiographs obtained from all 12 patients had abnormal findings. Major diagnoses were pneumonia, bronchitis, and bronchopneumonia. Collectively, these findings support the notion that HBoV infection may be associated with lower respiratory diseases, as suggested by Allander et al. (*1*). We did not find any association between the viral loads and disease severity and could not explain the difference in viral loads among specimens. Nevertheless, the viral loads in serum specimens were similar to those from NPA specimens in the 2 HBoV-positive patients who had both serum and NPA specimens. In our study, 29% of patients had diarrhea, which was also reported in 16% of HBoV–positive patients in the United States (*10*). Unfortunately, we did not collect stool specimens from HBoV-positive patients for viral detection.

Detection of HBoV in serum specimens from 2 patients suggests that HBoV may cause viremia, which was supported by the occurrence of intracranial infection in 1 patient. However, further studies are required to confirm whether HBoV indeed causes viremia. In addition, coinfection with human coronavirus 229E was identified in 2 of the 21 HBoV-positive children in our study. Although RSV, HMPV, and human coronavirus 229E were detected in 13.5%, 7.9%, and 6.0%, respectively, of the patients, no other children were found to be coinfected with HBoV and another virus. This rate of coinfection is lower than that reported for other countries (*1*,*6*,*7*,*10*). Whether seasonal or other factors might account for this difference remains to be determined.

In agreement with previous findings in other countries (*1*,*6*,*7*,*10*), results of our study indicate that HBoV is a conserved virus. Additional epidemiologic studies in different regions and sequence analysis of other genes are required to investigate the overall distribution, seasonality, and genetic variations of HBoV and to examine the origin of current HBoV endemics.

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Salmon Aquaculture and Transmission of the Fish Tapeworm

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Aquaculture of salmon constitutes a rapidly growing worldwide industry with an expanding globalized market (*1*,*2*). Although this industry has several economic benefits, according to recent reports it is also accompanied by effects that are detrimental to human and animal health and the environment (*1*,*2*). Aquaculture has been implicated in the transmission of infectious diseases. For example, in caged fish aquaculture, bacterial and parasitic diseases can be transmitted to wild fish (*1*,*2*). Furthermore, aquaculture-raised fish may be susceptible to the microorganisms and parasites of wild fish (*1*,*3*). However, in spite of the accepted fact that parasitic worms can be transmitted to humans by free-ranging fish (*4*), until recently, few examples have been reported of pathogens that could be transmitted to humans directly by the products and subproducts of salmon aquaculture. I discuss here information indicating that salmon aquaculture may be involved in expanding the range of fish tapeworm infections in nature and to humans.

Several recent publications report outbreaks of human cases of infection by the fish tapeworm *Diphyllobothrium latum* in Brazil (*5*–*9*). These infections have been epidemiologically linked to consumption of raw salmon produced by the aquaculture industry in southern Chile, thousands of miles away (*5*–*9*). Infections by *D. latum* have been detected in several cities in Brazil (*5*–*9*), and in a tourist who traveled there from Europe (*10*). These cases of diphyllobothriasis are noteworthy because this parasite was totally unknown to clinicians and parasitologists in Brazil, where it does not appear to have an endemic life cycle (*5*–*9*).

D. latum is transmitted to humans by plerocercoid larvae present in fish meat and visceral organs (www.dpd.cdc.gov/dpdx). *D. latum* and the closely related sea gull tapeworm, *D. dendriticum*, have well-established endemic life cycles in a series of glacial lakes that dot Region XIX and Region X in northern Chilean Patagonia. Infections with these parasites have been detected in this geographic area since the 1950s in persons who ingested uncooked fish from these lakes and also in animals (*11*–*14*). The link that closes the epidemiologic chain between the Brazilian outbreak of fish tapeworm infections and the aquaculture of salmon in southern Chile is that some of the freshwater lakes where *D. latum* and *D. dendriticum* are endemic are used to grow the freshwater stages of juvenile salmon, or smolt, in cages (*15*). Smolt are temporarily grown in these lakes to accelerate their growth before they are transported to cages in the sea where the salmon will reach adult stages. The practice of growing smolt in freshwater lakes appears to be unique to Chilean salmon aquaculture; in other salmon aquaculture settings, smolt are grown in tanks containing filtered water.

During the past 55 years, work by Chilean parasitologists has demonstrated that native species and introduced salmonid fish are infested with *Diphyllobothrium* plerocercoids in these lakes (*11*–*14*). Moreover, the other intermediary hosts of the fish tapeworm, the calanoid copepods *Diaptomus diabolicus* and *Boeckela gracilipes*, are also abundant (*16*). Native and introduced fish ingest copepods containing procercoid larvae that develop into plerocercoids (*16*). The fish tapeworm life cycle is subsequently closed in these lakes when humans and animals, the definitive hosts of these fish tapeworms, ingest infested fish (*11*–*16*). The persistence of the cycle of *D. latum* in these lakes is facilitated by the release of untreated sewage, which deposits stools of infected humans containing high concentrations of fish tapeworm eggs in the water (*11*–*17*).

The Brazilian studies did not detect *Diphyllobothrium* plerocercoids in several samples of Chilean salmon tested after the first human cases of diphylobothriasis appeared (*7*). However, this failure may have resulted from limited sampling or temporal and spatial variability in the infestation of the salmon with plerocercoids (*11*–*17*). Nonetheless, recent work in Chile has demonstrated the presence of *Diphyllobothrium* plerocercoids in rainbow trout raised in aquaculture, which suggests that aquacultured fish can

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become infected with these parasites (*18*). In Chile, infestation with *Diphyllobothrium* plerocercoids has also been detected in coho salmon living in the wild, a nonindigenous species raised originally in aquaculture that escaped from pens (*19*). Larvae of another fish tapeworm, *D. pacificum*, whose definitive hosts are large marine mammals such as sea lions and fur seals, have been detected in marine fish in Chile (*20*). Salmon aquaculture sea cages attracts these large mammals, creating the possibility for the parasite life cycle to occur in the environment around the salmon cages (*20*). However, this is an unlikely scenario for the spread to human populations, because the fish tapeworms identified in the patients in Brazil had the morphologic characteristics of *D. latum*, which as discussed above is one of the diphyllobothrium endemic in the lakes of southern Chile (*5–9*). These findings suggest that the aquaculture of salmon in southern Chile has expanded the species range of infestation by diphyllobothrium to nonindigenous salmonid fish species introduced by the aquaculture industry (*18*,*19*) and that the escape of infected fish from aquaculture sea cages has probably resulted in the expansion of the geographic range of the disease in Chile (*19*). In turn, the marketing of Chilean aquacultured salmon in Brazil has expanded the range of this human disease to a geographic region where this pathology was until now absent (*5–9*).

Traditionally in Europe and North America, infections with fish tapeworms were incurred during the preparation of gefilte fish by Jewish women who tasted bits of uncooked freshwater fish and thus ingested plerocercoids (*21*). In Chile, infestation of humans with the fish tapeworm in the *D. latum*–endemic area results from ingestion of raw and smoked fish, and in the Brazilian outbreak all the case-patients had previously eaten salmon sushi. Marinated ceviches may also be able to transmit infecting plerocercoids (*5–9*,*11–17*). The disease in humans can be prevented by cooking the fish at a temperature of 54°C to 56°C for 5 minutes (*21*). Alternatively, the plerocercoids can be destroyed by blast-freezing the fish at –35°C for 15 hours and by regular freezing at –20°C for 7 days before consumption (*22*).

Thus, to avoid new human outbreaks of fish tapeworm in other geographic areas where this parasite does not exist, salmon originated from aquaculture should not be eaten raw, at least not until it has been frozen under the conditions discussed above. Assuming the epidemiologic information presented here explains the appearance of the fish tapeworm outbreak in Brazil, it would be preferable, in terms of sanitation, for the Chilean aquaculture industry to stop growing salmon smolt in the lakes in the areas where diphyllobothriasis is endemic in humans and animals (*11–17*).

This epidemiologic event may also be understood as a cautionary tale and an additional example of the dangers entailed by the globalization of food supply and of the rapidly changing global eating habits that facilitate the distribution of human and animal pathogens worldwide. The expansion of diphyllobothriasis-endemic areas in Chile may, in turn, facilitate the appearance of future outbreaks of this disease as the aquaculture industry expands to these new infested areas and the market for Chilean salmon enlarges worldwide. The increased popularity of eating uncooked fish in sushi and ceviche will also be a factor in the emergence of future outbreaks of this disease (*4*,*21*). As has been the case with other human infectious diseases disseminated by the industrialization of animal husbandry, this outbreak of diphyllobothriasis could have been prevented by use of existing information, including that concerning the endemic nature of diphyllobothriasis in the lakes of southern Chile and its transmission by raw fish.

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Thermal Death Point of *Baylisascaris procyonis* Eggs

To the Editor: In the past 20 years, *Baylisascaris procyonis*, the common intestinal roundworm of raccoons, has increasingly been recognized as a source of severe human neurologic disease that particularly affects children (*1*,*2*). Although human baylisascariasis appears to be rare, the devastating neurologic disease caused by this infection and the lack of effective treatment make it a disease of public health importance (*3*).

Adult raccoons infected with *B. procyonis* can shed millions of unembryonated eggs in feces daily (*4*). Once infective, eggs can remain viable in the environment for years and are resistant to most decontamination methods (*5*). Given the severe and untreatable nature of baylisascariasis, and the hardy nature of *B. procyonis* eggs, information on optimal methods to inactivate *B. procyonis* eggs is essential. To guide attempts at environmental decontamination as well as personal protection in the case of accidental or intentional contamination of drinking water supplies, we attempted to determine the thermal death point of *B. procyonis* eggs.

Experiments were conducted in which 150 μL each of embryonated eggs, at a concentration of 100 eggs per μL, were added to six 1-mL polypropylene tubes of sterile water. The 6 tubes were then added to a water bath at 35°C and allowed to sit for 10 min to equilibrate. Then the temperature of the water bath was slowly increased at a rate of ≈5°C per 7 min, and 1 tube was removed at each 5° increment from 37°C to 62°C. Eggs were then examined by light microscopy to determine whether the larvae were still viable, as judged by larval motility (Figure). The experiment was repeated by using a more objective assessment of viability through examination of hatched larvae. Inactivation was measured with a viability dye (methylene blue) exclusion method in which uptake of dye by larvae indicates cell death and inactivation. After the eggs were removed from the heat, the mammilated layer was removed through exposure to undiluted chlorine beach and then washed 5 times in 0.85% saline for 1 min at 600×*g*. Hatching was achieved by the glass bead method (*6*,*7*). Hatched larvae were then removed and mixed 1:1 with a 1:10,000 dilution of methylene blue. Viable larvae remained motile and had an intact cuticle that could not be penetrated by the stain, whereas nonviable larvae took up the methylene blue along the cuticle and stained blue (*8*).

The experiment was repeated by adding the heated water, in 5° increments between 37°C and 62°C, directly into the tube containing the eggs. The duration of exposure of the eggs to the water was <1 min. The eggs were then processed in the same manner as previously described and examined by light microscopy. All experiments were replicated.

All larvae remained viable in water up to 47°C; >75% of the larvae were viable at 52°C and 57°C; complete inactivation occurred at 62°C. When the heated water was added directly to the infectious eggs, all larvae remained viable up to 42°C, and most larvae were observed to be viable at 47°C and 52°C; complete inactivation occurred at 57°C.

These preliminary findings indicate that *B. procyonis* eggs have a thermal death point, ≤62°C, very similar to the thermal death point of *Ascaris lumbricoides* and *A. suum* (*9*). Given the widespread prevalence of *B. procyonis* in raccoons, the close association of raccoons with human populations, and the serious nature of infection, identification of the thermal death point of infectious *B. procyonis* larvae has important implications. Potential for human infection can be mitigated by decontaminating areas where *B. procyonis* eggs are known to be found. Health authorities and parasitologists are routinely contacted by citizens and organizations regarding concerns about areas that have been contaminated with raccoon feces, including yards, pools, and homes. Unfortunately, no comprehensive studies have been published that describe practical and effective methods for decontamination of areas where *B. procyonis* eggs are present. The recognition of complete inactivation of eggs at relatively low temperatures will provides guidance in circumstances in which natural or intentional contamination with *B. procyonis* eggs requires disinfection efforts and indicates that approaches well

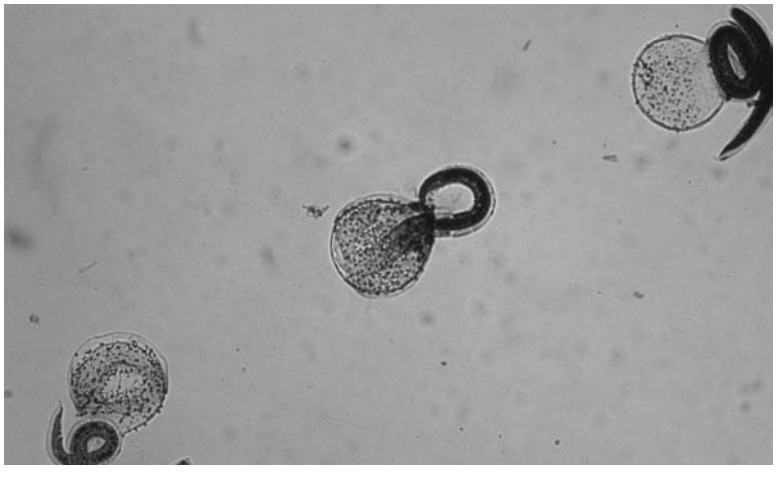

Figure. Hatched, stained, nonviable *Baylisascaris procyonis* larvae (magnification ×10).

short of incineration or boiling will be effective. Furthermore, these results suggest that temperatures achievable in point-of-use hot water heaters (household units) can deactivate infectious *B. procyonis* eggs, thus providing an option for maintaining safe drinking water during a possible event of bioterrorism or a "boil water advisory." However, further efforts are needed to determine the effectiveness of heat and other disinfection methods on inactivation of eggs in natural circumstances such as in feces or contaminated play areas including soil.

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Questioning Aerosol Transmission of Influenza

To the Editor: We have reviewed the literature cited in Tellier's Review of Aerosol Transmission of Influenza A Virus (*1*) and disagree that it supports the conclusions drawn regarding the importance of aerosols in natural influenza infection. In certain cited studies, researchers recovered viable virus from artificially generated aerosols; this is not evidence that aerosol transmission leads to natural human infection (*2*,*3*). By standard definitions, the rarity of long-range infections supports the conclusion that effective aerosol transmission is absent in the natural state (*4*) (www.cdc.gov/ncidod/dhqp/gl_isolation_hicpac.html). The superior efficacy of inhaled versus intranasal zanamivir is referenced as support for the idea that the lower respiratory tract is the preferred site of influenza infection; however, 1 study cited is insufficiently powered, and the other 2 do not compare the intranasal and inhaled routes (*5*–*7*). The major site of deposition of inhaled zanamivir is the oropharynx (77.6%), not the lungs (13.2%) (www.gsk.ca/en/products/prescription/relenza_pm.pdf). In another flawed study (*8*), study participants naturally infected with wild-type virus are compared with study participants experimentally infected with an attenuated strain.

In a review of such relevance, critical analysis of confounding factors is necessary. The Alaska Airlines outbreak (*9*) is presented as proof of airborne influenza transmission; however, droplet/contact transmission remains plausible because passenger movement was not restricted and the index patient was seated in high-traffic area. In the Livermore Hospital study (*10*), serious confounders such as bed arrangements, number of influenza exposures, patient mix, and ventilation were not accounted for.

We encourage readers of Teller's article to review the relevant primary literature. We believe that the only reasonable conclusion that can be drawn at this time is that aerosol transmission does not play a major role in natural influenza epidemiology. Whether aerosols play any role in the transmission of influenza is a question demanding an answer; it is clear that we do not yet have that answer.

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In response: Coughing and sneezing during influenza produce virus-containing aerosols. In the laboratory, influenza virus in homogeneous aerosols, free of large droplets, can infect volunteers at very small doses; studies of infectivity decay in aerosols show persistence for hours. These observations required the generation of artificial aerosols but were performed under conditions that do not enhance stability or virulence (*1*,*2*). Therefore, they have great relevance for natural infections.

The scarcity of infections that are transmitted long range in well-ventilated areas does not rule out infectivity of aerosol-size particles near patients. That only 13% of inhaled zanamivir is deposited in the lungs is not important: after inhalation, the zanamivir concentration throughout the respiratory tract is >10 μmol/L, orders of magnitude above the 50% inhibitory concentration (*3*). Intranasal zanamivir is protective against large droplets (*4*), which are trapped in the nose (*5*). The requirement for inhaled zanamivir in natural infections (*6*,*7*) points to aerosol contribution and to the lower respiratory tract as the preferred site.

Little et al. (*8*) compared the severity of natural illness caused by H3N2 strains from 1974 and 1975 to that caused by experimental intranasal inoculation from H3N2 strains from 1972, 1974, and 1975. The challenge strains underwent few passages; characterizing them as "attenuated" is incorrect.

Although large droplets probably accounted for some cases in the Alaska Airlines outbreak (*9*), this outbreak was remarkable for its high attack rate (72%) and for deficient ventilation, which would increase transmission by aerosols but not by large droplets. Passengers with influenza are common, yet with proper ventilation such an attack rate is uncommon.

During the Livermore Hospital study (*10*), respiratory infections other than influenza occurred in both groups. It was assumed that visitors and staff would provide equivalent introductions of the virus during the several months of the study; 4 study participants in the irradiated building seroconverted, but the virus did not propagate. The concern by Lemieux and colleagues about ventilation is odd because it would affect mostly aerosol transmission.

I concur with encouraging readers to review the original references. They make a compelling case for the importance of aerosol transmission. In contrast, no convincing data rule it out.

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Parvoviruses PARV4/5 in Hepatitis C Virus–infected Patient

To the Editor: Parvoviruses are small, nonenveloped DNA viruses that infect both vertebrate and invertebrate hosts. Until recently, parvovirus B19 and adeno-associated viruses, which belong to the genera *Erythrovirus* and *Dependovirus*, respectively, were the only known members of the family *Parvoviridae* that infected humans (*1*). However, 2 recent publications have identified 2 distinct, novel parvoviruses in humans by using the DNase sequence–independent single-primer amplification technique and a related method (*2*,*3*). The first of these viruses, termed PARV4, was observed in a patient with symptoms of acute viral infection syndrome after high-risk behavior for infection with HIV-1, although the patient was subsequently confirmed as negative for HIV-1 (*2*). The second parvovirus was identified in respiratory samples from children with lower respiratory tract infections and termed human bocavirus (*3*).

Parvovirus B19 is a frequent contaminant of plasma pools that are used in the manufacture of blood products, which results in high viral loads in pools and viral transmission in recipients of clotting factors (*4*). We identified PARV4 in such pools (*5*), albeit at a lower frequency and titer than parvovirus B19, when parvovirus B19 was not excluded by screening with nucleic acid amplification techniques. Sequence analysis identified a second genotype of PARV4, which we have termed PARV5, that shares 92% nucleotide identity with PARV4 (*5*).

PARV4 was originally identified in a plasma sample from a homeless, injection drug user with fatigue, night sweats, pharyngitis, neck stiffness, vomiting, diarrhea, arthralgia, and confusion (*2*). This person was coinfected with hepatitis B virus. In this study, we looked retrospectively for PARV4 and PARV5 in blood samples from a similar cohort of persons, many of whom were known to be infected with hepatitis C virus (HCV) (as determined by the presence of both HCV RNA and antibodies to HCV), and some of whom were intravenous drug users (IVDUs) (*6*).

Blood samples were collected from 26 cadavers in London and the surrounding area as part of a study to investigate the inhibition of nucleic acid amplification techniques for bloodborne viruses in tissue samples (*6*). The cohort was composed of 10 HCV RNA–positive IVDUs, 8 HCV RNA–positive non-IVDUs, 4 HCV RNA–negative IVDUs, and 4 HCV RNA–negative non-IVDUs (Table). Nucleic acid was extracted as previously described (*4*) by using the MagNA Pure LC instrument (Roche, Basel, Switzerland). PCR was performed with primers specific for the second open reading frame (ORF2) in the PARV4 genome (*2*), which is homologous to the VP1 capsid of parvovirus B19. Primers PVORF2F (5′-AGGAGCAGCAAACAAACTCAGAC-3′) and PVORF2R (5′-TCCTTCATCGCGGCTGTCACTAA-3′) amplify a 268-bp region of ORF2 (nucleotides 2710–2977, GenBank accession no. AY622943). The PCRs were performed and analyzed as previously described (*5*). The assay is highly specific (no cross-reactivity with parvovirus B19) and sensitive (detects 5–10 copies of PARV4 virus DNA per reaction).

PCR products were cloned, sequenced, and compared with the prototype PARV4. Two blood samples were positive for PARV4, and a third sample was positive for PARV5, with 99%–100% nucleotide identity. These positive samples were from HCV RNA–positive IVDUs (Table). The titer of PARV4 and PARV5 DNA in the positive samples was low and did not exceed >700 copies/mL of plasma, as determined by using a consensus TaqMan assay (J. Fryer, unpub. data). None of the other blood samples tested was positive for PARV4 and PARV5, including those for persons who were HCV RNA negative and not IVDUs.

In our previous study (*5*) of >130 fractionation pools (composed of thousands of units from screened healthy donors) for PARV4, the only positive pools were from North America and no European pools were positive for PARV4 or PARV5. These viruses may be present in such pools but diluted to undetectable levels. In

Table. Analysis of 26 cadavers for parvoviruses PARV4 and PARV5*

	No. positive/no. tested	
Group	PARV4 and PARV5 in HCV RNA–positive cadavers	PARV4 and PARV5 in HCV RNA–negative cadavers
IVDUs	3/10	0/4
Non-IVDUs	0/8	0/4

*HCV, hepatitis C virus; IVDUs, intravenous drug users.

the present study, PARV4 and PARV5 have been identified in blood samples obtained from persons from the United Kingdom. For parvovirus B19, there is evidence of persistent virus infection, at low levels, in bone marrow of previously exposed persons (*7*) and in plasma of immunocompromised and immunocompetent persons (*8*,*9*). There is also evidence for the lifelong persistence of parvovirus B19 (genotypes 1 and 2) in tissues such as skin and synovia (*10*). PARV4 and PARV5 virus genomes share only limited homology with parvovirus B19 (<30% amino acid similarity). Although they have been detected in blood and plasma, nothing is known about the role of these viruses in human disease or their ability to persist in infected persons, healthy or otherwise. Further studies will be required to determine the prevalence of PARV4 and PARV5 in healthy persons compared with its prevalence in those with chronic infections and at high risk, such as IVDUs, and to investigate the nature of persistence of these novel viruses.

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Saint Louis Encephalitis Virus, Brazil

To the Editor: Saint Louis encephalitis virus (SLEV), a member of the *Flaviviridae* family, is widely dispersed in the Americas (*1*,*2*). In Brazil, SLEV was first isolated in the 1960s from a pool of mosquitoes at the Amazon Basin. Subsequently, the virus was repeatedly isolated from animals and arthropods in the Amazon region and São Paulo state (*3*). Nonetheless, isolation of SLEV from humans is rare; only 2 isolates from humans were described before 2005. Each isolate was from a patient who had jaundice and febrile illness without any neurologic symptoms (*1*,*3*). Recently in São Paulo, SLEV was isolated from a patient who had an incorrect diagnosis of dengue fever (*2*,*4*).

Despite the rare isolation of SLEV from humans, antibodies to this virus have been found in ≈5% of studied populations in the north and southeast regions of Brazil. However, because of antibody cross-reactivity among different flaviviruses and the fact that this population is vaccinated against yellow fever and exposed to dengue virus (DENV), such results should be interpreted carefully. Nevertheless, in these areas, SLEV may circulate and infect humans, although most infections are undiagnosed (*1*,*3*,*5*).

In contrast to previous instances in which the disease was detected in only 1 patient, we describe the first community outbreak of SLEV in Brazil. The outbreak was detected in São José do Rio Preto (population 400,000), in northwest São Paulo state. This outbreak was concurrent with a large outbreak of DENV serotype 3 (DENV-3), which occurred during the first half of 2006, with >15,000 possible cases reported to public health authorities. During this time, we were involved in an epidemiologic study to monitor the disease. We tested ≈250 samples for DENV, and 65% were positive. We tested for SLEV only those patients who were in our hospital or those who were referred to us for SLEV testing after an initial diagnosis of SLEV or DENV. The protocol approved by our ethical committee allowed us to test only samples from these patients (process no. 300/2004).

We used a multiplex nested reverse transcription–PCR (RT-PCR) assay to identify the most common flaviviruses in Brazil (DENV-1, DENV-2, DENV-3, yellow fever virus) as well as DENV-4, Ilheus virus, Iguape virus, Rocio virus, and SLEV. Of 54 samples (49 serum and 5 cerebrospinal fluid [CSF]) that were negative for DENV and yellow fever virus, SLEV RNA was detected in 6 (4 serum and 2 CSF) (*6*). RT-PCR results were negative for all other tested flaviviruses. Sequences of the amplified SLEV cDNAs from the 2 CSF samples were determined by using an ABI377 automated sequencer (Applied Biosystems, Foster City, CA, USA). The resulting sequences (GenBank accession nos. DQ836336 and DQ836337) were identical and showed 96% homology to an Argentinean SLEV isolate (AY6-32544). All 6 SLEV-infected patients had an initial diagnosis of dengue fever or viral encephalitis; 3 had a diagnosis of viral meningoencephalitis, and the other 3 had signs of hemorrhagic disease (Table).

Dengue is widely disseminated in Brazil and causes large outbreaks almost every year. The high prevalence of antibodies in the Brazilian population (*1*,*3*,*6*) suggests that SLEV infections are being misdiagnosed; its importance is underestimated. Brazil has no SLEV surveillance programs, and health professionals do not usually consider SLEV among their differential diagnoses. This SLEV outbreak was detected in a large urban center and was not specifically linked to patients who dwell in pockets of tropical forests, as previously reported (*1*–*4*).

This outbreak may represent the first time that hemorrhagic signs have been linked to SLEV infections. SLEV-associated hemorrhagic manifestations have not been reported in the literature. However, of our 6 SLEV-infected patients, 3 had hemorrhagic signs. Substantiating a causal link between SLEV infection and such clinical manifestations is difficult because DENV is endemic in the studied region (*7*). Possibly, SLEV-infected patients with hemorrhagic signs may have been previously infected by DENV. No reports have linked hemorrhagic manifestations to sequential DENV and SLEV infections; this possible link needs to be carefully evaluated.

In Argentina, SLEV has been isolated several times from animals (*8*). In some regions, SLEV seroprevalence in humans is ≈13% (*9*), but the number of documented human infections is small (*10*). These findings indicate either that SLEV is more prevalent than reported or that SLEV is reemerging. The Brazilian cases may parallel the situation in Argentina.

Our results clearly indicate an SLEV outbreak among this local population in Brazil. This outbreak differs from isolated infections previously described and indicates that this disease may be more prevalent in Brazil. In fact, the number of samples tested for SLEV during this DENV outbreak was relatively small. Had more samples been investigated, more cases of SLEV infection might have been found. A more comprehensive epidemiologic study is required to fully assess the magnitude of SLEV infection in Brazil.

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Table. Clinical data, 6 patients with Saint Louis encephalitis, Brazil, 2006*

Patient no. (age)	Sample tested by RT-PCR	Date of hospital admission	Initial diagnosis at admission	Signs, symptoms, selected laboratory results
1 (27 y)	Serum	Feb 25	Dengue fever	Clinical: fever, abdominal pain, diarrhea Serum: AST 58 IU/mL, ALT 69 IU/mL
2 (7 mo)	Serum	Mar 06	Dengue hemorrhagic fever, viral encephalitis	Clinical: fever, abdominal pain, melena, petechiae, positive tourniquet test Serum: platelets 311,000 /mm^3, hematocrit 29% CSF: 13 cells/mm^3, lymphocytes 86%, monocytes 14%
3 (37 y)	Serum	Apr 22	Dengue hemorrhagic fever	Clinical: fever, headache, chills, myalgia, maculopapular rash, positive tourniquet test Serum: hematocrit 43%, platelets 280,000/mm^3 History: previous DENV infection (2002)
4 (34 y)	Serum	Apr 23	Dengue hemorrhagic fever	Clinical: fever, headache, chills, myalgia, maculopapular rash, positive tourniquet test Serum: platelets 141,000/mm^3, hematocrit 38%, AST 81 IU/mL, ALT 56 IU/mL
5 (5 y)	CSF	Jun 05	Viral meningoencephalitis	Clinical: fever CSF: 286 cells/mm^3, lymphocytes 60%, polymorphonuclear cells 37%, eosinophils 3%
6 (11 y)	CSF	Jun 07	Viral meningoencephalitis	Clinical: fever, facial palsy CSF: 12 cells/mm^3, lymphocytes 100%

*RT-PCR, reverse transcription–PCR; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CSF, cerebrospinal fluid; DENV, dengue virus.

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Cryptococcus gattii Risk for Tourists Visiting Vancouver Island, Canada

To the Editor: An unprecedented outbreak of *Cryptococcus gattii* genotype amplified fragment length polymorphism (AFLP) 6/VGII on Vancouver Island, British Columbia, Canada, is affecting both human and animal hosts with normal immunity (*1–3*). So far, >100 human cases, including at least 6 fatalities, have been reported by the British Columbia Centre for Disease Control (*4*), (www.bccdc.org, www.cbc.ca). Vancouver Island is a major tourist destination, with ≈7.5 million visits each year (www.bcstats.gov.bc.ca). We report the first known intercontinental transmission of *C. gattii* from this outbreak in a tourist from Denmark who visited Vancouver Island. This case indicates a potential risk for tourism-related acquisition.

A 51-year-old, HIV-negative, apparently immunocompetent man from Denmark, with known psoriatic gout and under treatment with a nonsteroidial antiinflammatory drug, was admitted to a hospital in Herning, Denmark, with chest pain radiating to the left shoulder and arm, lasting for 1 day. Six weeks before his admission, he returned to Denmark from a 3-week trip to Canada, during which he visited Victoria and surrounding areas on the eastern coast of Vancouver Island for 7 days. During their stay, the patient and his 3 fellow travelers visited gardens and studied the local natural vegetation.

During his stay in Canada, the patient had no symptoms, and symptoms had not developed in any of his family members as of October 2006. On admission to the hospital, his temperature was 38.2°C, and a chest radiograph showed 3 large nodular infiltrates suspect for malignancy or abscesses. Neither bacterial nor

fungal pathogens could be isolated from sputum by classic and molecular methods. After 4–5 days, his temperature was 40°C, a productive cough with dyspnea was noted, and his condition deteriorated. A chest radiograph showed progression of the infiltrates, and a computed tomography scan of the abdomen and chest showed infiltrates near the pleura, suggesting encapsulated fluid (Figure). An ultrasound-guided lung biopsy was performed, and mucoid material was aspirated. Microscopy and a culture from the aspirate showed a cryptococcal isolate. This isolate was further identified by internal transcribed spacer and D1/D2 sequencing, as well as amplified fragment-length polymorphism analysis (*2*). In addition, detailed genotyping was performed by using sequences of 7 genes (*IGS*, *CAP10*, *GPD1*, *LAC1*, *MPD1*, *PLB1*, and *TEF1*; GenBank accession nos. DQ861593–DQ861599) (*5*).

Extensive molecular research showed that this isolate belonged to the highly virulent AFLP genotype 6A (VGIIa) of *Cryptococcus gattii*, which is the major genotype involved in the Vancouver Island *C. gattii* outbreak (*1–4*). All 7 sequenced genes had a complete match with the sequence types specific for isolates involved in the Vancouver Island outbreak (*5*). Thus, we conclude that the pathogen was acquired during the patient's visit to Vancouver Island and imported to Denmark. The presence of 3 cryptococcal masses of more or less equal size suggests that the patient was exposed to a high concentration of infectious cells of *C. gattii*. The observed incubation time of 6 weeks is shorter than that was previously reported for infections related to the Vancouver Island outbreak (2–11 mo) (*4*). These observations, in combination with the absence of any known predisposing factor in this patient, such as smoking or treatment with corticosteroids, suggest that this specific AFLP6 genotype of *C. gattii* is highly virulent (*4*,*5*).

This case suggests a potential risk of tourists acquiring cryptococcosis while visiting Vancouver Island. Therefore, we recommend tourists and medical staff of healthcare centers worldwide be alert for symptoms of cryptococcosis after travel to Vancouver Island.

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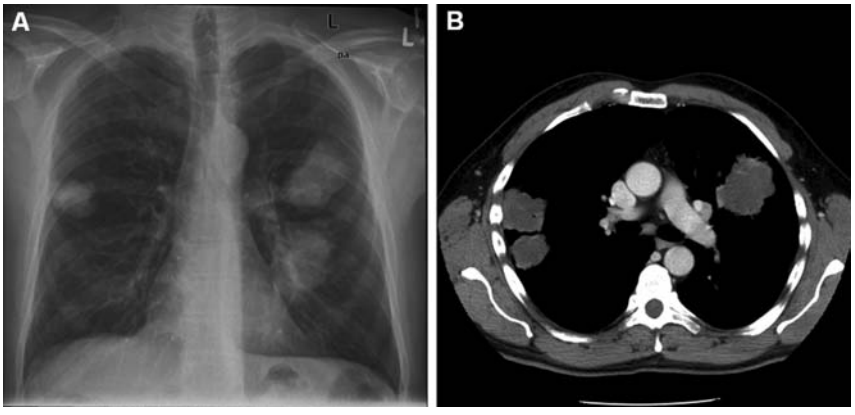

Figure. A) Chest radiograph and B) computed tomographic scan of the patient showing 3 nodular *Cryptococcus gattii* infiltrates near pleura.

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Clostridium difficile in Discharged Inpatients, Germany

To the Editor: Using discharge diagnoses from US hospitals in 2000–2003, McDonald et al. recently documented a dramatic increase in the rate of *Clostridium difficile*–associated disease (CDAD) (*1*). During the same period, a new strain of *C. difficile* was identified; this strain appears more virulent, at least in part because it produces higher levels of toxin (*2*).

To our knowledge, this strain has not been identified in Germany. However, to address this emerging threat, we conducted a similar analy-

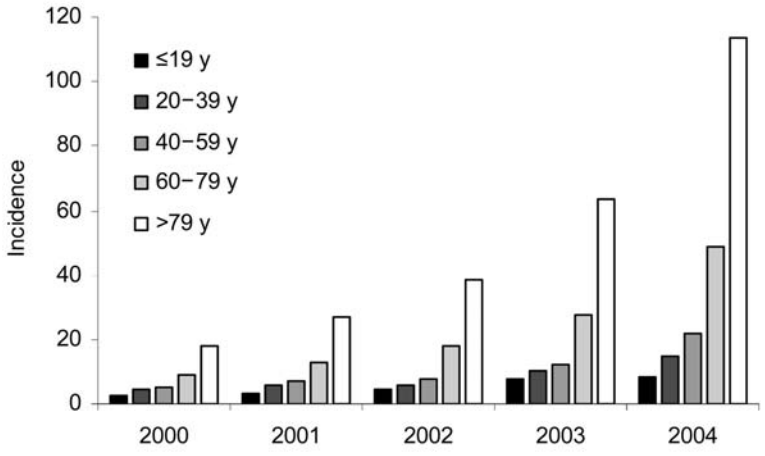

Figure. Incidence of *Clostridium difficile*–associated disease per 100,000 inpatients upon discharge from hospitals in Germany.

sis of discharge data to compare findings from the United States with data from Germany. We therefore determined the absolute number of inpatient discharges from all hospitals in Germany with the number of discharge diagnoses of CDAD reported in the national Statistische Bundesamt for the years 2000–2004. We then calculated the incidence of CDAD as a discharge diagnosis for each year and stratified our results by age groups (Figure).

Our results confirm the observations from the United States. The effect of *C. difficile* on illness of patients in hospitals in Germany has escalated dramatically. This is true especially for patients ≥60 years of age. This trend indicates the need for increased awareness of this pathogen and a concerted effort to control CDAD by reducing unnecessary antimicrobial drug use and implementing currently recommended infection control measures. It also highlights the need to develop more rapid and accurate diagnostic tools and more effective prevention and treatment strategies.

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Human Bocavirus in Febrile Children, the Netherlands

To the Editor: Human bocavirus (HBoV) is a recently discovered virus of the family *Parvoviridae*, genus *Bocavirus*, which appears to cause widespread respiratory tract infections (RTI) in children. In selected groups of children with RTI, detection rates have varied from 2.8% to 11.3% (*1–9*). However, the exact prevalence and pathogenic effects of this virus remain to be established.

During a prospective cohort study to evaluate the prognosis of fever at a general practice after-hours service in Rotterdam, nasopharyngeal swabs were collected from febrile children and tested for respiratory viruses, including HBoV. We report the incidence and clinical features of HBoV infection in these children.

From June 1, 2005, through January 16, 2006, all children 3 months to 6 years of age whose parents contacted the after-hours service because of fever, as reported by parents and not further defined, were eligible for inclusion in the study. Children were excluded when the parents could not communicate in Dutch (n = 77) and if the child had already been included within the past 2 weeks (n = 11). A research nurse visited the child at home within 24 hours of inclusion. The child was physically examined, and a nasopharyngeal swab and a blood sample for C-reactive protein measurement were collected. The parents subsequently recorded the child's symptoms in a diary for 7 days. The Central Committee on Research Involving Human Subjects, the Netherlands, approved this study.

Nucleic acids were isolated on a MagnaPure isolation station (Roche Applied Science, Penzberg, Germany) and subsequently analyzed by real-time assays. Detection of HBoV was performed by using a primers set and a

fluorescein amadite–labeled TaqMan probe directed against sequences of the NP1 gene. (Sequences are available from the corresponding author.) Testing was routinely done for the following viruses: influenza virus types A and B, parainfluenza virus types 1–4, respiratory syncytial virus (RSV) types A and B, adenovirus, coronavirus (OC43, 229E, and NL63), and rhinovirus.

Nasopharyngeal swabs were collected from 257 (81%) of 319 enrolled children. The overall virus detection rate was 52.9%; most frequently detected were adenovirus (11%), RSV-A (10.5%), parainfluenza virus type 1 (8.5%), and rhinovirus (8%). Five children were included twice; none of them was HBoV positive. The PCR for HBoV was positive in 4 children (1.6%), all boys. The characteristics of these children are shown in the Table.

All 4 children reported rhinorrhea and cough. Patient 1 reported abdominal pain, diarrhea (more than twice daily, with mucus), dyspnea, and a skin rash, along with respiratory symptoms. All symptoms lasted for >1 week. At physical examination, the research nurse evaluated the children to be not ill or (slightly) ill, based on standard criteria. Patient 1 had a skin rash and palpable cervical lymph nodes. Patient 4 had palpable lymph nodes and red tonsils.

Patient 1 was given amoxicillin for otitis media, and patient 4 received amoxicillin for tonsillitis, as diagnosed by the general practitioner on the basis of the patient's clinical symptoms, without bacteriologic confirmation. During a 1-week follow-up period, none of the patients sought further medical advice.

Our finding that HBoV may cause RTI is in accordance with the literature (*1–6*). Our findings support those of others in suggesting a role for HBoV in systemic infection, causing gastrointestinal symptoms and skin rash (*6,8*).

Our detection rate, in general practice, is lower than the rates reported from former studies of children with RTI (3%–10%) (*1–8*). Coinfection of HBoV and other viruses was found among 3 (75%) of 4 children (Table). In other studies, coinfection was found in 17.6%–55.6% (mainly adenovirus, RSV, and human metapneumovirus) (*1,2,5,7–9*). The other detected viruses could have caused the symptoms of patients 2–4. However, HBoV was the only detected virus in 1 child with respiratory symptoms, gastrointestinal symptoms, and rash, and therefore might be the pathogen. Considering the high amount of HBoV in patient 3, symptoms were likely caused by HBoV in this patient as well.

Our ability to analyze severity of disease in our study population was limited because all children had mild disease, and no child was hospitalized. However, all children reported a prolonged course of fever, >7 days or recurrent within 1 week. This finding is in contrast with the mean duration of fever of 2.6 days in the study of Arnold et al., which was based in a hospital setting (*6*). In our study, none of the HBoV-positive children received a diagnosis of bronchiolitis, pneumonia, or bronchitis, as in previous studies. None of the 4 children in our study was born preterm, compared with 19%–44% of HBoV-positive children in previous studies (*5,6*). None had a positive history for asthma or other underlying diseases, as did up to 50% of the children in previous studies (*1,6*).

In conclusion, HBoV was detected in nasopharyngeal swabs of 4 (1.6%) of 257 children <6 years of age whose parents contacted a general practice after-hours service. Our results suggest that HBoV might cause mild disease with respiratory and gastrointestinal symptoms and skin rash. Further research, not restricted to susceptible or hospitalized patients, is needed to clarify the prevalence and pathogenicity of this new virus in the general population.

Acknowledgments

We thank Ann Vossen for the analysis of the swabs.

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Table. Characteristics of children with human bocavirus infection, the Netherlands*

Patient no.	Age, y	Month detected	Symptoms	Body temperature (°C)	CRP (mg/L)	Positive PCR (log copies/mL)
1	2.3	Nov 2005	Earache, rhinorrhea, cough, sore throat, abdominal pain, diarrhea, skin rash, dyspnea, increased breathing rate	36.2	9	Bocavirus (4.20)
2	1.9	Nov 2005	Rhinorrhea, cough, sore throat, vomiting, increased breathing rate	38.8	26	Rhinovirus (7.57), RSV B (5.51), bocavirus (2.88)
3	1.3	Dec 2005	Earache, rhinorrhea, cough, headache, vomiting, skin rash, increased breathing rate	38.6	26	Bocavirus (6.72), parainfluenza virus 4 (4.11), adenovirus (2.00)
4	1.0	Jan 2006	Earache, rhinorrhea, cough, sore throat, abdominal pain, diarrhea, vomiting	37.7	88	Adenovirus (6.90), bocavirus (2.88)

*CRP, C-reactive protein; RSV, respiratory syncytial virus.

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Dengue Virus Serotype 3, Karachi, Pakistan

To the Editor: The global prevalence of dengue fever (DF) has grown dramatically in recent decades; DF is now endemic to >100 countries (*1*). Dengue hemorrhagic fever (DHF), a potentially lethal complication of dengue virus infection, was first recognized in Asia in the 1950s and is now a leading cause of hospitalization and death among children (*1*). During the past decade, DHF epidemics have occurred in China, Sri Lanka, India, the Maldives, Bangladesh, and Pakistan (*2–4*).

In Pakistan, an outbreak of DHF was first reported in Karachi in 1994 (*4*). Through mid-2005, 15–20 patients with DF or DHF were admitted each year to the Aga Khan University Hospital (AKUH), a tertiary care referral center in Karachi. Many more cases, however, may have gone unrecognized. Ours is the first report of dengue virus serotype 3 in Pakistan.

From September through December 2005, at least 3 major hospitals in Karachi, including AKUH, had a sudden increase in the number of patients with signs consistent with the World Health Organization definition of DHF: high fever, rash, epistaxis, gum bleeding, liver dysfunction, and thrombocytopenia (platelets <100,000/mm^3); most had evidence of capillary leakage in the form of raised hematocrit and pleural effusion with or without ascites (*5*). Because in Pakistan, Crimean-Congo hemorrhagic fever (CCHF) is an important differential diagnosis for hemorrhagic fever, most patients seen at AKUH received care in strict isolation and were empirically treated with ribavirin. At time of admission, blood samples were collected for serologic testing for dengue virus and reverse transcription (RT)–PCR testing for CCHF virus. The first 5 samples, collected during the initial 2 weeks of the outbreak, were also sent to the Special Pathogens Reference Unit, Centre for Emergency Preparedness and Response, Health Protection Agency, Salisbury, United Kingdom, for diagnostic confirmation. In the absence of a local surveillance and disease notification system, the number of patients with suspected DHF at different hospitals in Karachi could not be ascertained.

Of the 106 patients who had a clinical diagnosis compatible with DHF (*5*), 9 (8.5%) died and 97 (91.5%) recovered. Patients with possible DF (fever, mild thrombocytopenia with platelets >100,000/mm^3) were not admitted and were treated as outpatients. Dengue virus infection was confirmed for 42 of the 106 patients. Serum samples from 39 patients contained anti–dengue virus immunoglobulin M (IgM) antibody (Chemicon, Temecula, CA, USA). Diagnosis for 6 of these patients was confirmed by using immunoblot tests (Dengue IgM Blot and Dengue IgG Blot, Genelabs Diagnostics, Singapore). Of the 9 patients who died, 6 had dengue IgM and IgG according to immunoblot testing, and 3 had dengue IgM according to ELISA. Diagnoses for 3 additional patients were confirmed by RT-PCR.

An RT-PCR assay specific for dengue viruses (*6*) was used to amplify the C/PrM/M region of the genome and produced PCR products of the expected size in 3 patient samples: 2 (K1 and 2) from Karachi and 1 (B) from Balochistan. The PCR products were sequenced, and data were subsequently placed in GenBank under accession numbers DQ469827 for D3418-05 (patient K1), DQ469828 for D3419-05 (patient K2), and DQ469826 for D3417-05 (patient B). These data were compared with those in databases by using the basic local alignment search tool for nucleotides (blastn), with default settings (*7*). For

each sequence analyzed, the lowest Expect (E) value showed significant similarity with a dengue serotype 3 isolate from India in 2004 (DQ323042). A phylogenetic tree was constructed with a collection of dengue sequences (online Appendix Table, available from www.cdc.gov/ncidod/EID/13/1/182-appT.htm). Phylo-genetic relationships between sequences are depicted in the Figure. Sequences from the 2005 outbreak are most similar to those from Indian strains of dengue serotype 3, which were isolated in Delhi.

An unexpected finding was the detection, at both AKUH and the UK Special Pathogens Reference Unit, of dengue-3 and CCHF virus RNA in the sample from patient B. CCHF is endemic to the rural Balochistan province of Pakistan, where DF has been documented (*8*). In the absence of information on the current dengue situation in Balochistan and given the increasing dengue activity in Karachi, a similar increase can be assumed for Balochistan. Possible introduction of dengue serotype 3 in a CCHF-endemic area resulted in dual infection in patient B, who essentially had clinical and laboratory features compatible with DHF. Patient B received ribavirin and recovered. Our results suggest that the 2005 outbreak of DHF in Karachi, Pakistan, was caused by strains of dengue virus serotype 3 related to those circulating in India (*9*).

Acknowledgments

We thank Aslam Khan, Maqsood Bhatti, and Roshan Hadwani for their contributions to this work.

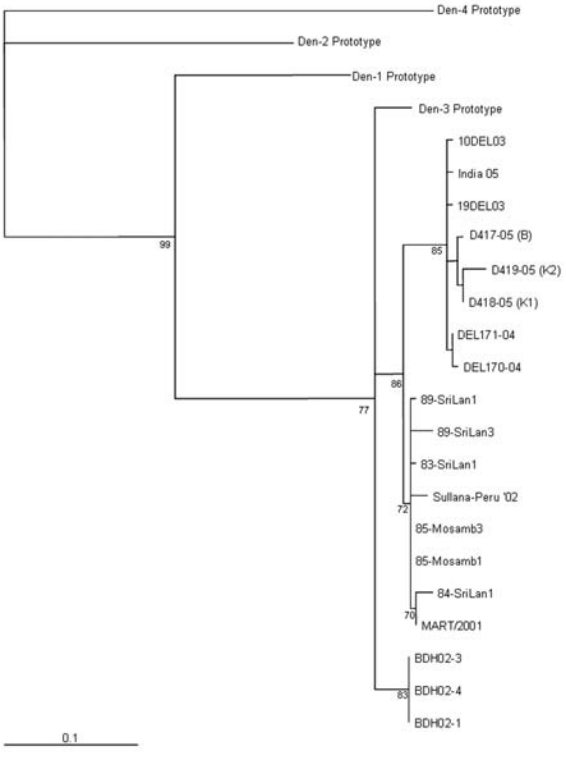

Figure. Maximum-likelihood phylogenetic tree of established dengue virus 3 serotypes and new sequences from Pakistan identified in this study. The tree is based on a 238-nt sequence alignment comprising the C/PrM/M gene (nucleotides: 179–417 dengue virus 3 prototype [NC_001475]). The bar shows the number of substitutions per bases weighted by the Tamura-Nei algorithm. Horizontal distances are equivalent to the distances between isolates; numbers at nodes indicate support values for the branch of the tree inferred at the node. The origins of the sequences used to reconstruct this tree are detailed in the online Appendix Table.

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Disease Ecology: Community Structure and Pathogen Dynamics

Sharon K. Collinge and Chris Ray, editors

Oxford University Press, Cary, North Carolina, 2006
ISBN: 0198567073
Pages: 227; Price: US $124.50

The disciplines of community ecology and epidemiology treat complex interactions among species, so a synthesis and integration of the 2 fields are long overdue. Because each field has insights and inferences to offer to the other, such an integration could be mutually beneficial and yield important steps toward a predictive and profound understanding. This book links an interesting framework for analyzing species' interactions (chapter by R. Holt and A.P. Dobson) with a series of case studies regarding many host-pathogen systems, including both well-known and more novel examples. As such, this volume is a ripe field for taking the first steps toward a synthesis.

Several of the case studies are nothing short of fascinating. For example, the studies of microbial communities in ticks (chapter by K. Clay et al.) and mosquito blood meal sources as indicators of arbovirus hosts (chapter by R.S. Unnasch et al.) are impressive demonstrations of the power of melding new molecular tools with more classical epidemiologic studies. Likewise, the studies of Nipah and Hendra viruses (chapter by P. Daszak et al.) and plague (chapter by C. Ray and S.K. Collinge) offer interesting views into complex disease transmission systems. Although a parallel chapter summarizing the complex community and environmental interactions underlying hantavirus transmission would have been a nice complement, the biggest shortfall is that few of the chapters manage to link strongly to the theoretical ecologic framework offered in the chapter by R. Holt and A.P. Dobson.

More generally, the book is attractively composed and appears to be bound well and printed on quality paper. For the size and content, though, the price is quite high—I suspect that this volume will be a valued addition to any library but is perhaps unlikely to be purchased by many people. This book will, I hope, be a first step toward a new synthesis of 2 seemingly distant but intimately related fields of inquiry, and at the very least represents an intriguing compendium of well-developed case studies of the complexities of disease systems.

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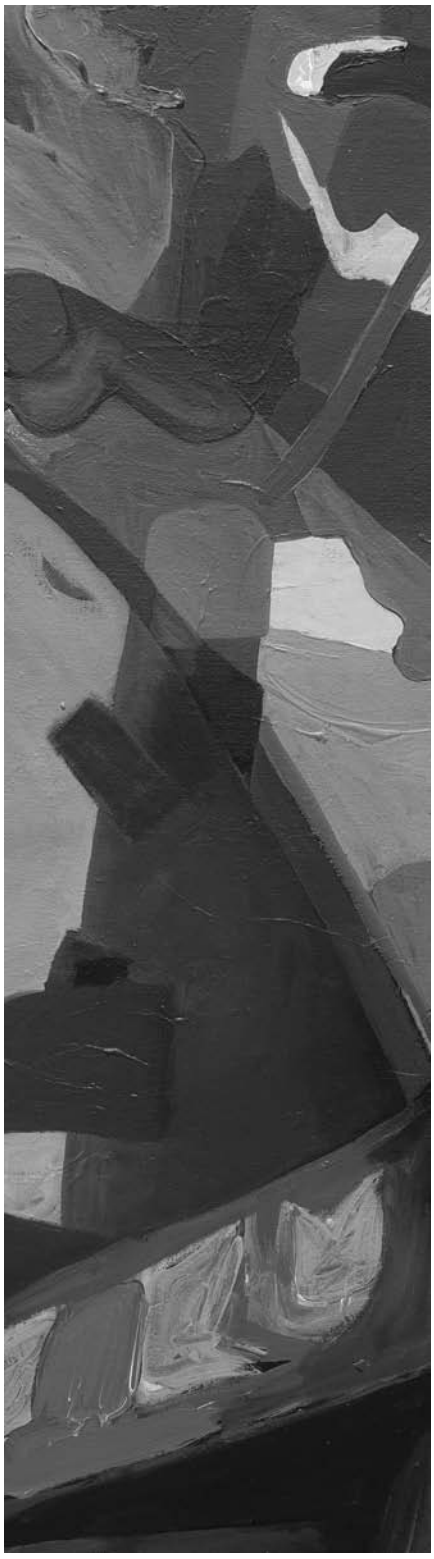
Correction: Vol. 12, No. 11
In "Susceptibility of North American Ducks and Gulls to H5N1 Highly Pathogenic Avian Influenza Viruses," by Justin D. Brown et al., an error occurred. In Table 1, morbidity, mortality, and virus isolation data for Mongolia/05 H5N1 HPAI virus from mallard ducks were omitted.

The corrected table appears in the updated article at http://www.cdc.gov/ncidod/EID/vol12no11/06-0652-T1.htm

We regret any confusion this error may have caused.



Jack Shadbolt (1909–1998). Toward Totem (1983–1986) (detail)**.** Acrylic paint on canvas (177.7 cm × 127.4 cm). UBC Museum of Anthropology, Vancouver, British Columbia, Canada. Photo: Bill McLennan

Exploding Totem in Art and Biology

Polyxeni Potter*

"What could be better than dying in the place you spent your life working…?" Jack Shadbolt's wife Doris mused, as she and friends moved his bed into the studio during the final days of his life. "When I can't paint, I don't want to live," he had said (*1*). Around the bed was Blue Breaking, a triptych he had painted, filled with shadowy forms, "like butterflies' footprints." A few days later, Shadbolt died of heart failure in Burnaby, British Columbia, where he had lived for more than 50 years.

Born in Shoeburyness, England, Shadbolt grew up in Victoria, Canada, to become an iconic figure, not only as artist but also as author and teacher. He began painting early in life, copying scenes from calendars with his father, a craftsman and amateur painter. He studied at Victoria College and Normal School, but his career took off when he started sketching the Victoria area with his friend Max Maynard, who introduced him to Emily Carr[1] (*2*). "To come under the immediate spell of a famous artist one admires tremendously and, at the same time, encounters personally in one's own local community," he wrote, "is the most compelling influence for an artist" (*3*).

Shadbolt traveled widely. He studied in London and Paris and at the Art Students' League in New York City. He became interested in the work of Pablo Picasso and Joan Miró and is linked to these and other modern masters. During World War II, at work with the Canadian Army War Artists in London, he sketched war scenes, barbed wire fences, watchtowers, and the bleak life of prisoners. This brush with darkness affected his individual brand of modernism, "…when the bomb blows the building apart it abstracts it, the pieces fall back together again and you get a memory image of what was there but vastly altered and psychologically made infinitely more intense than the original thing" (*2*). Yet, throughout his long career, his main inspiration was the regional West Coast culture and indigenous arts, "The nearest symbolic mythology at hand" (*3*).

"The shapes of knowledge are always ineluctably local," wrote anthropologist Clifford Geertz (*4*). The artist draws strength from the community. The more intensely William Faulkner focused on his unpronounceable county in Mississippi, the more intelligible he became to readers in all corners of the world (*5*). Shadbolt knew that his authenticity was linked to his region.

Originated in his initial contact with Emily Carr, his complex relationship with Coastal Indian art was described in a journal entry, "The Indian mode of expressing things from the inside out, out of deep interior identification with the spirit of the image portrayed, gave me my inventive impetus as well as helping me with my personal mode of abstraction" (*6*).

Along with a New York group that included Barnett Newman, Mark Rothko, Adolph Gottlieb, and Jackson Pollock, Shadbolt saw tribal art as "timeless and instinctive, on the level of spontaneous animal activity, self-

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[1]See www.cdc.gov/ncidod/EID/vol10no8/about_cover.htm

contained, unreflective, private, without dates and signatures, without origins or consequences except in the emotions" (*7*). This group, the primitivists of the 1940s, promoted a genre that was not, as it appeared, about tribal art but about what Shadbolt called "the act of art" (*8*).

"Creating is not an exercise in aesthetics but an act of sensory discovery. Having made a right move, one parlays it to the next move, and the next, and the next, until the (visual) reality grows into an unexpected **thing** under one's hands" (*3*). Shadbolt explains the process, "I have very little of the mystic in me, but if there is any it is in my rapture when, in the work process, that state is attained where everything seems a part of everything, each form existing for itself but not being itself until it also answers every other form in a perfect coordination of reciprocal intent….The 'whole is greater than the sum of the parts' is what art is all about" (*3*).

Caught in a blur of vibrant color, Toward Totem, on this month's cover, seems a mirage of its original intent. In the forefront, jumbled parts—a raven's beak, a glaring eye—jut off a degenerating totem pole. Superimposed on a solid diagonal on the left, the explosive image lights up a new pole, defined by the glare on surrounding flat surfaces. The abstraction of an old artifact, Shadbolt's fantastic modern equivalent sprouting in the background seems unconstrained by tradition, animal, clan, or taboo, even as its emotional charge betrays the artist's affection for the original.

In traditional terms, "totem," most often an animal, or class of animals, has a special relationship with the clan. Common ancestor, guardian spirit, and helper of the clan, totem is dangerous to outsiders but recognizes and spares its own, thus earning emblematic status as family crest, symbol, or historical record. Abstracting the essence but not the naturalistic image of Northwest Coast totem, Shadbolt created a modern form, which unbeknownst to him, may be closer to biological reality than the original.

Microorganisms are the microbial equivalent of totem. Though originating with and in general supportive of the clan, they do not linger to protect their own. Under evolutionary, ecologic, and societal pressures, they disperse. *Cryptococcus gattii* is a case in point. Infections away from Vancouver Island (initial focus), in other parts of the Pacific Northwest, indicate that away from the clan, the organisms have been spreading to humans and animals through common agricultural practices (*9*).

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the February issue for the following topics:

Immune Cell Apoptosis Prevention as Potential Therapy for Severe Infections

Ineffectiveness of Insecticide-treated Nets and Indoor Residual Spraying for Malaria Vector, Benin

Invasive Group B Streptococcal Infection in Infants, Malawi

Deaths from Cysticercosis, United States

Human African Trypanosomiasis in Rural Community, Democratic Republic of Congo

Subclinical Infection with Avian Influenza A H5N1 Virus in Cats

Meningococcal Disease in South Africa, 1999-2002

Neutralizing Antibodies after Infection with Dengue 1 Virus

Avian Influenza Risk Perception, Europe and Asia

Waterborne Toxoplasmosis, Northeastern Brazil

Rickettsia parkeri Infection in Serviceman after Tick Bite

No Evidence of Avian Influenza A H5N1 among Returning US Travelers

Disseminated Neonatal Herpes Caused by Herpes Simplex Virus Types 1 and 2

West Nile Virus Surveillance in Clinic-admitted Raptors, Colorado

Complete list of articles in the February issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

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IMED 2007: International Meeting on Emerging Diseases and Surveillance
Vienna, Austria
Contact: info@isid.org or
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617-278-9113 fax
http://imed.isid.org/

February 27–March 2, 2007
5th Annual American Society for Microbiology (ASM) Biodefense and Emerging Diseases Research Meeting
Renaissance
Washington, DC, USA
http://www.asmbiodefense.org/7200.asp

March 7–9, 2007
6th International Symposium on Antimicrobial Agents and Resistance (ISAAR 2007)
Raffles City Convention Centre
Singapore
http://www.isaar.org

March 20–23, 2007
ISOPOL XVI: 16th International Symposium on Problems of Listeriosis
Marriott Riverfront Hotel
Savannah, GA, USA
Contact: 240-485-2776
http://www.aphl.org/conferences/ISOPOL.cfm

April 30–May 2, 2007
10th Annual Conference on Vaccine Research
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
http://www.nfid.org

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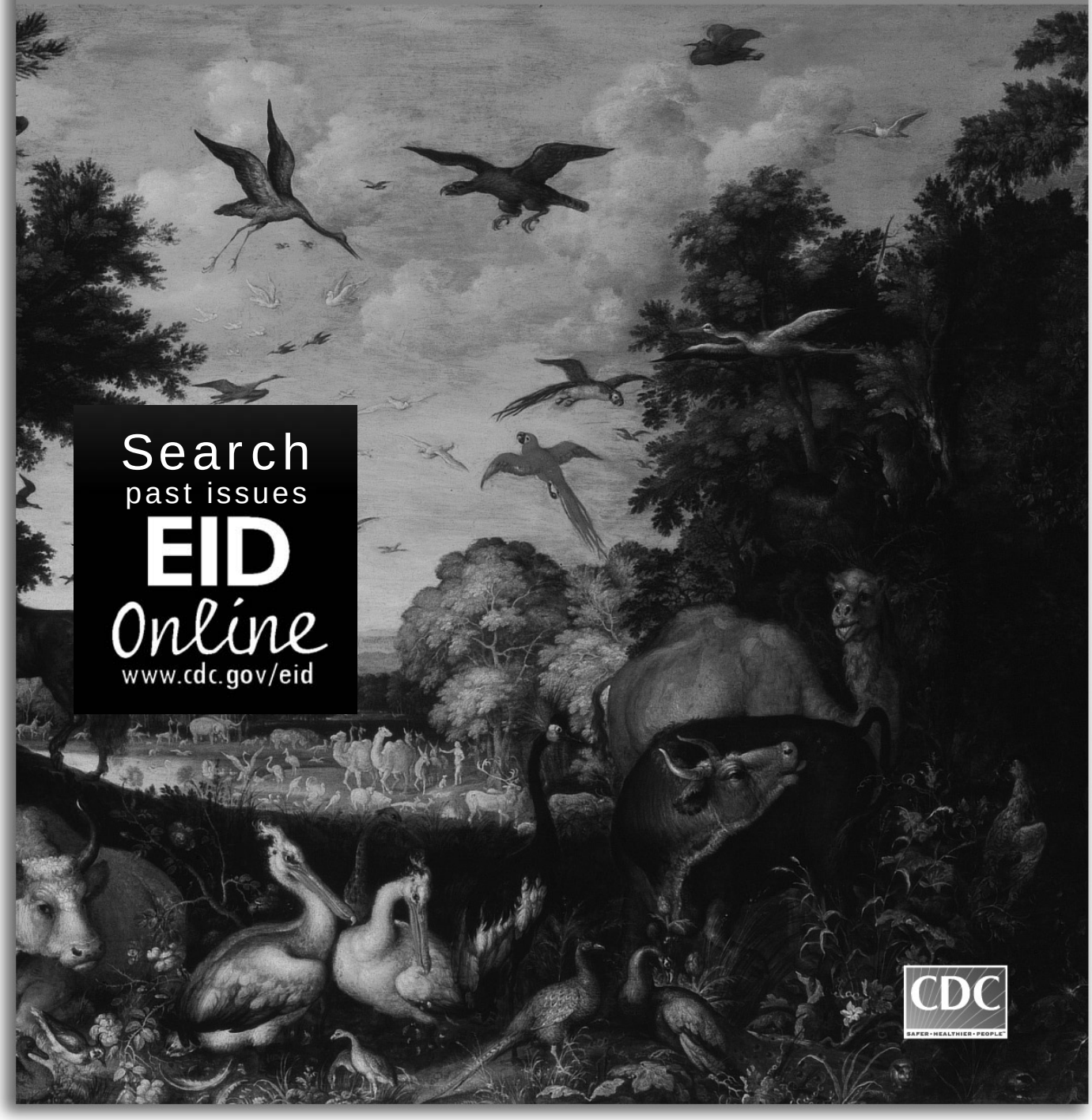
December 2006



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JOURNAL BACKGROUND AND GOALS

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Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- ★ New infections resulting from changes or evolution of existing organisms.
- ★ Known infections spreading to new geographic areas or populations.
- ★ Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- ★ Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - ★ Investigates factors known to influence emergence: 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.
   - ★ Reports laboratory and epidemiologic findings within a broader public health perspective.
   - ★ Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

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Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

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Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/ EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and 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. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. 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. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches 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.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.