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On the Cover

Alexis Rockman (b. 1962)
Manifest Destiny
Oil and acrylic on 4 wood panels
(2.44 m × 7.32 m)
Brooklyn Museum, New York
Courtesy of the artist

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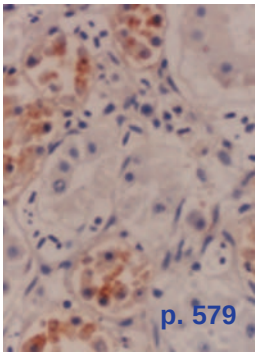

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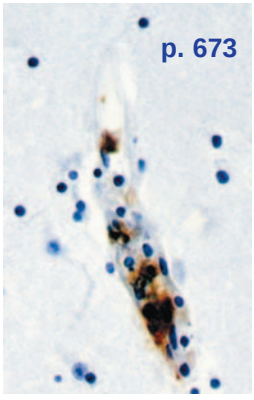

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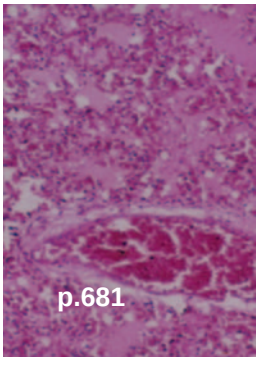

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Negligible Risk for Epidemics after Geophysical Disasters

Nathalie Floret,*† Jean-François Viel,*† Frédéric Mauny,*† Bruno Hoen,*† and Renaud Piarroux*†

After geophysical disasters (i.e., earthquakes, volcanic eruptions, tsunamis), media reports almost always stress the risk for epidemics; whether this risk is genuine has been debated. We analyzed the medical literature and data from humanitarian agencies and the World Health Organization from 1985 to 2004. Of >600 geophysical disasters recorded, we found only 3 reported outbreaks related to these disasters: 1 of measles after the eruption of Pinatubo in Philippines, 1 of coccidioidomycosis after an earthquake in California, and 1 of *Plasmodium vivax* malaria in Costa Rica related to an earthquake and heavy rainfall. Even though the humanitarian response may play a role in preventing epidemics, our results lend support to the epidemiologic evidence that short-term risk for epidemics after a geophysical disaster is very low.

Natural disasters are defined as "a disruption of human ecology which exceeds the community's capacity to adjust, so that outside assistance is needed" (*1*). Their classifications are geophysical (earthquakes, volcanic eruptions, tsunamis), hydrometeorologic (floods and wind storms), and geomorphologic (landslides). When covering these events, media outlets almost always mention the risk for epidemics that could raise the death toll well above an already staggering number of victims. According to the Centers for Disease Control and Prevention (CDC), an epidemic is the occurrence of more cases of disease than expected in a given area or among a specific group of persons over a particular period of time. For many, the word epidemic is associated with large numbers of deaths and poor living conditions, such as those that sometimes occur in refugee camps (*2*). The term outbreak is synonymous with epidemic and is sometimes preferred because it may not evoke the sensationalism associated with the word epidemic.

In addition to the media, other outlets draw attention to the risk for epidemics. In a letter published 3 weeks after the earthquake in Bam, Iran, in December 2004, the World Health Organization (WHO) warned that potential outbreaks of cholera, typhoid fever, malaria, and leishmaniasis were a major concern (*3*). WHO also issued a warning about the risk for epidemics that could develop after the 2004 tsunami: "There is an immediate INCREASED RISK of waterborne diseases, i.e., cholera, typhoid fever, shigellosis and hepatitis A and E.… Outbreaks of these diseases could occur at any moment" (*4*). The high risk for epidemics in areas affected by the tsunami was also pointed out by several papers published during the weeks after the disaster (*5*,*6*). Responding to WHO announcements, humanitarian agencies invested effort, time, personnel, and money in gearing up for potential epidemics, and considerable stocks of antimicrobial drugs, rehydration fluids for cholera patients, and vaccines were sent to the field.

However, not all experts support these alarming predictions. Some experts hold that disasters do not usually result in disease outbreaks but may increase disease transmission under certain circumstances (e.g., fecal contamination of water, spread of respiratory diseases in evacuation camps) (*7*). A similar point of view was published by VanRooyen and Leaning (*8*) and by de Ville de Goyet (*9*), who spoke of the myths propagated after disasters, some of which lead to an overestimation of the risk for epidemics.

No article has systematically reviewed published reports dealing with epidemics after geophysical disasters. The role played by outbreaks of infectious diseases in causing illness after geophysical disasters must be identified so that priorities can be defined and resources can be appropriately allocated. A systematic review of medical literature could help answer the question, "Is the risk for epidemics high after a geophysical disaster?" Consequently, we analyzed medical literature of the past 20 years and data provided by several websites and databases that compile outbreak alert messages and situation reports after disasters.

Materials and Methods

Literature Review

We screened Medline for articles that described outbreaks and epidemics, in both English and French, published from January 1985 through December 2004. We

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used the following search terms: (natural disaster* OR seism* OR earthquake* OR volcano* OR tsunami*) AND (infectious disease* OR communicable disease* OR epidemic* OR outbreak* OR vector-borne disease* OR arboviruses OR cholera OR malaria OR dengue OR West Nile virus OR Rift Valley fever OR hepatitis OR leptospirosis OR typhoid fever OR measles OR shigellosis OR scrub typhus OR plague OR diarrhea). We first selected all articles related to a specific earthquake, volcanic eruption, or tsunami, and then we examined them for any quantitative data on at least 1 infectious disease.

Screening Databases on the Internet

Data on epidemics and geophysical disasters were collected from the following databases: Emergency Disasters Data Base (Em-Dat, www.em-dat.net), WHO websites (http://who.int/), Disease Outbreak News (http://who.int/csr/don/en/), Centers for Disease Control and Prevention (http://www.cdc.gov/), Morbidity and Mortality Weekly Report (http://www.cdc.gov/mmwr/), and the Pan America Health Organization (http://www.paho.org). Research focused on events that occurred from January 1985 through December 2004. For disasters that were responsible for >100 deaths, we systematically screened reports of humanitarian agencies available on Reliefweb (http://reliefweb.int/).

Results

Literature Review

Although we found 233 articles in the Medline database related to our query, only 18 (7.7%) actually reported on infectious disease data collected after geophysical disasters. Common respiratory tract infections and diarrhea were the most frequently reported diseases. After the Bam earthquake in December 2003, a survey of 75,586 displaced persons described the main health problems encountered (*10*). Respiratory tract infections (mainly upper respiratory tract infections) were most frequently encountered; 11,320 cases were seen in the 10 weeks after the disaster. Researchers attributed the high number of respiratory infections to the freezing winter nights. Diarrhea was commonly diagnosed (1,224 cases with 174 cases of bloody diarrhea), but *Vibrio cholerae* infection was not observed. Similar findings were reported after the Chi-Chi earthquake in Taiwan in September 1999. An epidemiologic survey conducted in shelters showed that acute respiratory infections and acute gastroenteritis were the most common illnesses reported (*11*). They increased during the first 4 weeks, were significantly higher than those in unaffected neighboring counties, and then declined to baseline levels afterwards. An increase in gastrointestinal and respiratory infections also followed the 2001 earthquakes in El Salvador (*12*). Woersching and Snyder. conducted a 32-question survey in 100 households (594 persons) severely affected by the earthquakes. These researchers found that 30% of households assessed experienced ≥1 case of upper respiratory infection, and 22% experienced ≥1 case of diarrheal disease. This study also showed a high frequency of skin infections (31% of households). In a figure, authors reported 6 cases of cholera but did not state whether the diagnosis was biologically confirmed (no cases of cholera were officially identified in El Salvador in 2001) (*13*). An increase in respiratory and intestinal tract infections was also reported after the eruption of the Cerro Negro volcano in Nicaragua in April 1992, although this increase was not declared an epidemic (*14*). An assessment of the health consequences of the disaster showed that acute diarrhea was 6 times more frequent after the eruption, and medical consultations for acute respiratory disease were 3.6 times more frequent than before.

Two studies were performed to assess medical records of inpatients hospitalized during the first 15 days after the Hanshin-Awaji earthquake in January 1995 (*15*,*16*). Among infectious diseases, pneumonia was the most frequent illness diagnosed in inpatients (13%–21% according to the 2 surveys). An increased number of inpatients were also recorded in Papua New Guinea after the tsunami in July 1998 (*17*). However, no outbreak of communicable disease occurred.

A few studies investigated the prevalence of some pathogens in persons living in shelters. After the earthquake in Turkey in August 1999, an analysis of 1,468 stool cultures taken from persons with diarrhea showed 92% negative results; the most frequently isolated pathogens were *Shigella* spp. (4.9%). Phenotypic and genotypic comparisons of strains showed no cloning among the *Shigella* strains (*18*). Another study was conducted to determine the influence of the earthquake on patient admittance to the outpatient dermatology clinic. The incidence of skin infections was higher after the earthquake than it was in the same period 1 year later (*19*). A third study was performed to assess the prevalence of hepatitis A and E among children living in camps in northwestern Turkey (*20*). Hepatitis A and E virus seroprevalence was higher in the camps around Golyaka (68.8% and 17.2%, respectively) than in camps around Düzce (44.4% and 4.7%). The authors suggested that these differences were possibly related to delays in obtaining toilet facilities and piped water. After the earthquake in Colombia in January 1999, a parasitologic survey was performed in transitory housing camps from January 2000 to July 2001 (*21*). A high prevalence of *Giardia* spp. (60%) was found in stool specimens of 217 randomly selected children, and this prevalence was significantly associated with the use of communal toilets instead of individual toilets and with drinking municipal

water instead of water from individual tanks. The authors also stated that no outbreak of diarrhea, dengue fever, or malaria had occurred.

Only 3 articles reported outbreaks after a geophysical disaster. An outbreak of malaria (due to *Plasmodium vivax*) was reported after an earthquake in April 1991 in Costa Rica (*22*). From June 1991 through May 1992, a total of 3,597 cases were recorded, compared to 549 and 681 cases for the same period during the 2 preceding years. Even though heavy rainfall occurred in August 1991, authors suggested that the earthquake may have played a role. An outbreak of coccidioidomycosis was described after the 1994 earthquake in Northridge, California. The attack rate reached 30 cases per 100,000 inhabitants. According to the authors, being in a dust cloud and the amount of time spent in a dust cloud were associated with an increased risk of diagnosis (*23*). An outbreak of measles occurred after the eruptions of Mt. Pinatubo in June 1991. By August, many children of the Aeta tribe, who usually lived in isolation on the slopes of Pinatubo, had died in evacuation centers. The death toll reached 349 in the first 12 weeks, accounting for a death rate of 26/10,000 by the seventh week after the eruption (*24–26*). Deaths were caused by measles (31%), diarrhea (29%), and respiratory infections (22%). Living conditions were extremely difficult in camps: tents provided only minimal shelter from the elements, and evacuees experienced extremely hot days and cold, damp nights (*26*). Malnutrition and lack of basic sanitation also contributed to high death rates among children (*24*).

Database Research

From 1985 to 2004, 516 earthquakes, 89 volcano eruptions, and 16 tidal waves or tsunamis (including the December 2004 tsunami) were identified in the Em-Dat database. Sixty-three of these geophysical disasters were responsible for >100 deaths each, and 26 of them were responsible for ≥1,000 deaths (Table). Most of them (55 of 63) were reported on the ReliefWeb site. However, only 21 descriptions included medical data that covered at least the 3-month period after the disaster. Only 1 outbreak was reported: 19 cases of Crimean-Congo hemorrhagic fever, including 12 fatal cases, occurring in mid-March 1998 in a village in the district of Rustaq, Afghanistan, where an earthquake had occurred in February 1998. This outbreak was not caused by the earthquake but was detected because of epidemiologic surveillance that was implemented after the earthquake. No outbreak was reported after the other disasters, even in reports published up to 3 months after the events.

Among alert messages reported on the WHO outbreak news website, >300 concerned new outbreaks detected from 1997 to 2004 (we could not access previous WHO archives), and 90 of these concerned cholera outbreaks. We also found 779 epidemics reported in Em-Dat from 1985 to 2004. However, only 1 outbreak (of Crimean-Congo hemorrhagic fever [previously mentioned]) occurred in an area affected by a recent geophysical disaster.

Discussion

Although >600 geophysical disasters were recorded in the 20-year period we studied, we found no report in the medical literature in which major epidemics occurred in their wake. Only 2 outbreaks, one of *Coccidioides immitis* infection and the other of measles, could clearly be related to a preceding disaster (*23–26*). Since this result is at variance with the fact that iterative warning messages are broadcast after each disaster, we enlarged our search of the past 20 years by checking for alert messages from various institutional disease control databases and by screening reports available on Reliefweb.

The lack of reported epidemics in all the sources we analyzed begs an essential question: If epidemics can be expected to occur after a geophysical disaster, why are they almost never detected or reported? In general, epidemiologic studies are rarely conducted after disasters, and when they are, their methods are open to criticism. Most investigations only use cross-sectional survey methods without any reference to baseline status or control areas (*27*). In remote, rural areas of developing countries and in areas affected by war, surveillance systems are often not functioning, and an epidemic may go unnoticed. In addition, medical humanitarian agencies mainly focus on short-term assistance to affected persons, and most volunteers and experts usually leave the area within 3 months (*1*). At that time, basic sanitation facilities and access to basic hygiene may still be unavailable because of economic consequences of the disaster, and some affected victims may have to stay in camps and shelters for prolonged periods. Given the flaws of epidemiologic surveys described above, the hypothesis that unreported outbreaks occur a considerable time after the onset of a disaster must be examined. However, for some diseases, such as cholera, meningitis, and dengue, a large-scale outbreak would likely be detected by local health authorities or by humanitarian agencies working after the emergency phase. In that case, WHO would be notified or a field report would be made, even though the outbreak might not be reported in a medical journal.

Many arguments are usually presented to show that a geophysical disaster is a high-risk situation for epidemics. First, water and sanitation systems may be destroyed during the disaster, increasing the risk for outbreaks of waterborne diseases. However, natural disasters do not import diseases, and even in areas where a given disease is endemic, the worst-case scenario does not always occur

Table. Infectious diseases and outbreaks after major disasters (>1,000 deaths), 1985–2004

Date	Type of disaster	Location	No. deaths	Infectious diseases and outbreaks*
September 1985	Earthquake	Mexico	9,500	No report
November 1985	Volcano eruption	Colombia	21,800	Giardiasis, no outbreak
August 1986	Volcano eruption	Cameroon	1,746	No report
October 1986	Earthquake	El Salvador	1,100	No report
March 1987	Earthquake	Ecuador	5,000	No report
December 1988	Earthquake	Armenia	25,000	No report
June 1990	Earthquake	Iran	26,796	No report
July 1990	Earthquake	Philippines	2,412	No report
October 1991	Earthquake	India	20,005	No report
December 1992	Earthquake	Indonesia	2,500	No report
September 1993	Earthquake	India	9,748	No report
January 1995	Earthquake	Japan	5,297	Pneumonia
May 1995	Earthquake	Russia	1,989	No report
February 1997	Earthquake	Iran	1,728	No report
May 1997	Earthquake	Iran	1,100	No report
February 1998	Earthquake	Afghanistan	1,000	No report
May 1998	Earthquake	Afghanistan	4,700	No report
July 1998	Tsunami	Papua New Guinea	2,182	No outbreak
January 1999	Earthquake	Colombia	1,186	No report
August 1999	Earthquake	Turkey	17,127	Diarrhea, hepatitis A and E, skin infections, no outbreak
September 1999	Earthquake	Taiwan	2,264	Diarrhea, RTI, no outbreak
January 2001	Earthquake	India	1,500	No report
March 2002	Earthquake	Afghanistan	2,323	No report
May 2003	Earthquake	Algeria	2,266	No report
December 2003	Earthquake	Iran	40,000	Diarrhea, RTI, no outbreak
December 2004	Tsunami	Bay of Bengal	>200,000	No outbreak

*RTI, respiratory tract infection.

(*9*). Cholera is endemic around the Bay of Bengal, but cases of cholera are not constantly diagnosed in each village around the bay. Even if brackish water in the estuaries is an environmental reservoir for *V. cholerae*, toxigenic bacteria do not necessarily spread from them, should a tsunami occur. Many ecologic, sociologic, and seasonal factors are involved in the emergence of *V. cholerae*, and these factors rarely converge (*28*). Another surprising assertion is that tsunamis increase water sources for mosquitoes and therefore enhance the risk for vectorborne disease outbreaks. Water is an essential component of the mosquito environment. The characteristics of the water habitat, whether it is running or standing, clean or polluted, fresh or brackish, shaded or sunlit, permanent or intermittent, are the predominant factors determining which species of mosquito breed in it. Transient, polluted salt water generated by a tsunami will not sustain most species involved in transmission of dengue fever and malaria (*29*).

Second, natural disasters arguably lead to population displacement, formation of camps, overcrowding, and therefore, propitious circumstances for an epidemic. Settlements for victims of natural disasters, however, are not synonymous with refugee camps created to cope with complex emergencies (e.g., war, oppression, famine). In such complex emergencies, refugees may live for a long time in overcrowded conditions with a poor water supply and bad sanitary facilities. Usually refugees have been malnourished for weeks or even months before they reach the camps. Conditions like this in Goma, Zaire, produced epidemics of cholera, shigellosis, and meningitis, which caused thousands of deaths (*30*). For natural disasters, the shock is short-term, and communities can cope with problems more easily; predisaster health and nutrition status are better than in complex emergencies. The camps are often much smaller, which limits the spread of pathogens; access to food, safe water, and sanitary facilities is usually better; and most people stay only a few days or weeks. Nevertheless, crowded conditions and, in some cases, cold weather, favor the transmission of airborne diseases. The first response in preventing an outbreak of respiratory disease is to provide adequate shelter as soon as possible to injured persons and to prevent overcrowding. In our study, however, measles outbreaks were far less frequent than expected. Early implementation of immunization campaigns probably has a protective effect, and vaccination is recommended each time nonimmunized populations are moved to camps. Vaccination against influenza is not recommended even though it is a highly contagious disease and has a shorter incubation period than measles. Surprisingly, despite the lack of immunization campaigns we observed in our study, we never found any report of an

influenza epidemic whose spread was aided by a preceding geophysical disaster.

The "fact" that dead bodies are a potential cause of epidemics after a disaster is also almost always broadcast after major disasters. This "fact" is a myth, and depriving survivors of appropriate burial ceremonies for their relatives may administer yet another blow to already injured or weakened persons (*9*,*31*,*32*). The only situation in which handling corpses is a risk is during epidemics of infectious diseases such as cholera. Even in these situations, no reason exists to totally deprive families from honoring their dead if they follow certain precautions (*33*).

Our results, in line with those of Noji and de Ville de Goyet, lend support to the epidemiologic evidence that no high, short-term risk for epidemics follows a geophysical disaster. While most medical topics are usually discussed in small task groups of highly specialized experts, the debate about risk for epidemics after natural disasters is usually conducted by the mass media. The news industry is prone to emphasizing more dramatic and simplistic messages, and unjustified warnings will likely continue to be spread on the basis of an approximate assessment of risks. To respond more effectively to the needs of victims of natural disasters, the public, mass media, humanitarian organizations, and policymakers must be accurately informed regarding what actions are effective and what actions are futile.

Dr Floret is a public health physician at the University Hospital of Besançon, France. Her research interests are in health risk assessment for environmental pollutants, particularly dioxins.

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Potential Arbovirus Emergence and Implications for the United Kingdom

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Arboviruses have evolved a number of strategies to survive environmental challenges. This review examines the factors that may determine arbovirus emergence, provides examples of arboviruses that have emerged into new habitats, reviews the arbovirus situation in western Europe in detail, discusses potential arthropod vectors, and attempts to predict the risk for arbovirus emergence in the United Kingdom. We conclude that climate change is probably the most important requirement for the emergence of arthropodborne diseases such as dengue fever, yellow fever, Rift Valley fever, Japanese encephalitis, Crimean-Congo hemorrhagic fever, bluetongue, and African horse sickness in the United Kingdom. While other arboviruses, such as West Nile virus, Sindbis virus, Tahyna virus, and Louping ill virus, apparently circulate in the United Kingdom, they do not appear to present an imminent threat to humans or animals.

More than 550 arboviruses have been identified (*1*). Many are mammalian and avian pathogens, 4 of which (West Nile virus [WNV], Usutu virus, Sindbis virus [SINV], and Tahyna virus) circulate in the United Kingdom in resident and migratory birds (*2*,*3*). The only zoonotic arboviruses isolated from field material in the United Kingdom are the flavivirus Louping ill virus (LIV) (*4*) and the bunyavirus Uukuniemi virus (*5*). LIV is included in the tickborne encephalitis virus (TBEV) complex that includes Kyasanur Forest disease virus and Alkhurma virus. These viruses can cause encephalitis and hemorrhagic disease (*6*). Other human pathogens in the genus *Flavivirus* that will be discussed in this review include yellow fever virus (YFV), dengue virus (DENV), Israel turkey meningoencephalomyelitis virus, and Bagaza virus. European human and animal pathogens in the genus *Alphavirus* include SINV, Ockelbo virus, and Chikungunya virus and in the family *Bunyaviridae*, sandfly fever Naples virus (often referred to as Toscana virus), sandfly fever Sicilian virus, Crimean-Congo hemorrhagic fever virus (CCHFV), Inkoo virus, and Tahyna virus, which is widespread throughout Europe. Rift Valley fever virus (RVFV) and Nairobi sheep disease virus (NSDV) could be introduced to Europe from Africa through animal transportation. Finally, the family *Reoviridae* contains a variety of animal arbovirus pathogens, including bluetongue virus and African horse sickness virus, both known to be circulating in Europe. This review considers whether any of these pathogenic arboviruses are likely to emerge and cause disease in the United Kingdom in the foreseeable future.

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Factors That May Determine Arbovirus Emergence

Transmission of arboviruses between invertebrates and vertebrates imposes constraints on evolution and dispersal, which are reflected in their phylogenetic relationships (*7*). However, the influence of modern life on arbovirus emergence cannot be overemphasized. The following human activities may influence arbovirus emergence: 1) increased transportation of animals, humans, plants, arthropods, and other materials; 2) increased outdoor leisure activities; 3) reduced or nonexistent arthropod control programs; 4) deforestation; 5) reforestation; 6) land reclamation; 7) altered farming practices; 8) urbanization programs; 9) irrigation projects, including building dams or creating reservoirs and lakes; 10) military activities; 11) movement of military personnel and local populations in war zones; 12) natural disasters, such as flooding; and 13) the early effects of climate change (*8*).

Examples of Emerging Tickborne Arboviruses

TBEV complex viruses rarely cause disease in indigenous forest animals but may emerge as pathogens when they infect introduced species. Members of the complex

have evolved and dispersed westward across Asian and European forests during the past 3–5 millenia. This dispersion is driven by *Ixodes* spp. vectors that inhabit the moist forest undergrowth. Nymphal ticks may infect newly hatched larvae when they co-feed on forest animals. Since these vertebrates do not become sick or have detectable viremia, this direct method of vector infection is known as nonviremic transmission. In contrast, LIV and related viruses in Spain, Turkey, Greece, and nearby regions produce viremia and fatal encephalomyelitis in domesticated animals when infectious ticks feed on them (*9*). These animals have no genetic resistance or immunity to the TBEV-related viruses that have emerged from the forests (*8*).

Kyasanur Forest disease virus emerged in Karnataka, India, in 1957 after forests were cleared for urbanization and farmland reclamation. Arboreal monkeys scavenging on the exposed forest floor became infested with Kyasanur Forest disease virus–infected ticks from the undergrowth. Local inhabitants and veterinary scientists who examined dead and dying monkeys also became infected. A closely related virus, Alkhurma virus, emerged in Saudi Arabia in 1992, causing hemorrhagic disease in butchers who handled sheep imported into Saudi Arabia for the Hajj (*10*). Alkhurma virus was detected in ticks removed from imported sheep, but the country from which these sheep originated has not been identified.

CCHFV emerges sporadically in Africa, Asia, and Europe. This arbovirus may be transmitted to animals by ticks, especially members of the *Hyalomma* genus. Emergence of CCHFV largely depends on the transportation of livestock such as cattle and goats, on which infected ticks feed. Most cases of hemorrhagic disease occur in humans involved with the livestock industry. Since ≥21 tick species are present in the United Kingdom, and since viruses such as CCHFV can infect a wide variety of ticks, the introduction and emergence of CCHFV are not inconceivable.

NSDV (family *Bunyaviridae*, genus *Nairovirus*) is transmitted to sheep by *Rhipicephalus appendiculatus* and causes fever and gastroenteritis; the infection is often fatal. NSDV is distributed widely throughout Africa (*1*) but not elsewhere. However, the closely related Ganjam virus is found in India, which may imply that NSDV has been transported in ticks on animals imported to India from Africa. Both NSDV and Ganjam virus produce febrile illness with polyarthritis in humans.

Examples of Emerging Mosquitoborne Arboviruses

YFV circulates among simians and *Aedes* and *Hemagogus* spp. mosquitoes in the forest canopy and adjacent savannah regions of Africa and South America. In Africa, nonhuman primates do not become ill from YFV infection, presumably because the virus has adapted to the simian hosts. Nevertheless, human yellow fever epidemics do occur in Africa because the virus emerges sporadically from the jungles and savannah into rural and urban areas, where most of the human population is susceptible. In South American jungles, moribund monkeys are usually found immediately before local epidemics of yellow fever, implying that the virus recently encroached into the region. YFV was introduced into South America on ships that sailed from Africa during the past few centuries (*8*). Many European and North American ports observed cases of yellow fever. In some cases, the virus was carried on sailing ships from Cuba to Europe, causing localized outbreaks where the ships docked (*11*). Infections in Swansea (Wales) and St. Nazaire (France) resulted from the bite of *Aedes aegypti* mosquitoes released from the newly arrived ships. However, the disease immediately disappeared when the local temperature dropped and the tropical mosquitoes died. Thus, such viruses are unlikely to emerge in the United Kingdom.

The 4 DENV serotypes emerged more recently than YFV (*8*) and can generate epidemics in humans without depending on a sylvatic cycle in the jungles. Therefore, in contrast to YFV, which is maintained in the jungles of Africa and South America, DENV continues to be dispersed by humans and mosquitoes throughout the tropics and subtropics worldwide (*8*,*12*). Presumably, as *Ae. aegypti* and *Ae. albopictus* continue to spread, the geographic distribution of these viruses will also expand.

Since birds are highly mobile and can travel great distances in a relatively short time, they play a role in arbovirus dispersal. SINV, for example, is carried annually between South Africa and Scandinavia by migrating birds (*12*). SINV is rarely associated with disease in Africa. However, when cases of arthritis, polyarthritis, and fever associated with rash were recorded in Scandinavia, the antigenically closely related Ockelbo virus was isolated. Presumably, Ockelbo virus evolved from introduced SINV and subsequently became established in a local transmission cycle. In the New World, St. Louis encephalitis virus is also carried long distances by migrating birds. It was probably introduced into South America from Africa during the past few centuries and gradually dispersed by migrating birds to North America (*13*). More recently, WNV was introduced into the New York area, probably in 1999. The virus became established in birds and was rapidly dispersed north to Canada; south to Florida, Mexico, and the Caribbean; and west, reaching California in 2004 (http://www.cdc.gov/ncidod/dvbid/westnile/). The WNV that was introduced into New York was related to an Egypt/Israel strain that is also present in the Volga River Delta. Despite its high virulence in birds (particularly corvids), horses, and alligators in North America, WNV

circulates relatively harmlessly in the Old World, presumably reflecting co-evolution of virus and host over a long period.

Other emerging flaviviruses include Usutu virus, which appeared unexpectedly in Austria in 2000 (*14*), killing birds in Vienna and surrounding areas. Previously, Usutu virus had only been found in Africa. Israel turkey meningoencephalitis virus, isolated in Israel, is phylogenetically closely related to the African Bagaza virus (*7*). Thus, Israel turkey meningoencephalitis virus may represent the bird-borne version of Bagaza virus.

RVFV (family *Bunyaviridae*, genus *Phlebovirus*) is usually transmitted by *Aedes* spp. mosquitoes and occasionally infects livestock, producing epizootics with high death rates in sheep and other ruminants. Humans in areas where RVFV is epizootic may also become infected. Until 2000, RVFV was most commonly found only in eastern, western, and southern Africa. However, in September 2000, an outbreak was reported in Saudi Arabia and subsequently in Yemen, representing the first cases identified outside Africa and showing the potential for global dispersal.

Bluetongue virus (family *Reoviridae*, genus *Orbivirus*) is transmitted to domestic and wild ruminants by culicoide midges. Bluetongue virus epidemics can threaten entire livestock populations and were first described in South Africa after Merino sheep were introduced from Europe in the late 18th century. The virus subsequently dispersed globally, recently reemerging in Italy, Spain, and Portugal (*15*), most likely through the transportation of ruminants. Whether bluetongue virus will emerge in the United Kingdom likely depends on the effects of climate change on vector dispersal.

Arboviruses Most Likely To Circulate in the United Kingdom

The English Channel and North Sea are not barriers to migrant birds, bats, or commercial transportation systems. Therefore, most mainland European arboviruses could be introduced and established in the United Kingdom. The Table presents a summary of all recognized zoonotic mosquitoborne and tickborne arboviruses circulating in western Europe. While Uukuniemi virus has not been shown to be a human pathogen, it circulates widely in Europe, and seropositivity in humans has been demonstrated many times. YFV, DENV, RVFV, Ganjam virus, and NSDV are excluded from the Table because they only arise as imported viruses. Only LIV and Uukuniemi virus have been isolated from field specimens in the United Kingdom, probably because of limited investigation, although the other viruses in the Table have been isolated in Europe. Serologic evidence obtained from bird sera (*2*) implies that at least 3 additional arboviruses, WNV, Usutu virus, and SINV, circulate in the United Kingdom. Currently, TBEV has not dispersed west beyond the Alsace forests in France (*29*). LIV, Spanish sheep encephalomyelitis virus, and Greek goat encephalomyelitis virus, on the other hand,

Table. Zoonotic arboviruses identified in western Europe (not including Russia) by serologic testing or isolation*

Country	Seropositive†	Virus isolation	Reference
Sweden	SINV, TBEV, INKV, BATV	SINV, INKV, BATV	(*12,16*)
Finland	SINV, TBEV, TAHV, INKV, BATV	TBEV, INKV, BATV, UUKV	(*12*)
Norway	LIV, TBEV, TAHV, INKV, BATV	SINV, LIV, TBEV, INKV, BATV, UUKV	(*12,17*)
Poland	SINV, WNV, TBEV, UUKV	UUKV	(*12,18*)
Estonia		SINV	(*12*)
Austria	SINV, SFV, CHIKV, TBEV, USUV, TAHV, BATV	TBEV, USUV, TAHV, BATV	(*12,19*)
Czechoslovakia	SINV, WNV, TBEV, TAHV, BATV, LEDV	SINV, WNV, TBEV, TAHV, BATV, UUKV	(*12,20*)
France	TBEV, WNV, TAHV	TBEV, WNV, TAHV	(*12,21*)
Germany	TBEV, TAHV, BATV	TBEV, TAHV, BATV	(*12*)
Portugal	SINV, CHIKV, WNV, TAHV, BATV, TBEV	WNV	(*12,22,23*)
Spain	SFV, CHIKV, TBEV, SSEV, TAHV		(*12,24*)
Italy/Sicily	SINV, CHIKV, WNV, TBEV, TAHV, SFSV	SINV, WNV, TBEV, SFSV, TAHV	(*12,25*)
Yugoslavia	SINV, TBEV, TAHV, BATV	TBEV, TAHV, BATV	(*25–27*)
Albania	TBEV, TAHV		(*12*)
Hungary	WNV, TAHV	WNV, TAHV, UUKV	(*20,25*)
Romania	SINV, SFV, WNV, TBEV	TBEV, WNV, TAHV, BATV, LEDV	(*12,17,27*)
Greece	SINV, TBEV, TAHV, GGEV, CCHFV	CCHFV	(*12,28*)
United Kingdom/Ireland	LIV, SINV, WNV, USUV, TAHV, UUKV	LIV, UUKV	(*2,5*)

*Adapted and modified from Lundstrom (*12*), which contains a more comprehensive list of relevant references. SINV, Sindbis virus; TBEV, tickborne encephalitis virus; INKV, Inkoo virus; BATV, Batai virus; TAHV, Tahyna virus; UUKV, Uukuniemi virus; WNV, West Nile virus; SFV, Semliki Forest virus; CHIKV, Chikungunya virus; USUV, Usutu virus; LEDV, Lednice virus; SSEV, Spanish sheep encephalomyelitis virus; SFSV, sandfly fever Sicilian virus; GGEV, Greek goat encephalomyelitis virus; CCHFV, Crimean-Congo hemorrhagic fever virus; LIV, Louping ill virus.
†Positive reactions to dengue virus or yellow fever virus are not included as they are assumed to represent cross-reactions with WNV or other Japanese encephalitis complex flaviviruses.

represent more recent lineages of western European and Far Eastern TBEV that have emerged on the moorlands after the introduction of sheep and goats for grazing (*8*).

Three alphaviruses are listed in the Table, SINV, Semliki Forest virus, and Chikungunya virus. SINV has been isolated from field material in Scandinavia, Estonia, Czechoslovakia, and Italy. This virus is widespread throughout the Old World. In Albania, seroconversion to Chikungunya virus was detected in 5 patients with mild flulike symptoms (*12*); other findings of antibodies to Chikungunya virus have been reported, but evidence of cross-reactivity with other alphaviruses was not always tested. Among the remaining zoonotic arboviruses included in the Table, Tahyna virus is the most common. This virus is widespread throughout western Europe in humans and many nonhuman mammalian species and birds (*12*). Inkoo virus has only been found in Scandinavia, but few investigations have taken place elsewhere. While investigations for Batai virus have not been extensive, this virus is also widespread throughout western Europe (*12*).

Probability That Arboviruses Will Emerge as Epidemic Pathogens

Tickborne Arboviruses

Many arboviruses emerged relatively recently (*30*) and have therefore had little time to be dispersed to new environments, such as the United Kingdom. Although birds carry millions of ticks north and south annually between Africa and Europe, tickborne African bunyaviruses and flaviviruses have not yet appeared as notable pathogens in the United Kingdom. European, Siberian, and Far Eastern strains of TBEV have been isolated in Estonia, probably introduced by migratory birds (*31*). However, they have not caused substantial disease in these regions. TBEV is less virulent than LIV for sheep (*6*) and might, therefore, become less dominant than LIV if it were introduced into the sheep-grazing moorlands of the United Kingdom. Whether such introduced viruses would emerge may reflect the level of contact between humans and farm animals. Although ticks are less susceptible to the effects of environmental disruption, the specific region into which TBEV-carrying ticks are introduced might determine whether they survived and then reproduced. The United Kingdom has an abundant supply of *Ixodes* ticks, but the risk that virulent Far Eastern TBEV would be introduced, become established, and emerge as an endemic pathogen in UK forests is probably low. Alternatively, in the case of CCHFV, the fact that this virus has been isolated from ticks in Greece, the Balkans, and western Russia in the Volga Delta cannot be ignored. Other tickborne virus pathogens are presumably subject to the same qualifications.

Mosquito-, Sandfly- and Midge-transmitted Arboviruses

When one takes into account the information in the Table and the serologic evidence of antibodies against SINV, WNV, Usutu virus, and Tahyna virus in sera of migrant and resident UK birds, the likelihood is high that mosquitoborne arboviruses, particularly those associated with ornithophilic mosquitoes, such as *Culex* spp., circulate, albeit harmlessly, in the United Kingdom. Other arboviruses circulating in Europe that are not recognized in the United Kingdom include Toscana virus, bluetongue virus, and African horse sickness virus, which was probably introduced into Spain after the importation of infected horses.

Tahyna virus, which causes encephalitis in humans, is widespread across western Europe (Table). Birds and rabbits are vertebrate hosts. Since the movement of rabbits between the United Kingdom and mainland Europe is extensive, and since many bird migratory routes from Africa pass over the United Kingdom, Scandinavia, and mainland western Europe, Tahyna virus is most likely to appear, if not emerge, in the United Kingdom.

Why has none of these viruses yet emerged as a noticeable pathogen in the United Kingdom? Perhaps they have, and we do not realize it. Recent reports state that ≤40% of human deaths from viral encephalitis are attributed to a specific etiologic agent in the United Kingdom (*32*). Moreover, no systematic studies have been conducted to determine the extent to which Tahyna virus is circulating, even though the virus is known to be present in UK birds (*3*). In addition, on the basis of serologic tests (*2*), a SINV-like virus also circulates among birds in the United Kingdom, possibly vectored by *Culex* mosquitoes. In Scandinavia, this virus causes rash, fever, and acute polyarthritis in humans.

Necessary Arthropods for Arbovirus Emergence

At least 21 species of hard ticks and 3 argasids are recognized in mainland Britain and its islands (*33*). Most parasitize mammals, but at least 12 prefer birds, and 2 are regularly found on reptiles. In many parts of the United Kingdom, LIV has been isolated from *I. ricinus* ticks, which are believed to maintain LIV in the wild by nonviremic transmission during co-feeding (*34*). Other tick species, such as *Ornithodorus maritimus* and *I. uriae*, transmit bunyaviruses, nairoviruses, and orbiviruses among seabirds (*5*,*35*) that inhabit the cliffs around the UK coastline. However, these seabird-tickborne viruses do not cause disease in mammals in the United Kingdom. Nevertheless, the United Kingdom has a wide range of ticks that can transmit arboviruses.

At least 33 mosquito species are endemic in the United Kingdom (*36*), but their susceptibility to infection by

arboviruses and ability to replicate and transmit them to vertebrate hosts have not been assessed. At least 9 of these mosquito species have been linked to transmission of WNV (*37*). Many related viruses cause encephalitic infections and are transmitted in nature between birds and mosquitoes, particularly *Culex* spp. Six of the 33 endemic mosquito species feed on humans and birds, which makes them potential bridge vectors (*36*). Eleven of the remaining 33 species bite humans. Clearly, the United Kingdom has a wide range of potential mosquito vectors. Moreover, if DENV vector species that survive in colder temperatures, such as *Ae. albopictus*, become established, the United Kingdom may be more vulnerable to outbreaks of dengue fever.

Several pathogenic mosquitoborne viruses have been isolated from animals, mosquitoes, and humans in Europe and Scandinavia (Table). The same viruses are likely also to exist in the United Kingdom, but why do these mosquitoborne viruses not cause disease? Possible explanations include the following: 1) the level of disease in the United Kingdom is low and therefore missed by surveillance systems; 2) the climate rarely satisfies the requirements for efficient arbovirus transmission by mosquitoes; 3) resident arthropods in the United Kingdom have low competence for arboviruses; 4) low-level immunity has been established among mammalian and avian species involved in virus circulation; and 5) viruses are circulating, but nonviremic transmission of the virus is occurring between mosquitoes co-feeding on nonsusceptible vertebrate hosts (*38*).

Some species of *Culicoides* (biting midges) are vectors of animal diseases, such as bluetongue virus (e.g., *Culicoides imicola* and *C. variipennis*) and African horse sickness. According to Campbell and Pelham-Clinton (*39*), 41 *Culicoides* spp. occur in the United Kingdom; dominant species include *C. impunctatus* in Scotland and *C. obsoletus* in southern England. In Scotland, 37 species occur, ≈20 of which are mammalophilic, but only 5 of which bite humans. In the highlands of Scotland, *C. impunctatus* can reach pest proportions and is associated with "nuisance biting." However, bluetongue virus and African horse sickness virus are not present in the United Kingdom, and because its climate is cooler than that of southern Europe, they are not likely to become established in the near future (http://www.defra.gov.uk/animalh/diseases/notifiable/disease/bluetongue.htm).

Sandflies are not known to be endemic in the United Kingdom, but they are present in all Mediterranean countries. As climate change takes effect, sandflies are expected to become established in the United Kingdom. Thus, sandfly fever/Toscana virus might emerge later in this century.

Finally, direct transmission of arboviruses between vertebrates (i.e., not requiring an arthropod vector) has been recognized for many years. For example, LIV and TBEV may be transmitted through the milk of sheep and goats (*6*). Moreover, evidence is accumulating that mosquitoborne arboviruses can be transmitted among vertebrate species by routes additional to those involving arthropods (*40*). While invertebrates almost certainly contribute to arbovirus transmission in northern Europe, the relatively lower numbers and density of mosquitoes in these regions might limit their efficiency in disease transmission. These alternative nonvectored modes of transmission could in part explain how such viruses might circulate between vertebrates in environments previously considered suboptimal for efficient circulation of arboviruses.

Conclusion

Arboviruses rarely cause disease in their maintenance hosts or vectors; consequently, viremic blood is not available for vectorborne transmission. Arboviruses have therefore developed other transmission strategies to ensure their survival. For years, the favorite mechanisms to explain virus survival in the absence of disease were vertical transmission, overwintering, or persistent infection in the arthropod or vertebrate host. We now recognize that the process of nonviremic transmission between co-feeding ticks or co-feeding mosquitoes, and perhaps ticks co-feeding with mosquitoes, is a powerful force for virus transmission even in the presence of immunity. Therefore, this repertoire of transmission strategies can ensure arbovirus perpetuation in the absence of disease.

Armed with this versatility, arboviruses can adapt to virtually any situation. Most arboviruses have evolved a sylvatic existence that is infrequently associated with disease in vertebrate hosts. However, in the presence of susceptible hosts, an environment that favors rapid arthropod amplification, an efficient mechanism for dispersal, and a suitable climate for transmission, DENV, RVFV, Japanese encephalitis virus, and YFV may cause epidemic outbreaks of immense proportions.

This variety of critical factors must come together at an appropriate time for an arbovirus to emerge as an epidemic pathogen, a situation that has rarely happened in the United Kingdom. Of course, if climate change has the effects that experts predict it will, the situation could change during the 21st century. In conclusion, despite compelling evidence that potentially highly pathogenic arboviruses are circulating or, alternatively, are being introduced annually into the United Kingdom, no imminent threat exists that they will cause epidemic outbreaks of serious concern to public or environmental health.

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Confronting Zoonoses, Linking Human and Veterinary Medicine

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Many of the emerging infectious diseases, including those caused by bioterrorist agents, are zoonoses. Since zoonoses can infect both animals and humans, the medical and veterinary communities should work closely together in clinical, public health, and research settings. In the clinical setting, input from both professions would improve assessments of the risk-benefit ratios of pet ownership, particularly for pet owners who are immunocompromised. In public health, human and animal disease surveillance systems are important in tracking and controlling zoonoses such as avian influenza virus, West Nile virus, and foodborne pathogens. Comparative medicine is the study of disease processes across species, including humans. Physician and veterinarian comparative medicine research teams should be promoted and encouraged to study zoonotic agent-host interactions. These efforts would increase our understanding of how zoonoses expand their host range and would, ultimately, improve prevention and control strategies.

Zoonoses are diseases that can be transmitted from wild and domestic animals to humans and are public health threats worldwide. Because these diseases come from animals, prevention and control strategies need to be innovative and require the combined efforts of many fields. For example, closer collaborations are needed between veterinarians, physicians, and public health professionals in 3 areas: individual health, population health, and comparative medicine research. In the individual health setting, assessing the potential for zoonotic disease transmission from animals to humans should include input from both physicians and veterinarians, especially for patients at high risk such as those who are immunocompromised. In population health, zoonotic disease threats should be addressed through surveillance systems that include domestic and wild animal and human populations, which would help lead to effective control measures. Since physicians and veterinarians would be the key professionals to recognize and report outbreaks, enhanced communications between hospital epidemiologists, veterinarians, and local public health officials would not only help expedite a local response, but also help identify whether unusual diseases or outbreaks involving animals and humans were related or separate events. In the research setting, collaboration between physicians and veterinarians in comparative medicine would improve our understanding of zoonotic agent-host interactions.

Individual Health Collaborations

At the individual health level, zoonotic diseases are a concern for all who live or work with animals. This risk is especially problematic for persons, such as companion animal owners, who are immunocompromised. Grant and Olsen found that physicians are generally not comfortable discussing the role of animals in the transmission of zoonoses and would prefer that veterinarians play a role (*1*). However, most patients do not view veterinarians as a source of information for human health. The authors found that only 21% of HIV patients asked their veterinarians about the health risks of pet ownership (*1*).

Zoonotic risks from companion animals are not limited to those living with HIV. One patient who was taking infliximab for longstanding rheumatoid arthritis became infected with *Cryptococcus neoformans* after cleaning a cockatiel's cage the week before hospitalization (*2*). Human lymphocytic choriomeningitis virus (LCMV) infection is associated with pet rodents and also causes serious infections in immunocompromised persons (*3*). These risks extend beyond the pet owners and can involve the recipients of the animal owners' donated organs. For example, LCMV has been responsible for the deaths of 3 organ transplant recipients who received their organs from donors who had owned infected pet rodents (*3*).

Exotic or unusual pets can pose a risk to the healthy. Salmonellosis developed in 4 children, 1 mother, and an 80-year-old woman after exposure to small pet turtles (*4*). Salmonellosis has also been associated with pet rodents. For example, during the summer of 2004, two young chil-

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dren became seriously ill with salmonellosis shortly after their families purchased pet rodents (*5*). A national search of the PulseNet National *Salmonella* Database from December 2003 to October 2004 found 28 matching human-case isolates of *Salmonella enterica* serovar Typhimurium. Of the 22 patients who were interviewed, 13 (59%) had been exposed to rodents during the 8 days before onset of illness (*5*).

Exotic pets can introduce pathogens previously unknown in the North American continent. For example, the 2003 monkeypox outbreak in the Midwestern United States originated after imported African rodents infected prairie dogs in pet distribution facilities (*6*). Laboratory-confirmed monkeypox developed in 35 persons (6). No one died, but the outbreak required vaccinating 30 persons with smallpox vaccine, 23 because of potential occupational exposure (*6*).

Occupational risks for exposure to zoonotic diseases are a concern for persons such as farmers, meatpackers, and pet shop employees who work with animals. For example, *Streptococcus suis* can cause meningitis or occasionally fulminant sepsis in pig farmers (*7*,*8*) *Campylobacter* infection is an occupational risk for packers in poultry factories, and *Streptobacillus moniliformis* can be an occupational risk for pet shop employees (*9*,*10*)

These examples illustrate that living and working with animals can impact human health at the individual level. Veterinarians who treat animals that suddenly become ill with confirmed infections should assess the risk for zoonotic potential and inform the animals' owners accordingly. From a medical-legal standpoint, veterinarians are obligated to do this, but the extent to which they should inform animal owners and ensure that they seek medical attention varies depending on the circumstances (*11*). The severity of the risk for zoonotic disease as well as the level of understanding by the animal owner in question would need to be considered (*11*). For example, the veterinarian may merely advise potentially exposed persons to seek medical attention or may strenuously urge and ensure that the person receives medical attention immediately. However, veterinarians' roles in assessing risk for potential zoonotic disease transmission could extend beyond this level of involvement.

Risk-benefit ratios for ongoing animal exposure could be weighed and discussed by both veterinarians and physicians. The roles in these veterinary-physician relationships would need to be established from the start so that the veterinarians would not be at risk of appearing to practice medicine. For example, veterinarians could provide an assessment of an animal's health status to a physician whose patient is immunocompromised and insists on keeping his or her companion animal. Since companion animal ownership has psychologic and physiologic benefits, this type of collaboration and cooperation between the 2 professions would be invaluable to patients. The veterinarian would provide regular checkups to the companion animal to ensure that its health status is closely monitored. In the occupational setting, regular veterinary monitoring of all involved animals' health may not be possible; however, if a worker were immunocompromised, then a careful assessment should be made about his or her continuing that line of work. Veterinary input might be helpful in these difficult decisions. Joint medical and veterinary medical workshops on zoonotic risks to human health could help forge ties and facilitate opportunities to establish these types of collaborative efforts.

Population Health Collaborations

Recognizing whether human and animal outbreaks were simultaneous would provide important information for identifying the causative pathogens and developing control strategies. For example, physicians treating the initial West Nile virus (WNV) patients in New York City in 1999 might have benefited if they knew that for the previous month and concurrently, veterinarians in the surrounding area had been seeing dozens of dying crows with neurologic symptoms similar to those of the affected humans (*12*). Depending on the state, animal disease surveillance can be fragmented. For example, in New York, human and animal rabies are the responsibility of local and state health departments, livestock are overseen by the state agriculture agency, and wildlife is the responsibility of the state environmental agency (*12*).

In New York, no local or state agency assumed full responsibility for the large wildlife die-off investigation in 1999 since which agency was responsible was not initially clear (*12*). This situation hindered communications between the veterinarians, public health officials, and physicians who were involved in the outbreak response at the local level. As an emergency, short-term measure, veterinarians could have expressed their concerns directly to the hospital epidemiologists in the area to be on the lookout for a possible human impact from an unknown disease that was causing widespread severe neurologic symptoms and death in wild birds. Such rapid, direct communication between veterinarians and physician epidemiologists could be particularly important in states in which local public health agencies either do not exist or are not involved in zoonotic disease reporting or investigation.

In some states, animal disease reporting and response are state level functions and are separate from human public health. I contacted state veterinarians in all 50 states about their states' animal disease reporting requirements. State veterinarians from 8 (19%) of the 43 responding states replied that veterinarians are required to contact their local public health agencies directly about reportable

zoonotic diseases. Of these, 2 require reports of rabies only. Names and contact information were obtained from the US Department of Agriculture Animal and Plant Health Inspection Service website (http://www.aphis.usda.gov/vs/sregs/official.html) and the Council of State and Territorial Epidemiologists Point of Contact Veterinarians website (http://www.cste.org/). State agencies such as departments of agriculture, environment, or boards of animal health are the usual primary recipients of animal disease reports. However, these agencies may not have the resources to conduct animal disease prevention and control activities at the local level. In addition, in the case of departments of agriculture, their mission, historically, has been to promote agriculture, not necessarily to control infectious diseases in all types of animals.

Animal disease reporting and oversight are split between different agencies in some states. This is the situation at the federal level and has prompted a recent National Academy of Sciences report to recommend that a federal-level, centralized coordinating mechanism be established to improve collaboration and cooperation among all the players in animal health oversight, including industry and local, state, and federal agencies (*13*). A similar mechanism for improving communication and collaboration across state agencies, such as between state animal health and public health veterinarians, would be important since evidence suggests that veterinarians preferentially report to more "animal-centric" state agencies.

For example, the Alaska Department of Health and Social Services and the Alaska Department of Environmental Conservation (DEC) mailed a laboratory usage and needs assessment survey to all 200 licensed veterinarians in Alaska. Of the 140 who responded, 95% stated that they would report to the state veterinarian at DEC, 4% to the state department of health, and 1% to the US Department of Agriculture when asked, "Who would you contact if you suspected or diagnosed a reportable animal disease?" (R. Gerlach, pers. comm.).

In 2004, I surveyed 4,144 randomly selected licensed veterinarians in 4 states: New Hampshire, New Jersey, New York, and Pennsylvania. When asked, "Which government agency would you first notify if your companion animal or livestock patient had an unusual infectious disease?" the largest percentage of the 1,070 respondents chose "State Agriculture Agency." Some veterinarians, ≈10% for companion animals and 14% for livestock, would skip the state and local agencies altogether and notify a federal agency. Twenty-eight percent of the veterinarians did not know if their community had a local public health agency. The survey did not include questions about wildlife. Veterinarians' names and addresses were obtained from each state's licensing boards except for New York State, which prohibits access to this information. For New York State, names and addresses were obtained from the American Veterinary Medical Association (Tables 1 and 2).

In addition to working with state officials during serious zoonotic outbreaks, veterinarians should also communicate and collaborate with local public health officials. During the 1999 WNV outbreak, the presumptive diagnoses for the initial human cases included Guillain-Barré syndrome, encephalitis, meningitis, and aspiration pneumonia. (*14*). Public health officials assumed the cause of the outbreak was St. Louis encephalitis (SLE) until a veterinary pathologist at the Bronx Zoo linked the animal and human outbreaks (*12*). She realized that crows and other birds ordinarily resistant to SLE were dying, so the agent was not likely SLE. Her work helped set the stage for the discovery of WNV in the Western Hemisphere (*12*).

At the population level, zoonotic pathogens cause foodborne, waterborne, and arthropodborne disease outbreaks. These pathogens include *Salmonella*, *Escherichia coli* O157:H7, *Cryptosporidium*, yellow fever virus, and *Borrelia burgdorferi* (*15*). Many of the category A, B, and C bioterrorist agents, such as *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, *Coxiella burnetii*, and Nipah virus, cause zoonoses (*16*,*17*).

The magnitude of the problem of zoonoses illustrates why the efforts of medicine, veterinary medicine, and public health need to overlap. Taylor and others identified 1,415 infectious agents and found that 868 (61%) could be transmitted between animals and humans (*18*). They found that zoonotic diseases were twice as likely to be associated with emerging or newly discovered infections than nonzoonotic pathogens and that viruses and protozoa were the zoonotic pathogens most likely to emerge. RNA viruses, in particular, have been identified as highly likely to emerge (*19*). These agents include WNV, avian influenza virus, hantavirus, and severe acute respiratory syndrome–associated coronavirus.

Joint surveillance of animal and human zoonotic disease outbreaks is already reaping benefits worldwide. For example, recognition of the first human case of H5N1 avian influenza in Hong Kong in 1997 was facilitated by the surveillance of ducks, geese, and chickens in southern China during the preceding decades (*20*). On the domestic front, in 1999, the Centers for Disease Control and Prevention established ArboNET, a cooperative surveillance system that monitors the geographic spread of WNV in humans, mosquitoes, birds, and other animals in response to the outbreak of WNV disease (*21*). ArboNET has provided an invaluable system for tracking the disease's spread and severity across the United States, identifying early WNV activity, and justifying continuing support for mosquito control (*22*). These types of surveillance systems should be continued and expanded to include other serious zoonotic diseases such as plague and tularemia.

Table 1. Veterinarians' choices of government agencies they would first notify regarding a companion animal patient with an unusual infectious disease*

Choice	No. (%)
State agriculture agency	326 (30)
State public health agency	241 (23)
Local public health agency	206 (19)
Other†	116 (11)
Federal agency (USDA, FDA, CDC)	111 (10)
Not sure	30 (3)
Do not care for companion animals	27 (3)
Did not answer question	13 (1)
Total	1,070 (100)

*USDA, US Department of Agriculture; FDA, Food and Drug Administration; CDC, Centers for Disease Control and Prevention.
†A total of 76 (66%) of 116 said, "state veterinarian," 20 (17%) of 116 gave a combination of government agencies, and 20 (17%) of 116 gave miscellaneous answers, including Animal Plant Health Inspection Service, state veterinary diagnostic laboratory, and animal hospital employer.

In addition to ongoing joint surveillance activities, researchers should collaborate in applied public health studies. For example, physician and veterinarian teams could conduct serosurveys of humans who live and work near high-risk animal populations to assess their risk of acquiring zoonoses. Long-term surveillance studies could be conducted on humans who are exposed to deer and elk, which are at risk of acquiring chronic wasting disease in disease-endemic regions of Colorado, Wyoming, and Nebraska (*23*). Surveillance studies on the role of vaccinated and unvaccinated horses in the amplification of WNV to humans would help improve our understanding of the epidemiology of virus activity (*24*).

Comparative Medicine Research Collaborations

The need for physicians and veterinarians to work together to control zoonoses extends beyond the individual and population health settings and should include collaborations in comparative medicine research. Comparative medicine is the study of the anatomic, physiologic, and pathophysiologic processes across species, including humans. Considerable attention is paid to infectious diseases, specifically the study of host-agent interactions.

As an academic discipline, comparative medicine is not new; the first chair in it was established in 1862 in France (*25*). The field has an illustrious history. In 1893, Theobald Smith, a physician, and F.L. Kilbourne, a veterinarian, published a paper establishing that an infectious agent, *Babesia bigemina*, the cause of cattle fever, was transmitted by an arthropod vector (*25*). Their seminal work helped set the stage for Walter Reed's discovery of yellow fever transmission (*25*). Another physician-veterinarian team, Drs. Rolf Zinkernagel and Peter C. Doherty, won the 1996 Nobel Prize in physiology or medicine for their discovery of how the immune system distinguishes normal cells from virus-infected cells (*26*).

These 2 examples illustrate that medicine and veterinary medicine are complementary; they are synergistic in generating new scientific insights across species. In essence, the 2 disciplines epitomize the philosophy of comparative medicine. And yet, as societies' needs grow to have scientists work together to understand and control emerging zoonoses, evidence suggests that the next generation of medical and veterinary medical scientists are not collaborating with each other. Biomedical and comparative medicine research is losing its appeal as a career among physicians and veterinarians.

On the physician side, the decline in physician-scientists is evidenced by several trends. First, from 1970 to 1997, the number of physician-scientists obtaining National Institutes of Health (NIH) support has been essentially flat and shrinking in proportion to doctoral recipients who seek and obtain funding (*27*). Second, from 1994 to 1997, the number of first-time physician-scientists seeking NIH funds dropped by 31%, and the percentage of medical school graduates interested in research careers fell from 14% in 1989 to 10% in 1996 (*27*). Medical school faculties now comprise 25% fewer physician-scientists than 20 years ago (*28*).

For veterinarian-scientists, the situation is considered dire. A 2004 National Academy of Sciences (NAS) report found that of American Veterinary Medical Association members, <1% were board certified in laboratory animal medicine and <2% were board certified in pathology (*29*). In addition, the total number of veterinarians who receive NIH grant funding is small. In 2001, only 4.7% of all NIH grants funded for animal research were awarded to veterinarian principal investigators (*29*).

Reasons for the lack of interest in research are similar for both medical and veterinary students: an emphasis on clinical care, educational debt, and a lack of mentors and research opportunities (*30*,*31*). Medical schools now

Table 2. Veterinarians' choices of government agencies they would first notify regarding a livestock animal with an unusual infectious disease*

Choice	No. (%)
State agriculture agency	422 (40)
US Department of Agriculture	141 (13)
Do not take care of livestock	135 (13)
State public health agency	122 (11)
Other†	118 (11)
Local public health agency	57 (5)
Not sure	42 (4)
Did not answer question	17 (2)
Other federal agency (CDC, FDA, FBI)	16 (1)
Total	1,070 (100)

*CDC, Centers for Disease Control and Prevention; FDA, Food and Drug Administration, FBI, Federal Bureau of Investigation.
†A total of 77 (65%) of 118 said, "state veterinarian," 25 (21%) of 118 gave a combination of government agencies, and 16 (14%) of 118 said that they do not see livestock.

emphasize primary care and care for the underserved, and while certainly important, this shift in priorities has been at the expense of encouraging biomedical research careers.

Veterinary schools have shifted their focus from comparative medicine research and livestock medicine to companion animal medicine to meet societal demand (*32*). However, similar to the situation with medical schools, this shift has caused fewer numbers of veterinary students to pursue research careers. In addition, comparative medicine programs have been shifting from a research to service orientation that limits veterinarians' research involvement to being primarily caretakers for laboratory animals (*32*).

This shift in comparative medicine orientation has discouraged many veterinary students from pursuing careers in research and hinders research on emerging zoonoses from diverse animal hosts.

What can be done? Although NIH has begun a roadmap to improve biomedical research into the 21st century, nowhere does the plan mention comparative medicine and the importance of veterinary involvement, which would certainly fit into its goals of promoting interdisciplinary research and new pathways to discovery (*33*). An NAS report recognizes the need for the roadmap initiative to address this issue and recommends creating integrated veterinary research through joint interagency collaborative programs at NIH (*34*).

One way to achieve this would be to offer jointly sponsored comparative medicine research grants from both the National Center for Research Resources (NCRR) and National Institute of Allergy and Infectious Diseases (NIAID). With NIAID's emphasis on zoonoses and cross-species investigations, comparative medicine research would fit in well with its mission of research on bioterrorist agents, emerging infectious diseases, and immunology. The NCRR and NIAID could offer research grants to medical and veterinary medical research teams that are promoting collaborative projects on zoonoses.

A second NAS report addressing this issue recommends that federal agencies involved in human and animal research coordinate their efforts. Jointly funded integrated and comprehensive animal health research programs should be established to ensure that veterinary and medical scientists work together as collaborators domestically and internationally (*13*).

Encouraging more veterinary school graduates to pursue careers in research is critical if partnerships are to be developed. A third NAS report recommends that the number of veterinarians serving as principal investigators should increase (*29*). This could be accomplished by increasing the number of NCRR-funded T32 training grants and making them available to persons who want to enter research training programs immediately after graduation from veterinary school (*29*). Finally, another way to encourage more veterinary students to pursue research careers would be for the National Institute of General Medical Sciences (NIGMS) to offer research training programs to them analogous to those offered to medical students. Currently, NIGMS research training programs are only open to holders of MD and PhD degrees (*35*).

Discussion

Since zoonoses are diseases of animals that can infect humans, veterinarians, physicians, and public health officials need to work more closely together to control, prevent, and understand them. In the individual health setting, collaborative input from both veterinarians and physicians would help assess a patient's potential zoonotic disease risks from animal exposure. For high-risk immunocompromised patients, these collaborative efforts could be tremendously important, not only for their personal well-being but also for their livelihoods.

Regarding population health, reporting of animal diseases varies considerably from state to state. Some states have 1 agency responsible for all animal disease reporting while others split the reports between various agencies. However, in many states, animal disease surveillance appears to be largely a state level function. In few states, local public health agencies are expected to receive zoonotic disease reports directly from veterinarians. If controlling zoonotic diseases is to be improved, greater communication and collaboration between veterinarians, physicians, and public health officials at the local level are needed. One NAS report recommends a federal level mechanism to promote greater collaboration among all the players involved in animal health (*13*); similar mechanisms could also be considered in states.

Joint disease surveillance efforts, which are proving to be extremely useful in the tracking of zoonoses, include ArboNet for WNV surveillance, the National Antimicrobial Resistance Monitoring System for enteric bacteria surveillance, and FoodNet for the population-based surveillance of foodborne pathogens (*36*,*37*). These programs should continue to be supported, and new surveillance programs for other serious zoonoses should be developed. Medical, veterinary, and public health schools should offer courses on zoonotic risks to human health that integrate all 3 perspectives.

Society would benefit if more collaborative comparative medicine research projects were conducted by physicians and veterinarians to investigate zoonotic agent-host interactions. Among the many ways to promote these projects are multiagency-sponsored comparative medicine research grants and more training grants for veterinarians interested in careers in research. These efforts would increase our understanding of how zoonoses expand their

host range and would, ultimately, improve prevention and control strategies.

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Human Influenza Surveillance: the Demand to Expand

Scott P. Layne*

The World Health Organization Influenza Program is one of the best developed and longest running infectious disease surveillance systems that exists. It maintains a worldwide watch of influenza's evolution to assist delivery of appropriately formulated vaccines in time to blunt seasonal epidemics and unpredictable pandemics. Despite the program's success, however, much more is possible with today's advanced technologies. This article summarizes ongoing human influenza surveillance activities worldwide. It shows that the technology to establish a high-throughput laboratory network that can process and test influenza viruses more quickly and more accurately is available. It also emphasizes the practical public health and scientific applications of such a network.

Influenza strikes persons in developing and industrialized countries alike and is capable of killing healthy persons of all ages. Among the hardest hit are infants <1 year of age and adults >65. During any given year, influenza epidemics kill 500,000–1,000,000 persons globally, and an unpredictable pandemic is capable of killing millions (*1*). Yet death rate statistics alone do not capture the full impact of influenza; it causes many hospitalizations, secondary bacterial pneumonias, and middle ear infections in infants and young children (*2*). Worldwide, literally tons of antimicrobial drugs are used to treat these complications, and the economic consequences are enormous. For large populations, the only way to deter influenza is to administer vaccines targeted against ever-mutating strains (*3*).

Current Surveillance

The World Health Organization (WHO) Influenza Program was established in 1952 to assist with public health threats associated with influenza. Today, its network of 112 national centers in 83 countries collects ≈160,000 samples each year from 600 to 1,200 million persons with influenza. As shown in Figure 1, the centers screen samples for influenza viruses and type- (A versus B) and subtype- (e.g., A/H1N1, A/H3N2) relevant samples (*4*). Certain influenza-positive samples are then forwarded to 1 of 4 WHO collaborating centers for further immunologic and genetic characterizations. Twice a year, WHO organizes a formal meeting with its collaborating center directors to review information on circulating influenza strains. This advisory committee identifies circulating strains that new vaccine formulations should target. Because influenza epidemics peak during the winter months, the committee offers its recommendations in February and September for the Northern and Southern Hemispheres, respectively. The findings are then reviewed by national health authorities who approve, and occasionally amend, implementation of the recommended vaccine strains (*5*). Surveillance for influenza requires global and national monitoring for both virus and disease activity to determine when, where, and which influenza viruses are circulating in the United States and globally, to determine the intensity and impact of influenza activity on defined health outcomes and identify unusual or severe outbreak, and to detect the emergence of novel influenza viruses that may cause a pandemic.

The Centers for Disease Control and Prevention's (CDC) WHO Collaborating Center for Reference and Research on Influenza supplies standardized reagents and test kits to all national centers for detecting influenza A and B strains, subtyping strains, and determining whether sample strains are immunologically related to recent vaccine strains. Typical circulating strains and atypical ones that appear to differ from vaccine strains are forwarded to 1 of 4 collaborating centers (in the United States, United Kingdom, Australia, and Japan) for further characterization (*5*). During the 2004–2005 influenza season, CDC's center in Atlanta received ≈3,500 strains from domestic and foreign surveillance. A partial summary of laboratory methods used is outlined below.

In most situations, 6–10 serum samples are used to compare sample strains against vaccine and reference strains, and during any given influenza season, >99% of

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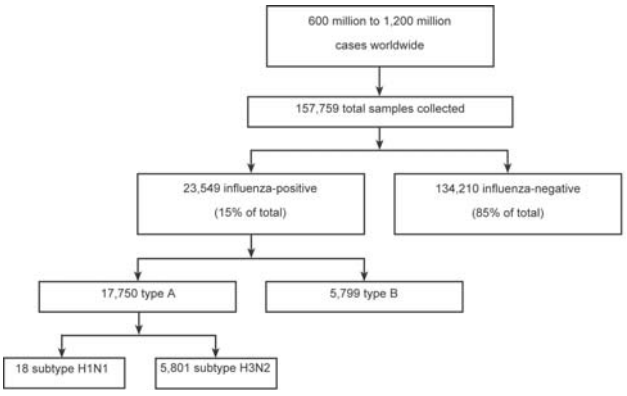

Figure 1. Global influenza surveillance for the 2004–2005 season. Respiratory samples were collected from persons with febrile respiratory illness worldwide. Approximately 15% of samples were influenza positive. Note that only some of the type A viruses were subtyped. Data are from the Centers for Disease Control and Prevention website (http://www.cdc.gov/flu/weekly/weeklyarchives2004-2005/04-05summary.htm).

sample strains are successfully matched by routine hemagglutination inhibition (HI) assays. Laboratory workers replicate sample strains in cell cultures or fertile eggs and adjust the resulting viral stocks to a standard hemagglutinin titer. They then use HI assays to determine whether sample strains are immunologically related (i.e., cross-reactive) to recent vaccine strains. Typing sera are added to wells in a series of 2-fold dilutions, and after the reactions stabilize, laboratory workers score assay wells (positive or negative) by looking for agglutinated erythrocytes (positive) that do not form buttons at the bottom of plates versus non-agglutinated cells (negative) that do form buttons. For sample strains that behave as variants, laboratory workers inject them into ferrets to produce a strain-specific antiserum. When the new antiserum is ready, HI assays are again performed as above. If the new sera show significant gaps in cross-reactivity (usually defined as a 4-fold difference between sample and vaccine strains), they are incorporated into the routine laboratory set and used to look for new epidemic strains. Examples of strains that were identified as variants include A/H3N2/Sydney, which spread rapidly among persons in 1997, and A/H5N1/Hong Kong, which jumped from fowl to humans in 1997. During the 2004–2005 influenza season, ≈3,500 HI assays were performed.

During the 2004–2005 influenza season, >600 cleaved hemagglutinin (HA1) domain sequences were analyzed. In this process, laboratory workers extract viral RNA from samples, convert viral RNA to cDNA, amplify cDNA with polymerase chain reaction (PCR) primers, and analyze DNA products with capillary array sequencers. They then compare sample strain sequences against vaccine and reference strain sequences to determine their phylogenetic relationships.

During the 2004–2005 influenza season, ≈350 neuraminidase (NA) sequences were analyzed to determine their phylogenetic relationships. For this process, laboratory workers follow similar preparative steps used for HA1 and then use multiple PCR primers to sequence the entire gene segment.

During the 2004–2005 influenza season, ≈3,500 single nucleotide polymorphism (SNP) profiles were analyzed from circulating strains. For this analysis, laboratory workers follow similar preparative steps used for HA1 and then use pyrosequencing to detect SNP that confer resistance to the antiviral drugs amantadine and rimantadine.

During the 2004–2005 influenza season, ≈120 whole genomes were sequenced from circulating strains. In this process, laboratory workers extract viral RNA from samples, convert viral RNA to cDNA, amplify cDNA with PCR primers, and analyze DNA products with capillary array sequencers. Amplification of all 8 gene segments (PB1, PB2, PA, HA, NP, NA, M1/M2, NS1/NEP) that have a combined length of ≈13.6 kb requires ≈30 type- and subtype-specific PCR primers.

These activities give ≈6 months to vaccine manufacturers on either side of the equator for scale up, production, and distribution. These activities also give healthcare services another 3 months to administer the ≈250 million doses of trivalent vaccine that are used globally. Despite its sophistication and scale, however, the WHO Influenza Program has several shortcomings (*6*).

First, surveillance gaps exist in many parts of the world for a variety of reasons, including limited funding, lack of infrastructure support for surveillance teams and preserving influenza samples, and intentional underreporting at the national level (*7*). Second, current laboratory methods for characterizing influenza are time-consuming and labor-intensive and, as a result, relatively few viral strains undergo definitive phenotyping and genotyping assays (*5,8,9*). During the 2004–2005 influenza season, for example, the 4 WHO collaborating centers analyzed ≈6,000 strains, representing only 1 sample per 100,000 influenza cases worldwide (Figure 1). Far fewer strains from domestic poultry and swine or from wild aquatic birds, which are thought to serve as precursors for pandemic strains, are analyzed comprehensively in a given year (*10*).

Third, with current methods, it can take weeks to months to generate laboratory data on influenza samples and understand their significance (Appendix available online at http://www.cdc.gov/ncidod/EID/vol12no04/05-1198.htm#app). Such prolonged times can impede vaccine strain selection activities. For example, the vaccine administered throughout North America in 1997 provided inadequate protection against the A/H3N2 Sidney strain

that spread rapidly from Asia and Australia (*11*). Public health officials ascribed the poor match between circulating strains and vaccine strains to many factors including 1) less than optimal surveillance, 2) time required to prepare and ship isolates, 3) lag time in laboratory testing with current manual methods, and 4) rapid spread of the Sydney variant.

Although the program has a remarkably good track record, it did not detect the Sydney variant in time to include it in the vaccine before the epidemic. Failure to detect an emerging influenza virus could prove disastrous should it be a novel strain with pandemic potential (*12*). The 1918 influenza pandemic is the biggest infectious disease catastrophe on record, topping even the medieval Black Death. Within months after the initial outbreak, the A/H1N1 virus struck 500 million persons and killed 40–50 million worldwide when the total population was only 2 billion (*13*). Subsequent pandemics, brought about by a shift to A/H2N2 in 1957 and A/H3N2 in 1968, had far lower death rates.

Between March and May of 1997, an outbreak of avian A/H5N1 in Hong Kong killed a child who was otherwise healthy and thousands of chickens (*14*). For the next 6 months, no new cases appeared. Between November and December of 1997, avian A/H5N1 infected 17 people and killed 5 of them (*15,16*). Confronted with a case-fatality ratio of 33%, health authorities took quick action and opted to destroy all 1.5 million chickens in Hong Kong. It took nearly 6 months to identify the index case; events transpired so quickly that manual laboratory methods were unable to generate all of the information that was needed.

Since 2004, avian A/H5N1 has caused additional outbreaks throughout Asia, resulting in >60 human deaths in Vietnam, Thailand, Cambodia, and Indonesia and the destruction of 150 million birds (*17*). In 2005, through a combination of wild bird migrations and farming practices, highly pathogenic avian influenza spread to northern China, Mongolia, Tibet, Kazakhstan, and Russia (*18*). At the time of this writing, it had further spread to several European countries (Turkey, Romania, and Greece) and threatened to spread to other continents, including Africa and North America through avian flyways.

The potential of avian A/H5N1 to cause a global human pandemic is uncertain because it cannot be predicted with current knowledge (*19*). Nevertheless, the anticipated economic, social, and political consequences are enormous (*20,21*). Therefore, we face a compelling demand to expand the current influenza surveillance system (*6,22*).

High-throughput Network

In August 2004, the US Department of Health and Human Services released a draft of its Pandemic Influenza Response and Preparedness Plan. The plan's surveillance annex offered specific recommendations for system enhancements and next steps (*5*). Many of these enhancements could be achieved by developing a high-throughput laboratory network that would expand the capabilities of the existing WHO collaborating centers on influenza (*6,22*). With such enhancements, WHO national centers would be able to collect samples from people with febrile respiratory illnesses, record epidemiologic observations, and send samples directly to the high-throughput network. At each site, high-throughput automated systems would collaborate, and epidemiologic observations and test results would appear in the laboratory's web-enabled database for analysis within days (*23*). Internet-based capabilities would allow WHO national centers to examine their own data and improve surveillance in an iterative manner (Figure 2). In tracking changes in epidemic strains, the new system would facilitate nonbiased proportional sampling of persons with febrile respiratory illnesses in an iterative fashion. In detecting the emergence of novel strains with pandemic potential, the new system would facilitate the use of rapid and more sensitive methods.

The plan integrates available biologic, engineering, and informatic technologies into a networked capability and

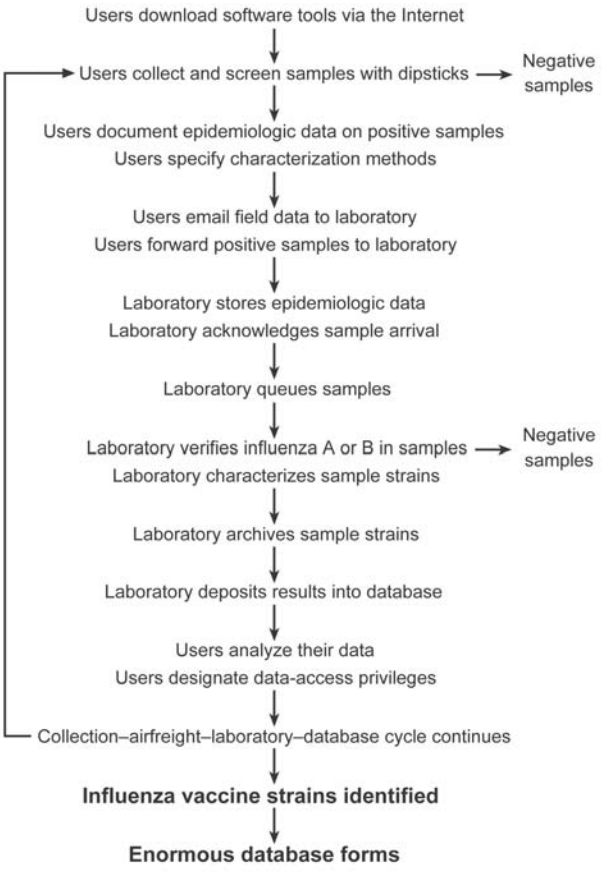

Figure 2. Flow chart for utilizing the high-throughput laboratory network.

makes them available through the Internet (*23*). Influenza is well suited to this approach because of its obvious public health implications, but also because a well-established infrastructure that includes global surveillance, standardized laboratory methods, surveillance-based recommendations, and targeted vaccines already exists (*6*,*22*). Key elements are shown in Figure 2.

Platform-independent software that facilitates influenza surveillance would be provided by the high-throughput network (*23*). Internet-based tools would manage laboratory access, epidemiologic questionnaires, testing instructions, sample submission, data analysis, and data privileges. The release of data to other users or entities would be controlled by the submitting organization or authority. More importantly, the WHO committee and national health authorities that recommend and review vaccine strains and antiviral drugs would have access to all the data.

On average, 1 of every 6 samples collected from persons with febrile respiratory illnesses contains influenza A or B viruses (*24*). The remainder contain other viral and bacterial pathogens. To expedite data collection, surveillance teams could use influenza dipsticks to screen samples on the spot. Several companies make diagnostic kits for influenza A and B; although these tests have certain disadvantages (limited sensitivity and specificity of immunoassays), their underlying technologies can form the basis for improved influenza screening and sampling devices (*2*). Such influenza dipsticks—or even portable PCR-based assays—would make it easier for teams in the field to screen out negative samples and focus on documenting epidemiologic information on positive ones.

An epidemiologic questionnaire would be provided to surveillance teams, with a menu that covers key questions (*23*). What are the collection date and location? Who is the host (human versus animal)? What is the age of the host? What is the physiologic source of the sample? What is the observed or reported severity of illness? What is the observed or reported outcome of illness? Which influenza vaccine or antiviral agents were administered? What are the likely exposures? Is there any recent travel history? The questionnaire would run on inexpensive handheld devices (e.g., personal digital assistants) or cell phones. Bar codes would be used to link samples to their corresponding questionnaires. Completed questionnaires would be sent by email to the high-throughput laboratory network, where questionnaire and laboratory data would form the basis for seeking associations on factors that influence virulence, transmissibility, and host range (*19*).

Current high-throughput automated laboratory systems are capable of operating 24 hours a day. At each networked site, epidemiologic questionnaires and instructions would arrive by the Internet, and bar coded samples would arrive by air freight. Larger sites could operate systems for genotyping, phenotyping, replicating, and archiving influenza viruses. Smaller sites could operate systems for genotyping and archiving viruses. In serving as resources, each site would provide reagents and supplies for analyzing all influenza subtypes. They would also perform control assays on a daily basis and maintain a quality assurance program, the documentation of which would be stored in the database. Automated laboratory methods would build upon manual methods currently in use and, because they can reduce working (liquid) volumes by at least 5- to 10-fold, they would enable economies of scale (*23*,*25*).

Genotyping systems would have flexibility to sequence all 8 RNA segments or any individual segment from influenza viruses. The various steps performed would include transcription viral RNA into cDNA, selection of optimal PCR primers, amplification of DNA by PCR, and analysis by capillary array DNA sequencers (*26*–*28*). Influenza viruses are often propagated in cell cultures or embryonated eggs to obtain enough viral RNA for sequencing. Newer methods that avoid this growth step, however, would facilitate direct high-throughput analysis of native samples from surveillance, including active samples preserved by cold chain as well as inactive samples preserved by ethanol fixation (*29*).

Phenotyping systems would conduct HI and neuraminidase inhibition (NI) assays. HI assays are easily adaptable to automation, but they require relatively large quantities of virus and typing sera (*8*). To overcome this drawback, automated methods that use flow cytometry are under development (*30*). They work by attaching monoclonal or polyclonal typing sera to a set of color-coded (multiplexed) beads and detecting the interaction of influenza with such beads. A high-throughput system that performs HI assays in parallel with flow cytometer–based assays, for example, may offer the best means to test and validate improved influenza serotyping methods. NI assays are also easily adaptable to automation, particularly newer ones that use a chemiluminescent sialic acid substrate instead of a fluorogenic substrate (*31*). They work by mixing substrate with neuraminidase from sample strains and measuring the chemiluminescent signal over time. When performed over a range of inhibitor or antiviral drug (oseltamivir and zanamivir) concentrations, they enable the determination of the 50% inhibitory concentration (IC_{50}) for individual drugs and strains (*32*).

A replicating system would verify that influenza A and B antigens are present in samples and set aside negative ones (*33*). Positive samples would be injected into cell cultures or embryonated eggs and, several days later, automatically harvested, assayed for HA titers, and adjusted to uniform concentrations. A part of this fresh stock would then go to the long-term storage system.

Archiving systems would store influenza samples for an extended time. The archiving system would take stocks from the replication system and place them into modular bar-coded storage containers, which would then be placed into freezers. Every step in the storage and retrieval process would be recorded by bar code scanners and managed by an inventory tracking program (*23*).

Expanded Surveillance

Influenza virus evolves through a combination of point mutations (drifts) and reassortment events (shifts) in its gene segments. For vaccine strain selection, laboratory methods for characterizing influenza have focused primarily on changes in hemagglutinin and neuraminidase (and to a much lesser extent on the M2 ion channel protein) because immunity against these surface proteins is protective (*2*). The emphasis on immune-inducing proteins is clearly practical, but it may overlook changes in the remaining gene segments (*26*).

Whole-genome sequencing and phylogenetic analysis of 156 A/H3N2 viruses that infected humans in New York from 1999 to 2004 shows 2 substantial findings (*28*). The first is that multiple influenza strains co-circulated in humans over time, with strains falling into 1 of 3 distinct clades. The second is that mixing between these clades occurred over short intervals of time, resulting in at least 4 reassortment events among the strains analyzed (*28*). One such reassortment event (shift), rather than a point mutation (drift), appears to explain the emergence of the A/Fujian/411/2002-like strain that caused an epidemic during the 2003–2004 influenza season. Similar findings on A/H3N2 viruses that infected humans in Australia and New Zealand from 2003 to 2004 have also been reported (*27*). In this instance, swaps of neuraminidase and 3 internal genes (NS1/NEP, NP, M1/M2) were found. Both independent findings show that influenza A virus is less restricted than previously believed and that reassortment events can occur without warning. Altogether, these new findings underscore the importance of rapid, whole-genome analysis for future influenza surveillance (*28*).

The high-throughput laboratory network would give rise to 3 domains of associated data from surveillance (*34*). Epidemiologic data would pertain to dates, locations, hosts, outcomes, histories, and exposures. Genotypic data would pertain to the exact sequence of nucleotides in all 8 viral RNA segments. Phenotypic date would pertain to immunologic pedigrees (HI titers) and antiviral drug sensitivities (IC_{50}) of sample strains. Some practical public health and scientific uses of such organized data follow.

New influenza vaccines are often introduced after 3 criteria have been met (*25*). First, a new strain is identified by laboratory-based methods. Second, geographic spread of the new strain is associated with human illness. Third, the most recent influenza vaccine stimulates a reduced immunologic response to the new strain. Given these criteria, the high-throughput laboratory network would help in 2 ways. It would provide faster information for vaccine strain selection, potentially saving 1–2 months in vaccine delivery. It would also continuously monitor for the emergence of escaping influenza strains and guide critical decisions to update pandemic vaccines or use them in combination with limited supplies of antiviral drugs (*19*). Researchers and drug companies are developing modern methods (based on reverse genetics and cell cultures, for example) to manufacture influenza vaccines that could cut delivery times in half (*2*,*35*). Within the next few years, these new methods, in combination with a high-throughput network, could save additional vaccine delivery time and save lives (*6*,*19*).

Fifteen hemagglutinin (H1–H15) and 9 neuraminidase (N1–N9) subtypes diverge by as much as 50% in their overall amino acid composition. Within each subtype, smaller amino acid substitutions (drifts) that enable influenza viruses to evade preexisting immunity exist (*2*,*3*). Although sequencing influenza viruses is useful for understanding viral mixing and evolution, it cannot delineate how immunologic (i.e., drift and shift) variants relate to one another at the amino acid and RNA coding levels. To develop such understanding, a large base of phenotypic data must be associated with its corresponding genotypic data. For each receptor subtype, phenotypic data would consist of HI titers and genotypic data would consist of RNA sequences from the same virus. Building a rough association matrix would be the first step in understanding how variants relate to one another at the amino acid and RNA levels.

Subsequently, a more complete association matrix would be used to develop models that could predict whether viral strains are immunologically related from sequences alone. Such efforts could help develop influenza vaccines that protect against a wider range of variants and establish a more fundamental molecular basis for influenza surveillance (*25*).

Researchers have proposed using antiviral drugs such as oseltamivir to halt an avian influenza outbreak in humans (*36*,*37*). The strategy would require stockpiling millions of doses and administering them to persons in the epicenter and surrounding areas within weeks. Immediate recognition of the outbreak and rapid surveillance to determine its size would be essential. Drug-resistant avian influenza viruses would likely emerge at some point, representing a potential threat to emergency control efforts, and health authorities would need real-time information on where the viruses were found and how many of them existed (*38*). Such emergency interventions would generate thousands of samples for laboratory analysis within days.

Given current laboratory surge capacity, a high-throughput laboratory network may be the only feasible means to meet the challenge.

Implementation

The spreading avian A/H5N1 outbreak poses serious threats to the health, economy, and security of the world (*22*). It has motivated political leaders and health officials to increase financial support for influenza surveillance and to seek agreements and incentives that promote information sharing and international cooperation (*39*). Effective measures will require real-time, accurate, and comprehensive information to make rapid public health decisions.

The first 2 sites in a high-throughput laboratory network could be up and running in 12–18 months at a cost of $15 million. It would generate epidemiologic, genotypic, and phenotypic data as described in this article. With available technologies and methods, each site would be capable of analyzing up to 10,000 samples per year, a substantial improvement over current capabilities. Implementing multiple sites worldwide (at the 4 WHO collaborating centers, for example) would enable regional collaborations and help encourage the timely sharing of samples and information (*40*).

Human history shows that an influenza pandemic is years overdue (*41*). Moreover, whole-genome sequencing of influenza virus shows dynamic RNA segments that are capable of epidemiologically meaningful reassortment events (*27*,*28*). Whether avian A/H5N1 will be the precursor strain is unknown. However, we must expand, speed up, and connect human and animal surveillance efforts today, which must be matched with an expanded capacity to produce and deliver influenza vaccines worldwide.

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Prospects for Universal Influenza Virus Vaccine

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The current vaccination strategy against influenza A and B viruses is vulnerable to the unanticipated emergence of epidemic strains that are poorly matched by the vaccine. A vaccine that is less sensitive to the antigenic evolution of the virus would be a major improvement. The general feasibility of this goal is supported by studies in animal models that show that immunologic activities directed against relatively invariant viral determinants can reduce illness and death. The most promising approaches are based on antibodies specific for the relatively conserved ectodomain of matrix protein 2 and the intersubunit region of hemagglutinin. However, additional conserved determinants for protective antibodies are likely to exist, and their identification should be encouraged. Most importantly, infection and current vaccines do not appear to effectively induce these antibodies in humans. This finding provides a powerful rationale for testing the protective activity of these relatively conserved viral components in humans.

Current influenza virus vaccines attempt to induce strong antibody responses against the viral glycoproteins hemagglutinin (HA) and, with lesser emphasis, neuraminidase (NA) because their protective efficacy is well documented. Thus, typical HA-specific antibodies neutralize viral infectivity and fully protect against infection when they are present at sufficient concentration in the lining fluid of the respiratory tract, and typical NA-specific antibodies inhibit the release of newly formed virus from infected host cells and thus limit the spread and shedding of virus during infection. Current vaccines are highly effective in children and adults (70%–90%), although not in those ≥65 years of age (30%–50%) (*1*). Apart from their limited efficacy in the elderly, a major drawback of current vaccines is that the principal vaccine targets, most notably the distal region of HA, are subject to continuous alteration in circulating epidemic virus strains (*2*,*3*). This process, termed antigenic drift, results from the high mutation rate of the viral genome and the continuous selection of mutants with improved replication characteristics in the immune human host population. On average, the prevalent influenza A virus strain acquires 3–4 amino acid changes per year in HA, with most being located in the regions recognized by protective antibodies. Every 2 to 5 years, the accumulation of mutations results in a major antigenic drift away from the previously circulating strains (*4*). A more drastic antigenic change, termed antigenic shift, occurs if a new HA subtype is introduced into the pool of human virus strains by reassortment of genes between animal and human strains or by direct transmission of strains from an animal reservoir to humans, as has occurred recently with strains of H5N1, H7N7, and H9N2 (*1*). Accordingly, the influenza vaccine must be updated on a regular basis to reflect the antigenic changes that occur in the pool of circulating virus strains. Because vaccines have to be manufactured before the actual epidemic strains are known, a failure to anticipate emergence of a strain with major antigenic drift or shift relative to the vaccine will result in a substantial reduction or abrogation of vaccine-mediated protection.

While antibodies to the immunodominant, but highly variable, regions of HA and NA can provide potent virus strain–specific protection, the existence of weaker and more broadly protective immune activities directed to less variable regions of viral proteins has long been known (*5*). These protective activities have collectively been termed heterotypic or heterosubtypic immunity because they provide a measure of protection against viruses of distinct subtypes. Because of their potential for broadening vaccine-mediated protection in humans, they have been studied extensively in animals and found to be mediated predominantly by virus-specific memory T cells (*6*,*7*), antibodies (*8–10*), or a combination of both (*11–13*). The reason for these differences in the relative strength of T-cell and antibody-mediated protection is not clear but could be attributable to differences in vaccination

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procedures, virus challenge, and read out (how protection was measured) between the various studies. Pros and cons of some of these activities in terms of their potential for development of a broadly protective, "universal" influenza vaccine are briefly discussed below.

Memory T Cells

A large fraction of the virus-specific T-cell response in mice and humans is directed to conserved determinants of viral core proteins, and many studies in mice have shown that memory T cells can accelerate recovery and reduce illness on virus challenge. Cytotoxic T (Tc) cells were found to be more protective than helper T cells, and among Tc cells, protective activity was shown to depend on their frequency (number of virus-specific cells/total cells), cytokine secretion profile, memory type (central vs. effector), and even fine specificity. However, in contrast to findings in mice, the protective value of memory Tc cells in humans remains controversial. The classic study by McMichael et al. (*14*) indicated that presence of memory Tc cells in blood, which could give rise to Tc cells on stimulation in vitro, correlated with reduced virus shedding 3–4 days after volunteers were challenged with a wild-type virus, but had no significant effect on illness. Subsequent studies performed in children found no significant difference in shedding of attenuated vaccine strains in patients who had recovered from previous infection with a vaccine or natural strain of a different subtype than did study participants who had no evidence of previous virus exposure (*15,16*). Similarly, children vaccinated with an H1N1 strain showed no difference in attack rate and febrile respiratory illness during exposure to natural epidemic H3N2 virus from controls who received a placebo (*17*).

Although the presence of memory Tc cells in the vaccinated children was not demonstrated experimentally, it can be implied based on findings that infection with a live, attenuated vaccine or natural virus strain typically stimulates a Tc-cell response in humans. Taken together with the observation that the degree of antigenic change (drift, shift) is a major determinant of epidemic severity, little evidence exists for a substantial protective role of subtype cross-reactive memory Tc cells in human influenza virus infection; the contribution of Tc cells per se in the control of the infection is not questioned, only whether memory Tc cells provide a further improvement.

Vaccine-induced or natural upper respiratory tract infection in humans may not engender an optimally protective memory Tc-cell population because of insufficient number or composition. However, a large number of memory T cells may also result in immunopathologic manifestations (*14,18*), which tend to be associated with excessive inflammatory responses in acute infections. Thus, a universal vaccine based on the induction of a strong memory-Tc response might necessitate a difficult balancing act between protection and immunopathologic changes. Unless one can identify a particularly protective memory Tc-cell population that is poorly induced by natural or vaccine-induced infection, the nondiscriminatory enhancement of memory T-cell populations may not be a promising approach for a universal influenza vaccine.

Antibodies Specific for Conserved Viral Determinants

A precondition for antibody-mediated protection is the accessibility of the viral antigen to antibody on infectious virus particles, intact infected cells, or both. This accessibility restricts the potential targets to conserved structures of the ectodomains of viral transmembrane proteins HA, NA, and M2, in the case of influenza A viruses, and HA, NA, NB, and BM2, in the case of influenza B viruses. Results of studies reported thus far have focused on M2 of influenza A and HA of influenza A and B viruses.

M2 of Influenza A Viruses

M2 forms tetramers that exhibit pH-inducible proton transport activity. It regulates the pH of the viral core after virus uptake into the host cell's endosomal compartment during initiation of infection and subsequently of vesicles that transport the viral transmembrane proteins to the cell surface during the late stage of infection. M2 tetramers are expressed at high density in the plasma membrane of infected cells and are well accessible to M2e-specific antibodies in this location, but only a few copies become incorporated into the envelope of mature infectious virus particles (*19,20*). M2 has a small, nonglycosylated ectodomain (M2e) of 23 amino acids (aa), not counting the posttranslationally removed N-terminal Met. This region has shown only limited variation among human influenza A viruses. This remarkable degree of structural conservation of M2e is attributable mainly to its genetic relation with matrix protein 1 (M1), the most conserved protein of influenza A viruses with which it shares coding sequences. Thus, aa residues 1–9 of M2e and M1 are encoded by the same nucleotides in the same reading frame and aa 10–23 of M2e and 239–252 of M1 in a different reading frame.

Studies by several groups conducted in mice and ferrets have shown that M2e-specific antibodies, while they did not prevent infection, restricted subsequent virus replication and reduced illness and proportion of deaths (*20–24*). This antibody response was only poorly induced by infection, both in mice (*22*) and humans (*24,25*). A likely reason for the poor M2e-specific antibody response is extensive antigenic competition with HA- and NA-specific responses (*26*). Thus, in view of the >10-fold difference in ectodomain size, the frequency of M2e-specific precursor

B cells must be orders of magnitude lower than the frequencies of HA- and NA-specific precursor B cells. Assuming that most immunogenic entities generated in the course of infection contain a mixture of all 3 transmembrane proteins, most M2e may be taken up by HA- and NA-specific B cells, leaving little or none for B-cell receptor–mediated uptake and processing by M2e-specific precursor B cells. Note that the same phenomenon results also in a suppression of the NA-specific antibody response by immunodominant HA-specific B cells (*26*). Such competition can be avoided by presenting individual antigens on physically distinct immunogenic entities to the immune system (*27*). The substantial M2e-specific antibody responses seen in mice after vaccination with dedicated M2e vaccines (*20–24*) supports the above explanation.

In view of the poor or absent M2e-specific antibody response in humans, confirming the genetic stability of M2e was essential when the virus was propagated in an immune environment. Replication of A/PR/8/34(H1N1) (PR8) virus for >3 weeks in severe combined immunodeficient (SCID) mice that were chronically treated with M2e-specific monoclonal antibodies (MAbs) resulted in the emergence of M2e-escape mutants (*28*). However, only 2 distinct escape mutants emerged, 1 with a replacement of Pro at position 10 by Leu (P10L) and the other with a replacement of the same Pro by His (P10H) (*28*). Each of these mutants was isolated repetitively from many distinct mice treated with distinct M2e-specific MAbs, which indicates that they represented essentially the entire range of escape mutants capable of arising from the PR8 wild-type virus under the given experimental conditions. No escape mutants emerged after 11 consecutive passages of PR8 in BALB/c mice that had been actively vaccinated with M2e (unpub. data). In addition, incorporating determinants of potential escape mutants into a polyvalent universal M2e vaccine would likely further impede emergence of escape mutants. Indeed, preliminary studies have shown that no escape mutants emerged in SCID mice treated with a combination of MAbs specific for M2e of wild-type PR8 and the P10H and P10L escape mutants (unpub. data). Thus, although M2e is not totally invariant, it is remarkably stable, even under immune pressure.

Several vaccination strategies have been evaluated in mouse and ferret models, including M2-expressing recombinant viruses, M2 recombinant proteins (*20*,*21*), M2-encoding plasmid DNA (*29*), and synthetic M2e peptides that were chemically linked to carrier proteins or synthetically linked to defined helper T-cell determinants (*22–24*). In most studies in which induction of an antibody response was confirmed, M2e-specific immunity reduced illness, but did not entirely prevent it. The best protection was reported for mice vaccinated by the intranasal route with an M2e-hepatitis B core fusion protein construct and detoxified heat-labile *Escherichia coli* enterotoxin adjuvant; almost none of these mice died after a virus challenge that killed 90% of control mice (*21*). However, in contrast to the significant protection seen in most mouse models, pigs vaccinated with recombinant M2e-hepatitis B core protein or plasmid DNA encoding an M2e-nucleoprotein fusion protein showed no protection or even had higher death rates, respectively, after virus challenge (*29*). This finding needs to be confirmed, and the explanation for it remains unknown. At this time, it serves as a reminder that immune phenomena are complex and that observations made in 1 species may not apply to another. By the same token, good protection in an animal model does not guarantee protection in humans.

Taken together, the observations that M2e shows minimal antigenic variability, even under antibody-mediated pressure in vivo, that M2e-specific antibodies typically restrict virus replication in vivo, and that humans exhibit low or undetectable M2e-specific antibody titers provide a strong rationale for further exploration of an M2e-based vaccine.

HA of Influenza A and B Viruses

The HA molecule has a large ectodomain of ≈500 aa. A posttranslational cleavage by host-derived enzymes generates 2 polypeptides that remain linked by a disulfide bond. The larger N-terminal fragment (HA1, 320–330 aa) forms a membrane-distal globular domain that contains the receptor-binding site and most determinants recognized by virus-neutralizing antibodies. The smaller C-terminal portion (HA2, ≈180 aa, excluding transmembrane and cytoplasmic domain) forms a stemlike structure that anchors the globular domain to the cellular or viral membrane. Sixteen HA subtypes have been identified among influenza A viruses (*30*); 3 of these (H1, H2, H3) have been associated with classic influenza isolates, and 3 (H5, H7, H9) have been associated with recent sporadic human isolates (*1*). Influenza B viruses possess only 1 HA subtype.

Although the degree of sequence diversity between subtypes is great, particularly in the HA1 polypeptides (34%–59% homology between subtypes), more conserved regions are found in HA2 (51%–80% homology between subtypes). The most notable region of conservation is the sequence around the cleavage site, particularly the HA2 N-terminal 11 aa, termed fusion peptide, which is conserved among all influenza A subtypes and differs only by 2 conservative aa replacements in influenza B virus. Part of this region is exposed as a surface loop in the HA precursor molecule (HA0) (*31*). However, when HA0 is cleaved into HA1/HA2, the newly generated terminals separate, and the hydrophobic fusion peptide becomes tucked into a cavity of the stem (*31*). As most HA subtypes are cleaved by extracellular enzymes, this surface loop may be accessible

to antibody, at least temporarily, on HA0 expressed in the plasma membrane of infected host cells. The protective potential of antibodies directed to this region of HA0 has been explored in 2 studies by immunization of mice with synthetic peptides spanning the cleavage site (*32*,*33*). Both studies found that mice vaccinated with a peptide spanning the HA1/HA2 joining region exhibited less illness and fewer deaths on virus challenge (*32*,*33*). Most importantly, HA1/HA2 joint-specific antibodies were undetectable in virus-immune human sera (*33*). These findings make the HA1/HA2 joining region another promising candidate for inclusion in a universal vaccine. Indeed, the authors of 1 study, some of whom had been involved in an M2e-vaccine study, commented that joint-specific immunity in the mouse model was more robust than M2e-specific immunity (*33*).

Although the HA1/HA2-joining region is the most broadly conserved HA sequence, other determinants on HA2 are shared between a restricted number of subtypes. For instance, a MAb that reduced illness and death in passively immunized mice against viruses of the H1, H2, and H5 subtypes has been described (*34*,*35*). This MAb was shown to recognize a conformational epitope of HA2 (*36*), but no immunogen that could selectively induce this response has been described. A search for determinants shared by a more restricted number of closely related subtypes such as H2 and H5, which display 85% sequence homology in HA2, or shared by members of the same subtype, which typically display >95% sequence homology in HA2 (*30*), would be worthwhile, particularly since the HA2-specific antibody response appears to be induced less effectively than the HA1-specific response by infection in humans (*37*). That many HA2-specific antibodies do not display substantial antiviral activities in vitro does not preclude protective activity in vivo because the mere binding of antibody to native HA expressed on infected cells and infectious virus could mediate protective activity by targeting Fc-receptor expressing cells or complement deposition to these structures.

Other Viral Transmembrane Proteins

To our knowledge, conserved determinants for protective antibodies have not been described for any of the other transmembrane proteins of influenza A and B virus. BM2 of influenza B virus, the homolog of M2, has only a 6-aa-long ectodomain (*38*). This ectodomain is most likely too small for formation of a BM2-specific epitope because protein epitopes have usually been found to comprise 12–17 contact residues. NB of influenza B virus also shows structural similarities with M2 of influenza A virus, including ion channel activity (*39*), and has an 18-aa-long ectodomain. However, NB2 has 2 attached carbohydrate chains that can be expected to mask the protein core from recognition by antibody. NA, however, is a good and not sufficiently explored target for cross-protective antibodies. Like HA, it displays a large ectodomain of ≈420 aa. Nine subtypes are recognized among influenza A viruses, while influenza B virus contains 1 subtype. The C-terminal of the polypeptide (≈380 aa) forms a globular head that is anchored to the viral membrane by a flexible stalk. The globular domain contains the enzyme-active site and all known antigenic sites.

Although no cross-protective NA-specific antibody population has been identified, indirect evidence supports the existence of cross-reactive determinants on N1 and N2, the subtypes found in classic human strains. Thus, mice vaccinated first with a mixture of purified N1 and N2 proteins and subsequently boosted with the individual antigens showed a small memory response also against the heterologous subtype (*40*). Given the ample expression and accessibility of NA on infectious virus and infected host cells, a search for determinants shared between or within subtypes would be worthwhile.

Conclusions

Studies in animal models have yielded clear evidence for the existence of antibody populations that are directed to relatively invariant determinants of the ectodomains of viral transmembrane proteins and are capable of substantially reducing, in some cases even preventing, clinical illness resulting from influenza virus infection. Additional highly conserved determinants likely exist, particularly on HA2 and NA polypeptides, which can serve as targets for protective antibodies. These targets should be identified for 2 reasons. First, with the exception of the fusion peptide, none of the presently identified "conserved" determinants is totally invariant, and each of these relatively invariant determinants may show increased variability under specific immune pressure. Second, incorporation of several conserved targets in a universal vaccine may decrease the likelihood and rate of emergence of escape mutants and increase the strength of protection.

None of the identified broadly protective antibody populations has been found consistently and at appropriate concentrations in human sera, which indicates that neither is effectively induced by natural infection or current vaccines. Therefore, the observation that heterosubtypic protection in humans tends to be low does not exclude the possibility of substantial protection by these antibody populations in humans if it can be induced by a specific vaccine. A focused search will likely show additional relatively conserved target structures for protective antibodies. Any of these responses, if not already induced effectively by infection or current vaccines, will be worth pursuing for incorporation into a universal vaccine. The main difficulty may be to develop in each case an immuno-

genic moiety that can effectively induce the desired antibody population. However, even if an appropriate vaccine for induction of a desired broadly protective antibody response cannot be developed, through this research, humanized antibody reagents may be generated that can be used to treat life-threatening human infections. In view of the potential rewards, the universal vaccine approach should be further explored in animal models and its protective efficacy assessed in humans.

None of the universal vaccines studied thus far in animal models has achieved the level of protection provided with current vaccines. Still, an optimized polyvalent universal vaccine, while not preventing infection, may prevent clinical disease, as has been reported already for 2 vaccination modalities (*21*,*23*). If the same results applied to humans, a universal vaccine might replace the current vaccine. Alternatively, if a universal vaccine can only reduce, but not prevent, clinical disease in humans, it could still be used as adjunct to current vaccines and provide increased resistance in case of the unanticipated emergence of a major drift variant or new subtype. Newborns, who are at risk for severe disease, would then receive at least some protection by maternal antibodies. In the elderly, another high-risk population, a universal vaccine may be particularly advantageous because the protective antibodies are generated by memory B cells that tend to be maintained into old age and can be recalled by booster vaccination. In contrast, the efficacy of current inactivated vaccines depends greatly on the ability to mount a strong response to novel (strain-specific) determinants generated through antigenic drift and shift on HA and NA. This response requires naive B cells, whose frequency tends to decrease with increasing age. When all factors are taken into account, protection against influenza virus infection likely can be improved by a universal vaccine.

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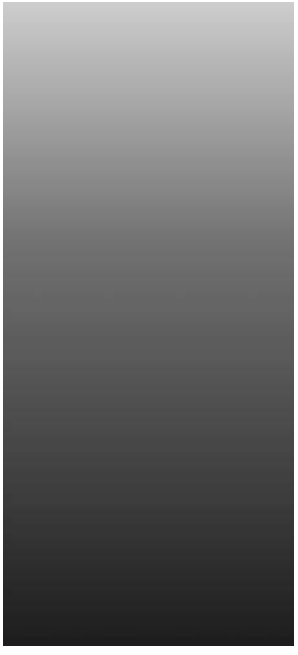

Domestic Ducks and H5N1 Influenza Epidemic, Thailand

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In addition to causing 12 human deaths and 17 cases of human infection, the 2004 outbreak of H5N1 influenza virus in Thailand resulted in the death or slaughter of 60 million domestic fowl and the disruption of poultry production and trade. After domestic ducks were recognized as silent carriers of H5N1 influenza virus, government teams went into every village to cull flocks in which virus was detected; these team efforts markedly reduced H5N1 infection. Here we examine the pathobiology and epidemiology of H5N1 influenza virus in the 4 systems of duck raising used in Thailand in 2004. No influenza viruses were detected in ducks raised in "closed" houses with high biosecurity. However, H5N1 influenza virus was prevalent among ducks raised in "open" houses, free-ranging (grazing) ducks, and backyard ducks.

The continuing spread of H5N1 avian influenza viruses from eastern Asia to domestic and wild birds in central Asian countries, including Mongolia, Kazakhstan, Russia, and Turkey, indicates the extent to which the geographic range of this highly pathogenic influenza virus has expanded. The highly pathogenic H5N1 viruses were first detected in 1996 in geese in Guangdong, China (*1*); they later spread to ducks in the coastal provinces of South China (*2*) and to Hong Kong's live poultry markets (*3*). These viruses infected at least 18 persons in Hong Kong, 6 of whom died (*4*). The viruses were eradicated in 1998 by the culling of all poultry in Hong Kong and by changing marketing practices. Although these particular genotypes have not been detected again, other H5N1 genotypes continued to emerge in 2000 and 2001 (*5*).

The biology of the H5N1 viruses changed dramatically for the first time in late 2002, when the viruses were isolated from dead wild aquatic birds in Hong Kong and from decorative waterfowl that died in Kowloon Park, Hong Kong (*6*,*7*). After the Z genotype of H5N1 influenza became established as the dominant H5N1 influenza virus in eastern Asia, it was transmitted to persons in Vietnam, Thailand, and Cambodia. In 2004, a distinguishable genotype was transmitted to persons in Indonesia (*8*). Most human cases have resulted from the direct transmission of virus from poultry to humans (*9*). To date, evidence for human-to-human transmission is limited (*10*,*11*). In Thailand, 13 persons infected with an H5N1 influenza virus died in 2004, and 2 additional human deaths occurred in October 2005. By contrast, in neighboring Vietnam, 42 human deaths caused by H5N1 influenza virus were reported in 2005. What accounts for these differences? Here we examine the hypothesis that the lower death rate in Thailand resulted in part from that government's recognition of the role of backyard chickens and domestic ducks in the spread and perpetration of H5N1 influenza virus and the government's aggressive culling of flocks in which the virus was detected (*12*).

Thai health officials recognized that the spread of H5N1 influenza viruses to domestic chickens correlated with the distribution of free-grazing ducks (*13*). At the beginning of the 2004 poultry outbreak, ducks were raised in 1 of 4 systems: 1) in high-biosecurity closed houses, 2) in moderately high-biosecurity open houses (ducks raised for meat and laying ducks); 3) in rice fields after harvest (free-range or so-called grazing ducks); or 4) in backyards (backyard ducks). We discuss each method, particularly emphasizing the role of grazing ducks in the perpetuation and spread of H5N1 in the country. We also describe the clinical and pathologic changes in ducks and consider the current policies regarding duck raising in Thailand. We conclude that the traditional methods of raising ducks in Thailand and the rest of Southeast Asia must be modified if we are to control the spread of avian influenza virus.

Methods of Duck Raising in Thailand

Four systems were in use during 2004 (Figure 1).

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Closed High-Biosecurity System

Pekin ducks and white Cherry Valley ducks are raised in closed sheds housing 5,000–6,000 birds each. Day-old ducklings are raised for meat in 50 to 55 days by using an "all-in/all-out" system. Before the ducks are sent to slaughter, 60 cloacal samples (≈1%) are collected for virus isolation. In the slaughterhouse, 60 additional samples are collected from the same flock for virologic analysis. At the end of every 50- or 55-day cycle, each poultry house is cleaned and disinfected. After 3 to 4 weeks, the farm is repopulated with day-old ducklings and the cycle is repeated. In 2005, ≈2–3 million ducks were raised in this system.

Open House System

In the open house system, ducks are raised for meat or as egg layers. The species raised for meat, Pekin and white Cherry Valley ducks, are raised essentially as in the closed-house system with the all-in/all-out strategy. Virologic sampling is conducted as described above. At present ≈1 million to 2 million ducks are being raised in this system. The species raised as egg layers are khaki Campbell, native laying ducks, and a crossbreed of the khaki Campbell and native laying duck. Layer ducks are housed in flocks of 3,000 to 4,000 birds. After they begin laying eggs (at 5 to 6 months of age), these ducks are kept for 12 to 13 months or until they stop laying, at which point they are sent for slaughter. After a short period for cleaning the houses, additional ducks are added as space becomes available. Presently, ≈5 million to 8 million laying ducks are raised in this system in Thailand. Laying ducks are sampled for virologic analysis every 3 months. Influenza-positive flocks are culled.

Grazing System (Free-range Ducks)

In 2004, ducks were also raised in the open on rice fields. Most free-range ducks are egg-laying ducks such as khaki Campbell or a crossbreed of khaki Campbell and native laying ducks. However, a small number of "meat" ducks, such as Pekin and white Cherry Valley ducks, are also raised in the open. After hatching and spending 3 weeks in a brooder, young female ducks are moved to rice paddy fields. For the next 5 to 6 months, they grow by eating snails and residual rice after the harvest. When the food supply in 1 field is exhausted, the ducks are moved by truck to another field, often over considerable distances, and even from 1 province to another (Figure 2). When the grazing female ducks are 5–6 months old, they are brought back to the farms, as in the open system described above. However, some flocks of female laying ducks are kept in the rice fields. Male ducks of the species, who are raised with egg-laying hens**,** and others that are produced for meat are raised in the grazing system for 2 months and are then taken to the slaughterhouses. If they have not reached the optimal weight for slaughter, they are fed supplementary rations for 1 to 2 weeks. During the nationwide surveillance campaign in 2004, 60 cloacal swab samples from each flock were collected for virologic analysis, and the whole flock was culled if a single duck was positive for H5N1 by virus isolation. Flocks that were negative for virus were monitored and put into houses. At the beginning of 2004, ≈10 million to 11 million grazing ducks were being raised in Thailand. Raising free-range ducks is currently illegal in Thailand; all are housed.

Backyard Ducks

Mixed species of ducks continue to be raised in the backyards of village homes together with other animals, including chickens, geese, and pigs. The duck species raised in backyards include Pekin, white Cherry Valley, Barbary Muscovy, khaki Campbell, native laying ducks,

Figure 1. Duck-raising systems in Thailand. A) Closed system with high biosecurity, an evaporative cooling system, and strict entrance control. B) Open system but with netting to prevent entrance of passerine birds. Biosecurity was not strictly enforced. This system is no longer approved for the raising of poultry. C) "Grazing duck raising." Biosecurity is never practiced in this system. D) Backyard Muscovy ducks raised for a family; no biosecurity is practiced in this system

Figure 2. Example of grazing-duck movement. A single flock of ducks was moved 3 times by truck in 1 season in 2004. The size of the flock is 3,000–10,000. The time spent at each site depends on the availability of rice fields at the site: an acre of rice could support 3,000 ducks for 1 to 2 days. The duck owners have agreements with the landowners regarding the time of harvest and the acreage available. One flock could spend as long as 1 month at a single site before being moved to the next.

and mule ducks (a sterile crossbreed of Muscovy ducks and native ducks). If a single case of H5N1 infection is detected in a village, all the poultry in the village are culled. Approximately 1.0 million to 1.5 million ducks were raised as backyard ducks at the beginning of the outbreak in 2004; culling reduced that number to <1 million by August 2005.

National Surveillance Program

In response to the H5N1 influenza outbreaks in 2004, the government of Thailand dispatched teams to villages to identify infected birds and cull flocks in which infection was detected.

Sample Collection, Histopathology, Virus Isolation, and Serology

During the study period (February to September 2004), our laboratory received 450 sick, moribund, or dead ducks from 25 flocks in the western and central provinces of Thailand. In the detailed studies (Table 1), blood was sampled for serologic analysis by the hemagglutination inhibition (HI) test. All moribund ducks were euthanized, and their internal organs were collected, fixed with 10% buffered formalin, and processed for histopathologic analysis. Additionally, parts of the brain, lung, trachea, intestine, liver, pancreas, kidney, ovary, oviduct, testes, heart, and tight muscle were collected for virus isolation. The tissues were ground and filtered through 0.2-μ filters. The filtrates of each organ were injected into 9- to 11-day-old embryonated chicken eggs and incubated at 37°C for 2 days. The eggs were observed daily to determine whether death occurred. The allantoic fluid was harvested and tested for influenza virus by HI assay. Any positive sample was then subtyped for H5N1. A second egg passage was performed if the embryonated eggs were still alive 72 hours after injection.

H5N1 Subtyping

Avian influenza virus was subtyped by HI assay by using antiserum specific against the H5 hemagglutinin. Reverse-transcription polymerase chain reaction (RT-PCR) analysis was used for H5 and N1 typing (*14*).

Immunohistochemical Testing

To evaluate histologic changes, we used immunohistochemical testing by indirect immunoperoxidase staining as described (*15*). Tissue was fixed in formalin before being embedded in paraffin, then cut in 5-μ-thick sections and mounted onto silanized slides.

Criteria for Culling Ducks

The criteria for culling duck flocks were based on H5N1 virus isolation and identification by serologic and RT-PCR analysis (*12*). During the screening of village poultry in 2004, a single positive virus isolation resulted in the culling of all poultry (e.g., chicken, ducks, geese, quail) in the entire village. If serologic evidence of infection was detected, cloacal swabs of 60 ducks in that flock were collected and processed for virus isolation in embryonated chicken eggs.

Results

Detection of Influenza Viruses in Different Duck-raising Systems

Closed High-Biosecurity System

As mentioned earlier, ≈1% of every duck flock was sampled for H5N1 detection before being sent to slaughter. More than 10,000 ducks were tested during the study period. No virologic or serologic evidence of H5N1 virus

Table 1. Studies of H5N1 influenza in grazing ducks in Thailand, February to July 2004*

Flock no.	Approximate no. ducks	Age when positive for H5N1 virus (d)	Duration of virus shedding before detection of illness or culling (d)	Highest viral titer (log_{10} EID_{50}/mL)	Antibody titers to H5N1 (HI) (log_2) before culling
1†	4,600	66	8	2.0	<1‡
2§	5,200	78	10	3.1	2
3†	8,000	42	5	2.0	<1
4†	6,800	74	7	2.5	2
5†	4,300	93	5	3.3	2
6¶	7,200	59	5	3.6	2
7¶	10,000	82	7	ND	ND
8†	6,300	60	9	3.8	2
9†	9,800	71	10	ND	ND
10†	5.500	51	6	3.4	ND

*EID_{50}, 50% egg infectious dose; HI, hemagglutination inhibition; ND, not done.
†Suphanburi Province.
‡Serum samples collected ≈12 d after flock moved to rice field.
§Nakornpathom Province.
¶Ayuthdhaya Province.

infection was detected in the birds raised in this closed system in western Thailand, including Nakornpathom and Kanchanaburi provinces, despite cocirculation of H5N1 influenza viruses in other duck-raising systems in the region.

Open House System

Most farms that raised ducks with the open house system are in western Thailand, including the 4 provinces of Nakornpathom, Kanchanaburi, Suphanburi, and Rachaburi. Birds from 17 farms were tested for infection with virus; in birds from 4 (23.5%), infection with the H5N1 virus was detected.

Grazing System

In 28 (45.9%) of the 61 free-range duck flocks tested, infection with H5N1 influenza virus was detected. Investigators studied H5N1 infection in 10 flocks of grazing ducks in Ayuthdhaya, Nakornpathom, and Suphanburi provinces between February and July 2004 to determine the biologic and pathologic features of H5N1 infection in the field (Table 1). No virologic or serologic evidence of H5N1 infection was detected in any of the flocks while they were located in the brooding houses. However, after they were moved outdoors to the rice fields, infection with H5N1 influenza was detected in all 10 flocks; the earliest infection was detected 12 days after the ducks left the brooding houses (flock 3, at 42 days of age). The interval between leaving the brooding houses and detection of H5N1 infection was 12–63 days. Of the 10 flocks, 3 (flocks 2, 8, and 9) showed disease signs; only a few birds (<1%) in each flock were clinically affected. However, the interval between initial detection of H5N1 viruses in the flock and culling was 5–10 days, which supports the contention that most ducks in the flocks showed no disease signs.

Serologic evaluation of the flocks showed that low titers of HI antibody were detected before culling, which indicates that an immune response had already begun without disease signs in most birds. Cloacal virus titers in individual ducks showing disease signs before culling were 2.0–3.8 log_{10} 50% egg infectious dose $(EID)_{50}$/mL which shows that virus was being shed in feces (Table 1). Similar virus titers were detected in asymptomatic ducks.

Signs of disease in flocks, 2, 8, and 9 were depression, lethargy, cloudy cornea, and blindness. However, no deaths were observed in the 10 days before culling.

Backyard Ducks

Of the backyard poultry, chickens were the most frequently infected; 56% of the chicken flocks tested were positive for H5N1 influenza (*12*). Ducks were the second most frequently infected; 27% of backyard duck flocks were positive for H5N1. During the second wave of H5N1 infection of poultry and humans in Thailand (August–November 2004), 47% of backyard duck flocks were H5N1 positive. During this time, scientists realized that most ducks infected with H5N1 were asymptomatic.

Pathologic Features

As previously mentioned, our laboratory received 450 sick, moribund, or dead ducks, which were studied for pathologic features of H5N1 infection. These birds had been raised in the open house system or were from backyard flocks. They exhibited signs of disease such as high fever, dyspnea, depression, and diarrhea, and nervous signs such as ataxia, incoordination, and convulsions (Figure 3A). Most had ocular and nasal discharge accompanied by conjunctivitis; 20%–100% of the birds in each flock from which these ducks originated were dead. All cloacal and tracheal swabs and tissue samples were positive for H5N1 by HI and RT-PCR (results not shown).

At necropsy, gross lesions were detected, including

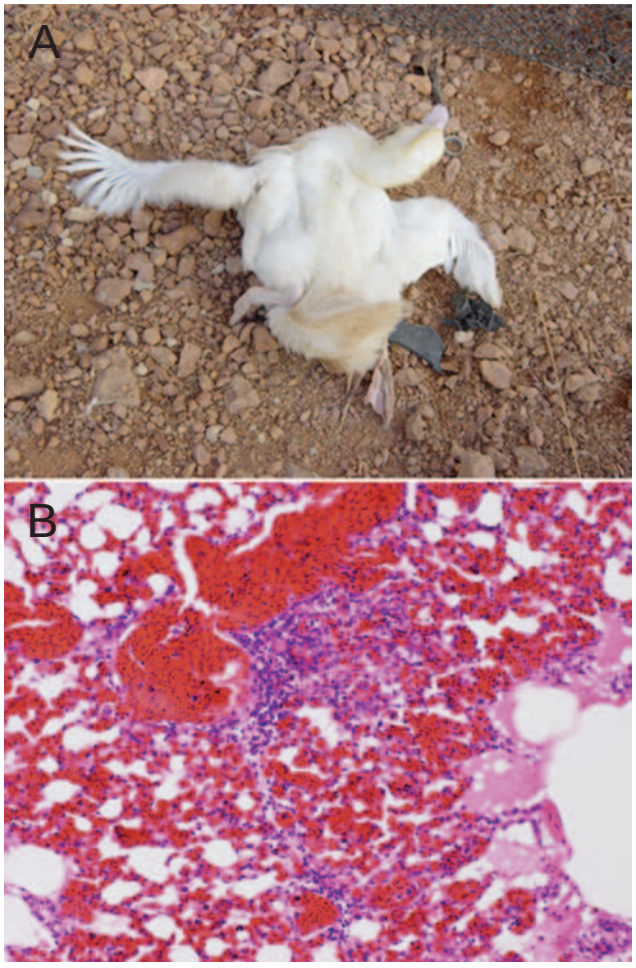

Figure 3. A) A White Cherry Valley duck (*Anas platyrhynchos*), infected with HPAI H5N1 displays nervous signs, convulsions. B) Histopathologic features of the lung of an HPAI H5N1–infected white Cherry Valley duck; infiltration of inflammatory cells in the lung parenchyma (magnification ×100).

ecchymotic or petechial hemorrhage of leg and footpad; serous fluid surrounding the heart, pancreas, liver, and abdomen; cyanosis of the oral cavity; and mild pleural effusion. On histopathologic examination, the most striking lesions were found in the lung, with extensive pneumonia and severe pulmonary edema with hyaline material in the alveolar space and slight mononuclear infiltration in the area surrounding congested vessels (Figure 3B). Nonsuppurative encephalitis with perivascular cuffing of mononuclear cells and gliosis were detected in the brains of ducks that displayed nervous signs. Hyaline degeneration and necrosis of myocardium with mononuclear infiltration were detected predominantly in dead ducks from fast-growing breeds such as the Pekin and white Cherry Valley ducks. Necrotizing pancreatis with mononuclear infiltration was detected in all affected ducks. Most affected ducks exhibited focal hepatitis, tubulonephritis, splenic lymphoid depletion or necrosis, and enteritis. Virus antigen was detected by immunohistochemical tests in all organs tested, including trachea, lung, liver, pancreas, rectum, bursa of Fabricius, spleen, brain, heart, and kidney (Figure 4).

Experimental Infection of Khaki Campbell Ducks

Because culling of all H5N1-positive ducks was mandated in Thailand, we could not determine the natural outcome of infection in birds raised in the open on rice fields. Therefore, khaki Campbell ducks were experimentally infected with 4 representative H5N1 viruses isolated in Thailand in 2004 and 2005. All animal experiments were performed in biosafety level 3+ facilities. All 4 viruses caused the deaths of infected ducks; however, their degree of lethality varied (Table 2). The most lethal virus tested was A/duck/Thailand/71.1/2004, which caused death in 10/10 of the infected khaki Campbell ducks, a death rate comparable to that previously reported for Mallard ducks

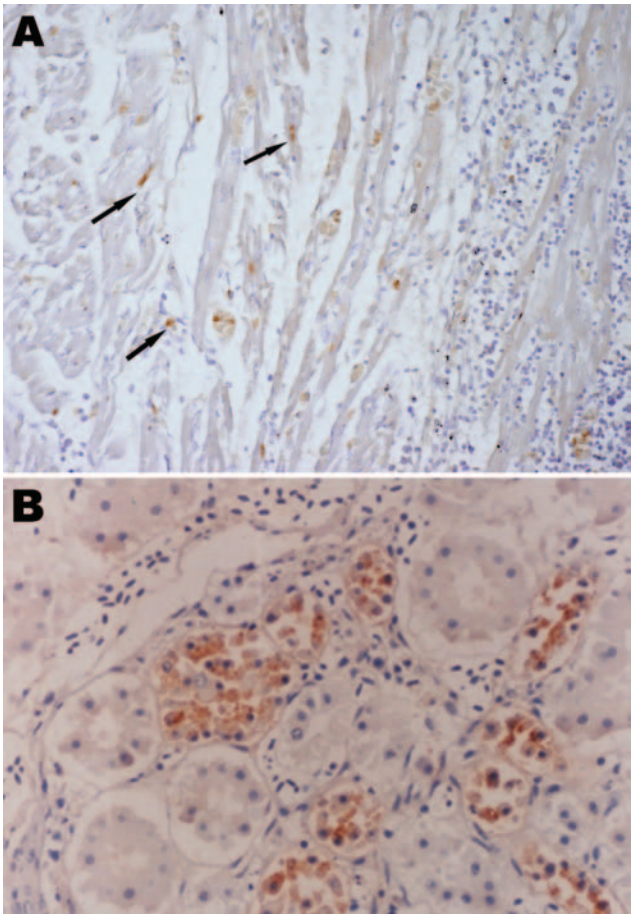

Figure 4. Immunohistochemistry of an HPAI H5N1–infected white Cherry Valley duck (*Anas platyrhynchos*). The viral antigen is detected in myocardial cells and lymphoid cells (arrow) (A) and renal tubular cells (B) (magnification ×100). The primary antibody used for immunohistochemistry in this study was a mouse anti–avian influenza H5 antibody (Magellan Biotechnology, Chunan, Taiwan).

Table 2. Experimental infection of khaki Campbell ducks with viruses isolated in Thailand, 2004–2005

Virus*	Deaths†	Illness†	Day detectable virus was shed‡				
			2	4	6	8	10
A/duck/Thailand/144/05	5/10	3/10	10/10	9/9	1/5	0/5	0/5
A/quail/Thailand/551/05	1/10	2/10	8/10	8/10	1/9	0/9	0/9
A/Thailand/MK2/04	2/10	2/10	9/10	10/10	6/8	2/8	2/8
A/duck/Thailand/71.1/04	10/10	10/10	10/10	1/1	–§	–	–

*Eight 4-week-old khaki Campbell ducks were injected with 10^6 50% egg infectious dose of virus by intranasal and intratracheal infection, and 2 contact ducks were introduced 1 day later.
†Total number of deaths or birds showing disease/total number of birds. Birds were observed daily for signs of infection or death.
‡Number of ducks shedding by the trachea or cloacae/total number of ducks remaining alive. Ducks were swabbed every other day beginning on day 2 postinfection.
§All of the ducks in this group died.

(*16*). Also tested was a human virus isolated in 2004, A/Thailand/MK2/2004, which resulted in the death of 2/10 khaki Campbell ducks. Of the two 2005 viruses tested, 1 caused very slight disease and resulted in only 1/10 deaths (A/quail/Thailand/551/2005) whereas the other (A/duck/Thailand/144/2005) resulted in 5/10 deaths. Ducks inoculated with A/Thailand/MK2/04 shed virus for the longest period of time (day 10 postinfection), whereas the 2005 virus isolates were shed only until day 8 postinfection. These results indicate that the H5N1 avian viruses recently isolated in Thailand can cause death in khaki Campbell ducks; however, several infected ducks remained completely healthy with no signs of disease throughout the study.

Current Status of Duck Raising in Thailand

As of October 2005, the government of Thailand forbids the practice of raising ducks in open fields and moving grazing ducks from 1 region to another. Farmers who do so are subject to fines and other punishments. Additionally, they receive no compensation if they raise ducks in the open free-range system, and the ducks become infected with H5N1. Farmers were initially compensated for the culling of their ducks. Duck raising is now confined to the high-biosecurity system.

After a lull of almost 1 year, a case of human H5N1 infection was reported in Thailand in October 2005. The report was preceded by the illegal grazing of 3 flocks of 3,000 to 5,000 free-range ducks in rice fields in the area (Kanchanaburi Province). Although no direct contact between the grazing ducks and backyard chickens was known, within 2 weeks of the arrival of the ducks, chickens in the area began dying, and a person who had direct contact with the diseased chickens died of H5N1 infection. Approximately 500 backyard chickens were culled in the village. Sequence analysis of the human isolate and avian isolates (duck and chicken) from this area would be essential to confirm the epidemiologic link between these cases and, coupled with the chronology of events, to assess whether free-grazing ducks were indeed the source of infection for this outbreak.

Discussion

The 4 duck-raising systems in wide use at the beginning of the 2004 Thai epidemic differed markedly in cases of influenza detected. No infections with H5N1 influenza virus were detected in ducks raised in the closed system, attesting to the effectiveness of the biosecurity employed. In contrast, H5N1 infection was detected in ducks raised in all 3 open systems. Notably, infection in the hatchery or during the 3 weeks of brooding was detected only after the ducks were released into the rice fields. The source of the H5N1 viruses infecting domestic ducks in the rice fields remains controversial. Because H5N1 viruses were detected in herons, storks, egrets, and other dead waterfowl in Eastern Asia, the initial spread of the highly pathogenic viruses in this region of the world has been attributed to wild migrating birds. What role wild migrating birds had in the spread of H5N1 influenza virus is now a moot question. The widespread outbreaks and massive die-off of barheaded geese and other species in western China (*17*,*18*), and the spread of H5N1 to central Asia (Kazakhstan, southern Russia, and Turkey) and more recently to Romania and Croatia in eastern Europe, are likely caused by wild migratory birds.

Detailed studies of 10 flocks of grazing ducks in Thailand in the present study showed infection with H5N1 influenza virus in all flocks. Although the ducks shed virus for 5 to 10 days, few ducks showed disease signs, and in some flocks, no ducks were symptomatic. Prolonged shedding of H5N1 viruses in experimentally infected ducks has been previously described (*16*,*19*), but prolonged shedding in free-range ducks has not. Therefore, free-range (grazing) ducks that are moved long distances by truck and that do not necessarily show disease signs are an optimal vehicle for the spread of H5N1 viruses throughout the country. These findings support the need for regulations that forbid the practice of raising ducks on the free range, a need underscored by the association of the recent human infection with illegal free-range duck grazing.

This study also points out the dangers of raising ducks in the open systems without complete biosecurity. Although stopping the commercial raising of ducks in open system may be impossible, the more problematic

issue is that of backyard ducks, which are part of traditional village livestock. Highly pathogenic H5N1 influenza virus is now likely endemic in poultry in Vietnam, Cambodia, China, and Indonesia. The vaccine option should be considered if backyard duck raising is to continue in Southeast Asia.

Although no human cases of H5N1 have been attributed to direct contact with ducks in Thailand, free-grazing ducks have been identified as a risk factor for the occurrence of H5N1 outbreaks among chickens (*13*). In Vietnam, however, reported human cases of H5N1 influenza have potentially been linked to the consumption of raw duck blood dishes (http://www.who.int/csr/don/2005_01_21/ en/index.html). Therefore, H5N1-infected ducks are a risk factor for both commercial and backyard poultry and potentially for humans as well. Since the introduction of the nationwide comprehensive surveillance program ("x-ray surveys") in Thailand (*12*) and the culling of all infected poultry, human cases of H5N1 infection have been markedly reduced. Traditional methods of duck raising in Thailand and in the rest of Southeast Asia must be modified if we are to control highly pathogenic H5N1 avian influenza.

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Recently Acquired *Toxoplasma gondii* Infection, Brazil

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The city of Erechim, Brazil, has a 17% prevalence of ocular toxoplasmosis, and type 1 *Toxoplasma gondii* predominates. To examine risk factors for acute *T. gondii* infection in this area, we administered a questionnaire to recently infected persons (n = 131) and seronegative controls (n = 110). Eating undercooked meat; having a garden; working in the garden or yard more than once per week; eating rare meat; eating cured, dried, or smoked meat; eating frozen lamb; and being male increased risk for *T. gondii* infection in univariate analysis. Risk factors independently associated with acute *T. gondii* infection in multivariate analysis were working in the garden (odds ratio [OR] 2.35, 95% confidence interval [CI] 1.27–4.33) and eating frozen lamb (OR 2.06, 95% CI 1.15–3.67). Among women (n = 86), having had children markedly increased the risk for *T. gondii* infection (OR 14.94, 95% CI 3.68–60.73).

In recent years, because of the large number of postnatal *Toxoplasma gondii* infections, researchers estimate that most ocular disease from *T. gondii* is caused by infection after birth (*1–3*). In Erechim, a city in southern Brazil, a representative population-based household survey showed that 17.7% of >1,000 persons examined had ocular toxoplasmosis (*4*). This high rate is believed to be due to acute infection after birth because the rate of infection in young children in this area is low (*4–6*). Erechim has a population of 96,310 and is located in northern Rio Grande do Sul (population 9,619,416), the southernmost state of Brazil, which borders Uruguay and Argentina. The area was mostly settled by Italian, German, and Polish immigrants in the early 20th century. Of the 3 primary restriction fragment polymorphism (SAG2) genetic types of *T. gondii*, investigators have primarily found the type 1 organism in southern Brazil (*7*) and have identified it from chickens (*8,9*). Type 1 *T. gondii* is uncommon in the United States and may be more virulent for ocular disease (*10,11*). However, with worldwide food and animal distribution it could become more common in the United States and other countries.

Although ocular toxoplasmosis has been a problem in southern Brazil for many years, no controlled studies have determined the sources of infection. Therefore, in 2003 and 2004, we conducted a case-control study at the principal ophthalmology clinic in Erechim to evaluate the risk factors responsible for *T. gondii* infection so that prevention messages could be tailored to the factors identified.

Methods

The study was conducted by ophthalmologists at Clinica Silveira and the Federal University at São Paulo, Brazil, in collaboration with researchers from the University of California, Los Angeles, California, and the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. The questionnaire was adapted from those used in previous *T. gondii* case-control studies (*12,13*), with modification and input from the study researchers. The questionnaire took ≈25 minutes to complete and inquired about demographic variables and a comprehensive set of risk factors related to meat, vegetables, food preparation, soil contact, drinking water, and animal exposure (especially cat and cat feces exposure). Information about the number of pregnancies and children was collected from women participating in the survey. Questions involving habitual behavior focused on the most recent 12 months. The questionnaire was self-administered at Clinica Silveira, the principal ophthalmology clinic in Erechim, with parental assistance for children <16 years of age.

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The study was reviewed and approved by the human subjects review committee at the Federal University of São Paulo and approved for analysis at CDC. Oral consent was obtained for completion of the questionnaire. All participants with ocular disease received care and follow-up at Clinica Silveira.

Case-patients with recent *T. gondii* infection were defined as visitors to the clinic from June 2003 to June 2004 with positive test results for both *T. gondii*–specific immunoglobulin G (IgG) and IgM. For each patient, an attempt was made to identify a control as the next patient with negative *T. gondii* antibody test results. Antibody testing was performed at the Fleury Laboratory by using the Abbott AxSYM system (Abbott Diagnostics, Abbott Park, IL, USA).

Data were first examined with univariate analysis; then factors with p value ≤0.05 for association with recent *T. gondii* infection (and sufficient sample size) were entered into logistic regression models for the sample as a whole (N = 241), for women ≥18 years of age (n = 86), and children <18 years of age (n = 106). Men ≥18 years of age were not examined as a subgroup in logistic models because the sample size was too small (n = 49). In odds ratio (OR) calculations with a zero value in ≥1 cells, a correction of 0.5 was added to every cell to avoid undefined results (logit method). Attributable risks were calculated from the logistic regression results for factors that significantly (p≤0.05) increased risk for *T. gondii* infection by using the following formula: attributable risk = proportion of cases exposed for each factor × ([OR − 1]/OR). Data analysis was conducted with SAS software (*14*).

Results

Of 140 persons who had positive *Toxoplasma* IgG and IgM test results, 131 (93.6%) agreed to participate and completed questionnaires. Of 121 persons who had negative *Toxoplasma* IgG and IgM test results, 110 (90.9%) agreed to participate and completed questionnaires. The demographics of patients and controls are presented in Table 1. Controls were more likely to be in the 16- to 20- and 21- to 30-year age ranges and female and less likely to be in the ≤5-, 31–40-, and ≥41-year age groups.

In univariate analysis among the group as a whole, patients were significantly more likely than controls to eat rare meat (this question referred to meat in general); eat cured, dried, or smoked meat given by a friend or relative (purchased cured, dried, or smoked meat was not significantly associated with case status); have a garden; work in the yard or garden; eat frozen lamb; and be male. Patients were less likely to eat raw beef, eat raw ground beef, eat raw ground chicken, and be born in Erechim or upper Uruguay (Table 2). Among women ≥18 years of age, having had ≥1 child (OR 14.68, 95% confidence interval [CI] 4.10–52.50, p<0.0001) (or ≥1 pregnancy, colinear with number of children) was strongly associated with risk for *T. gondii* infection (Table 3). Women infected with *T. gondii* were also more likely than those not infected to have lived in their present home >12 months, work with animals (all types), work in the garden, and have ≥1 cat or kitten living at home. They were less likely to be infected if they ate raw ground beef, raw chicken, rare or raw ground chicken, and fed their cat dried food. In addition, the trend in risk for recent *T. gondii* infection increased from having no children (4 [17.4%] of 23), to 1 child (11[68.8%] of 16), to 1 child (11 [68.8%] of 16), to ≥2 children (23 [79.3%] of 29) (p<0.0001).

Table 1. Demographics of *Toxoplasma gondii* patients and controls, Erechim, Brazil, 2003–2004, N=241

Factor	Patients, no. (%) (n = 131*)	Controls, no. (%) (n = 110*)	χ^2 p value
Age (y)			<0.0001
≤5	14 (10.7)	1 (0.9)	
6–10	16 (12.2)	16 (14.6)	
11–15	19 (14.5)	14 (12.7)	
16–20	12 (9.2)	31 (28.2)	
21–30	21 (16.0)	31 (28.2)	
31–40	28 (21.4)	11 (10.0)	
≥41	21 (16.0)	6 (5.5)	
Sex			0.01
Male	72 (55.0)	42 (38.2)	
Female	59 (45.0)	68 (61.8)	
Race			0.54
White	127 (97.0)	105 (95.5)	
Other	4 (3.1)	5 (4.5)	
Ethnicity			0.51
German, Polish, or Italian	117 (94.4)	102 (96.2)	
Other	7 (5.7)	4 (3.8)	

*Numbers within each category may not add to total n because of nonresponse.

Among children <18 years of age, in univariate analysis those with recent *T. gondii* infection were more likely than those without infection to have a cat that catches its own food; feed the cat raw food; work in the yard more than once per week; eat cured, dried, or smoked meat given by a friend or relative; eat frozen lamb; eat rare meat; eat rare pork; and be male. They were less likely to be born in Erechim or upper Uruguay or wear gloves while working in the yard (Table 4).

In multivariate analysis for the complete group, persons recently infected with *T. gondii* were significantly more likely than those who were not infected to work in the garden (OR 2.35, 95% CI 1.27–4.33, p = 0.006) and eat frozen lamb (OR 2.06, 95% CI 1.15–3.67, p = 0.02) but less likely to be born in Erechim or upper Uruguay (OR 0.19, 95% CI 0.09–0.39, p<0.0001) (Table 5). In multivariate analysis, for women ≥18 years of age, having had ≥1 child compared to having no children markedly increased risk for

Table 2. Factors associated with risk for acute *Toxoplasma gondii* infection, Erechim, Brazil, 2003–2004, N=241 (univariate factors shown with p≤0.05)*

Factor	No. with factor/ no. patients† (%)	No. with factor/ no. controls† (%)	OR (95% CI)	p value
Have a garden (excludes those living on farms)	59/69 (85.5)	41/61 (67.2)	2.88 (1.22–6.78)	0.01
Born in Erechim or upper Uruguay	78/130 (60.0)	98/110 (89.1)	0.18 (0.09–0.37)	<0.0001
Work in garden	57/130 (43.9)	30/110 (27.3)	2.08 (1.21–3.59)	0.008
Eat cured, dried, or smoked meat given by friend or relative	60/130 (46.2)	33/110 (30.0)	2.00 (1.17–3.41)	0.01
Eat rare meat	73/131 (55.7)	47/110 (42.7)	1.69 (1.01–2.81)	0.045
Of those that eat raw meat, eat raw beef	18/31 (58.1)	14/16 (87.5)	0.20 (0.04–1.02)	0.042‡
Of those that eat raw meat, eat raw ground beef	11/31 (35.5)	12/16 (75.0)	0.18 (0.05–0.71)	0.01
Of those that eat raw meat, eat raw ground chicken	2/30 (6.7)	5/15 (33.3)	0.14 (0.02–0.86)	0.02
Eat frozen lamb	37/131 (28.2)	19/110 (17.3)	1.89 (1.01–3.52)	0.045
Male sex	72/131 (55.0)	42/110 (38.2)	1.98 (1.18–3.31)	0.01
Work in yard >1×/wk	65/90 (72.2)	35/71 (49.3)	2.67 (1.39–5.15)	0.003

*OR, odds ratio; CI, confidence interval.
†Totals vary because some questions applied to a subset of participants and because response rates varied.
‡Exact method, OR 0.20, 95% CI 0.02–1.14, p = 0.078.

recent *T. gondii* infection (OR 14.94, 95% CI 3.68–60.73, p<0.0002) (Table 5). For children <18 years of age, being born in Erechim or upper Uruguay was associated with a reduced risk for recent *T. gondii* infection compared to those born elsewhere (OR 0.04, 95% CI 0.01–0.22, p = 0.0002), and male sex (OR 5.70, 95% CI 1.95–16.66, p = 0.002) was associated with an increased risk for *T. gondii* infection (Table 5).

The attributable risks for the factors that significantly increased the risk for recent *T. gondii* infection in logistic regression for the group as a whole (from Table 5) were working in the garden (0.25) and eating frozen lamb (0.15). Among women ≥18 years of age, having had ≥1 child was associated with a high attributable risk (0.84). Among children <18 years of age, male sex was associated with an attributable risk of 0.64.

Discussion

In the study group overall (Table 2), we found that a number of meat- and soil-related factors were associated with recent *T. gondii* infection, which included eating cured, dried, or smoked meat given by a friend or relative; eating rare meat; eating frozen lamb; and having a garden and working in the yard or garden (soil contact). The presence of meat-related factors emphasizes the importance of cooking meat well (>67°C) and not assuming that all home freezing methods will kill *T. gondii* cysts. Having a garden or working in the yard increases the chances of oocyst exposure through soil contact. Men were at increased risk for recent *T. gondii* infection, which could also be related to increased soil contact. To minimize *T. gondii* exposure, gloves should be worn while gardening, and hands should be washed thoroughly afterward with soap and water. Although sample size limitations precluded us from including all the variables in multivariate analysis, working in the garden remained significant in multivariate analysis.

Although the number of persons who ate raw meat was small, eating raw ground chicken was associated with a decreased risk for *T. gondii* infection. Chickens grown

Table 3. Factors associated with risk for acute *Toxoplasma gondii* infection in women ≥18 years of age, Erechim, Brazil, 2003–2004, N=86 (univariate factors shown with p≤0.05)*

Factor	No. with factor/ no. patients† (%)	No. with factor/ no. controls† (%)	OR (95% CI)	p value
Lived in present home >12 mo	46/46 (100)	35/40 (87.5)	14.41 (logit) (0.77–269.25)	0.01
Work with animals	16/46 (34.8)	6/40 (15.0)	3.02 (1.05–8.71)	0.04
Work in garden in past 12 mo	26/46 (56.5)	14/40 (35.0)	2.41 (1.01–5.78)	0.05
Eat rare ground chicken	1/23 (4.4)	4/15 (26.7)	0.13 (0.01–1.26)	0.05
Eat raw ground beef	4/12 (33.3)	6/7 (85.7)	0.08 (0.01–0.95)	0.03
Eat raw chicken	2/12 (16.7)	4/6 (66.7)	0.10 (0.01–0.98)	0.04
Eat raw ground chicken	0/12	4/6 (66.7)	0.02 (logit) (0.00–0.58)	0.001
≥1 cat living at home	20/46 (43.5)	9/40 (22.5)	2.65 (1.03–6.81)	0.04
≥1 kitten living at home	9/46 (19.6)	2/39 (5.1)	4.50 (0.91–22.26)	0.05
Feed cat dried food	29/46 (63.0)	33/40 (82.5)	0.36 (0.13–0.995)	0.05
≥1 pregnancy	34/37 (91.9)	10/28 (35.7)	20.40 (4.98–83.64)	<0.0001
≥1 child	34/38 (89.5)	11/30 (36.7)	14.68 (4.10–52.50)	<0.0001

*OR, odds ratio; CI, confidence interval.
†Totals vary because some questions applied to a subset of participants and because response rates varied.

Table 4. Factors associated with risk for acute *Toxoplasma gondii* infection among children <18 years of age, Erechim, Brazil, 2003–2004, N=106 (univariate factors with p≤0.05)*

Factor	No. with factor/ no. patients† (%)	No. with factor/ no. controls† (%)	OR (95% CI)	p value
Born in Erechim or upper Uruguay	32/58 (55.2)	46/48 (95.8)	0.05 (0.01–0.24)	<0.0001
Cat at least occasionally catches own food	25/26 (96.2)	16/21 (76.2)	7.81 (0.83–73.15)	0.04
Feed cat raw food	15/26 (57.7)	6/21 (28.6)	3.41 (1.00–11.61)	0.04
Work in yard >1 ×/wk	35/43 (81.4)	18/32 (56.3)	3.40 (1.21–9.61)	0.02
Wear gloves when working in yard	0/49	6/35 (17.1)	0.05 (logit) (0.00–0.84)	0.003
Eat cured, dried, or smoked meat given by friend or relative	20/58 (34.5)	7/48 (14.6)	3.08 (1.17–8.11)	0.02
Eat frozen lamb	12/58 (20.7)	3/48 (6.3)	3.91 (1.03–14.80)	0.03
Eat rare meat	33/58 (56.9)	17/48 (35.4)	2.41 (1.10–5.29)	0.03
Of those that eat rare meat, eat rare pork	10/32 (31.3)	0/17	16.33 (logit) (0.89–298.33)	0.01
Male sex	45/58 (77.6)	20/48 (41.7)	4.84 (2.09–11.26)	0.0002

*OR, odds ratio; CI, confidence interval.
†Totals vary because some questions applied to a subset of participants and because response rates varied.

commercially are probably not involved in the transmission of *T. gondii* because modern methods reduce soil exposure, chickens are usually frozen for storage before purchase, and they are thoroughly cooked (*15*). Eating chicken could be associated with a decrease in risk because those who eat chicken may be less likely to eat other meats that are associated with a greater risk. However, free-range chickens in Brazil can be infected with *T. gondii* (9), and eating fresh, undercooked meat from free-range chickens could pose a risk for infection. In addition, eating raw beef or raw ground beef decreased the risk for *T. gondii* infection in univariate analysis, although the sample size was small for this variable as well. Beef is rarely contaminated with *T. gondii* and is not believed to be a consequential source of infection (*15*), but the role of beef in transmission to humans has not been completely determined. Eating raw ground beef that is contaminated with pork because the grinding machine was not cleaned after grinding pork could increase the risk for *T. gondii* infection. Eating rare meat (all types combined) was a risk factor for *T. gondii* infection. Pork and lamb are the most likely meats to be contaminated with *T. gondii* (*15*). Although these data are not specifically from Erechim, researchers in southern Brazil have recently reported that 8.7% of 149 fresh pork sausage samples were positive for *T. gondii* by bioassay in mice in the state of Parana (*16*), and 17% of 286 finishing pigs tested positive for *T. gondii* antibodies with the modified agglutination test in the state of São Paulo (*17*). Dubey et al. determined that tissue cysts survive in pork for ≤3 minutes if heated to 64°C (*18*), so

Table 5. Factors associated with risk for acute *Toxoplasma gondii* infection in multivariate analysis, Erechim, Brazil, 2003–2004

Factor	OR (95% CI)*	p value
All persons (N = 241), factors representing ≥100 cases and p≤0.05 in univariate analysis		
Born in Erechim or upper Uruguay	0.19 (0.09–0.39)	<0.0001
Work in garden	2.35 (1.27–4.33)	0.006
Eat cured, dried, or smoked meat given by friend or relative	1.58 (0.86–2.91)	0.14
Eat rare meat	1.35 (0.76–2.41)	0.31
Eat frozen lamb	2.06 (1.15–3.67)	0.02
Male sex	1.21 (0.59–2.49)	0.60
Women ≥18 y (n = 86), factors representing ≥30 cases and p≤0.05 in univariate analysis†		
≥1 child	14.94 (3.68–60.73)	0.0002
Work with animals	1.74 (0.24–12.86)	0.59
≥1 cat living at home	0.63 (0.05–7.35)	0.71
≥1 kitten living at home	2.95 (0.21–41.03)	0.42
Feed cat dried food	0.86 (0.04–19.10)	0.93
Work in the garden in past 12 mo	1.09 (0.29–4.05)	0.90
Children <18 y (n = 106), factors representing ≥50 cases and p≤0.05 in univariate analysis		
Born in Erechim or upper Uruguay	0.04 (0.01–0.22)	0.0002
Eat cured, dried, or smoked meat given by a friend or relative	1.75 (0.51–6.02)	0.37
Eat frozen lamb	4.52 (0.85–23.97)	0.08
Eat rare meat	2.52 (0.93–6.81)	0.07
Male sex	5.70 (1.95–16.66)	0.002

*Referent is absence of factor. OR, odds ratio; CI, confidence interval.
†The factor "lived in present home >12 mo" had a p value of 0.01 in univariate analysis, but 95% CI calculated with logit crossed 1; therefore, it was not included.

cooking meat (especially pork or lamb) below this temperature could lead to infection.

The strongest factor in analysis of the group overall was the reduced risk for *T. gondii* infection among persons born in Erechim or upper Uruguay compared to persons born elsewhere. This reduction in risk was also significant in multivariate analysis and in children and is likely due to referral of patients with ocular disease from outside the area. Risk for *T. gondii* infection was also reduced among women born in Erechim or upper Uruguay, but the reduction was not significant (data not shown).

For women ≥18 years of age, having had ≥1 child was strongly associated with recent *T. gondii* infection in both univariate and multivariate analysis (Tables 3 and 5). Having had ≥1 pregnancy also showed a strong association with recent *T. gondii* infection in univariate analysis, but we did not examine it in multivariate analysis because it is highly correlated with the number of children. Pregnancy and number of children were risk factors for *T. gondii* infection in other studies, including several from Brazil (*19–22*). Avelino et al. (*20*) suggest that the greater vulnerability of pregnant women to *T. gondii* infection is probably due to alterations in immune mechanisms associated with gestation. In some settings, pregnancy could affect culinary habits. In our study, the number of children and past pregnancies increased risk for recent *T. gondii* infection. Another possible explanation for this finding is that children bring, or track, contaminated soil into the house, increasing risk for infection.

Among children <18 years of age, in univariate analysis many of the meat- and soil-related risk factors were associated with an increased risk for infection that was seen in the group as a whole (Table 4). In addition, feeding cats raw food and having cats that catch their own food were associated with increased risk. To prevent cats from becoming infected with *T. gondii*, they should be fed only dry, canned, or well-cooked food and kept indoors when possible to discourage hunting. In multivariate analysis of the risk factors for children, male sex was the principal factor that significantly increased risk for infection, although eating rare meat and frozen lamb approached significance.

One of the strengths of our study is that serologically defined acute *T. gondii* infection was required for the case definition. Most *T. gondii* IgM- and IgG-positive persons are infected for ≤1 year, so their more recent behavior correlates with the time of their infection. Our study also has a number of limitations. The Clinica Silveira is known for expertise in ocular toxoplasmosis and draws patients with toxoplasmosis-related eye disease from the surrounding area. This fact is the likely reason why patients with acute *T. gondii* infection were more likely to come from outside the Erechim area than from Erechim. Although the sample size was adequate to identify numerous risk factors for *T. gondii* infection, it was not large enough to allow analysis for all subgroups (for example, multivariate analysis of adult men). Some associations in our study may have been due to confounders that we did not consider in the analysis (especially in univariate analysis); however, we inquired about a comprehensive set of variables, including those previously known to be associated with *T. gondii* infection, and performed multivariate analysis. In addition, our study was limited to 1 area of Brazil, and the results cannot necessarily be applied to other areas of the country.

In conclusion, our study identified a number of risk factors for *T. gondii* infection among persons with ocular disease. Because *T. gondii* infection often leads to ocular disease in this region of Brazil, avoiding infection can improve ocular health. Proper meat and soil- and water-related hygiene could reduce the risk for infection with *T. gondii*, especially with preventive education efforts based on risk factors identified in this case-control study. The association of *T. gondii* infection in women with the number of children they have had (and their pregnancies) requires further study to fully elucidate the factors that contribute to this increase in risk. In the future, type 1 *T. gondii* could spread to other areas of the world and increase the risk for ocular disease in those regions.

Dr Jones has worked at CDC for 15 years, first in the area of HIV/AIDS and most recently in parasitic diseases. His current research focuses on toxoplasmosis.

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Reducing *Legionella* Colonization of Water Systems with Monochloramine

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Monochloramine disinfection of municipal water supplies is associated with decreased risk for Legionnaires' disease. We conducted a 2-year, prospective, environmental study to evaluate whether converting from chlorine to monochloramine for water disinfection would decrease *Legionella* colonization of hot water systems. Water and biofilm samples from 53 buildings were collected for *Legionella* culture during 6 intervals. Prevalence ratios (PRs) comparing *Legionella* colonization before and after monochloramine disinfection were adjusted for water system characteristics. *Legionella* colonized 60% of the hot water systems before monochloramine versus 4% after conversion (PR 0.07, 95% confidence interval 0.03–0.16). The median number of colonized sites per building decreased with monochloramine disinfection. Increased prevalence of *Legionella* colonization was associated with water heater temperatures <50°C, buildings taller than 10 stories, and interruptions in water service. Increasing use of monochloramine in water supplies throughout the United States may reduce *Legionella* transmission and incidence of Legionnaires' disease.

Legionnaires' disease, named after an outbreak of severe pneumonia at a legionnaires' convention in 1976, is a form of community-acquired and nosocomial pneumonia. It is caused by inhalation of aerosols or microaspiration of water containing *Legionella* bacteria. *Legionella* spp. are ubiquitous in fresh water and occur naturally as intracellular parasites of amebae (*1*). Potable hot water systems provide environments for amplification of *Legionella pneumophila*, the most common species isolated from patients with Legionnaires' disease. *L. pneumophila* grows optimally at 35°C and multiplies between 25°C and 42°C. Investigations of outbreaks of Legionnaires' disease in hospitals and other community settings have implicated potable hot water systems as sources of transmission (*2–5*).

No strategies have been proven to prevent community-acquired Legionnaires' disease. Prevention of transmission within healthcare facilities focuses primarily on preventing or limiting *Legionella* colonization of plumbing systems through temperature control or use of biocides (*6*). Healthcare facilities are of special concern because of increased susceptibility to and a high case-fatality ratio of Legionnaires' disease among immunocompromised patients and those with underlying illnesses (*5,7*). Because colonized water distribution systems are often implicated in *Legionella* transmission (*2,5,8,9*), effective water disinfection strategies could provide the best measure to prevent Legionnaires' disease.

Chloramination is a method of drinking water disinfection that provides a lasting residual disinfectant in the distribution system. The process involves adding ammonia to chlorinated water; aqueous chlorine reacts with ammonia to form inorganic chloramines (*10*). Monochloramine is the most active compound and forms preferentially at certain ratios of ammonia to chlorine. Approximately 55% of 11.8 million people living in the 25 largest cities in California currently receive water disinfected with monochloramine (unpub. data). A survey in 2004 of municipal water utilities in the United States found that 30% used monochloramine for residual disinfection (*11*). The Environmental Protection Agency estimates that municipal

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water utilities using surface water sources will increasingly convert to monochloramine to meet federal regulations that limit disinfection byproducts in drinking water (*12*).

Use of monochloramine for residual disinfection compared with chlorine was associated with a lower prevalence of *Legionella* colonization in plumbing systems (*13*) and decreased risk of nosocomial outbreaks of Legionnaires' disease in cross-sectional and retrospective case-control studies (*14*,*15*). The planned conversion to monochloramine for municipal drinking water disinfection in San Francisco, California, provided an opportunity to prospectively investigate the effect of chloramination on *Legionella* colonization in potable hot water systems. We report here on the results of a 2-year environmental study.

Methods

Study Site

The San Francisco Public Utilities Commission provides an average of 250 million gallons (950 million liters) of water per day to ≈2.4 million residents in northern California, including 750,000 in the city and county of San Francisco. Surface water makes up >99% of the water supply. Chlorine was added to kill microorganisms present in source water (primary disinfection) throughout the study period. Chlorine concentrations are monitored at several locations throughout the distribution system. Chlorine used for residual (or secondary) disinfection was replaced with monochloramine on February 2, 2004.

Buildings with ≥3 stories in San Francisco were identified from lists of commercial customers of the San Francisco Public Utilities Commission and real property owned by the city and county of San Francisco. Building managers and owners gave permission for sample collection inside the buildings for the duration of the study. Results of *Legionella* cultures were provided only at the completion of the study. Standardized questionnaires were administered to building engineers and facilities managers to obtain information on the age of the building, capacity of water heaters and hot water storage tanks, type of water heating system (boiler, heat exchanger, or instantaneous heaters), and type of pipe material used throughout most of the building. At the completion of the study, building engineers were surveyed about routine maintenance plans for the potable hot water system, standard procedures for flushing outlets after a disruption of water service, and knowledge of industry guidelines for controlling *Legionella* growth in building water systems (*16*).

Environmental Sampling

Samples from each building were collected 6 times during the 2-year period, 3 times before and 3 times after conversion to monochloramine disinfection. Each round of sampling lasted 8–10 weeks. Preconversion and postconversion rounds of sampling were conducted at corresponding seasonal intervals.

Nine samples were collected from each building during sampling rounds, including a 1-L water sample from a water heater or heat exchanger, four 1-L samples of hot water, and 4 swabs of biofilm at point-of-use outlets (faucets or shower heads). Water samples were collected in sterile, 1-L plastic bottles (Nalge Nunc International, Rochester, NY, USA) containing 0.5 mL 0.1 N sodium thiosulfate solution to neutralize free chlorine and chloramines. Water heater samples were drawn from the drain valve, pressure relief valve, or from the closest outlet to heat exchangers. Point-of-use outlets were selected at farthest points from water heaters when possible. Biofilm samples were collected from shower outlets and faucets by inserting a sterile, polyester-tipped applicator swab (Falcon, Becton Dickinson and Company, Sparks, MD, USA) and rotating it firmly against the interior surface. Biofilm swabs were placed in sterile, screw-capped test tubes containing 0.1 mL sodium thiosulfate solution in 5 mL of water from the same site. Hot water taps were run until the temperature reached a maximum for collection of water samples. The same locations were sampled in each round. When sampling could not be performed at the selected site, the nearest substitute site was sampled; however, only samples collected from the same site before and after monochloramine conversion were included in analyses.

Water temperature, pH, and free (disassociated) and total chlorine concentrations were measured in a separate sample bottle. Total chlorine includes free chlorine plus monochloramine. Temperature was measured with a handheld thermometer. pH was measured with a digital meter (pHep 3, Hanna Instruments, Leighton Buzzard, UK). Free and total chlorine residuals were measured by using the N, N-diethyl-p-phenylenediamine method with a colorimeter and test kit (Model DR/890, Hach Chemical Co., Loveland, CO, USA). Building engineers were asked about any interruptions in water service affecting the building or specific sites in the 3 months preceding the sampling date.

Laboratory Procedures

All culturing for *Legionella* species and amebae was performed in the Legionella Laboratory at the Centers for Disease Control and Prevention in Atlanta, Georgia, following standard procedures (*17*). *Legionella* organisms were speciated or serogrouped by macroscopic slide agglutination with a panel of polyclonal rabbit antisera against *Legionella* species and *L. pneumophila* serogroups (*18*). Laboratorians were blinded to the identity of buildings from which samples were obtained, and buildings were assigned different identification numbers in each

round. Samples were transported at ambient temperature and processed a mean (± standard deviation) of 3 (±2) days after collection. Water samples from point-of-use outlets were concentrated 100-fold by filtration through a 0.2-µm polycarbonate filter (Nucleopore, Pleasanton, CA, USA). Biofilm swab samples were placed on a lawn of *Escherichia coli* for detection of ameba and treated with diluted acid (0.1 mol/L KCl, 0.005 mol/L HCl) to reduce the number of non-*Legionella* bacteria before plating.

Concentrations of *Legionella* spp. in water samples are expressed as CFU/mL based on plate counts of *Legionella* colonies grown from a known volume of original sample. Concentrations determined by this method are approximate. The upper and lower limits of detection were 0.05 and 25 CFU/mL for point-of-use outlets and 10 and 5,000 CFU/mL for water heaters. Plate counts were not determined for samples overgrown with non-*Legionella* organisms.

Surveillance for Legionnaires' Disease

Active, laboratory-based surveillance for culture-confirmed *Legionella* infections in San Francisco residents was conducted from January 1, 2003, through December 31, 2004, through the Active Bacterial Core surveillance activity of the California Emerging Infections Program (*19*). We reviewed legionellosis case report forms from the national passive surveillance system for cases among San Francisco residents or persons with a history of travel to San Francisco during the incubation period. Surveys were sent to infection control departments at all San Francisco hospitals to identify cases of probable or confirmed Legionnaires' disease during 2003 and 2004. Information was solicited from hospitals about environmental testing for *Legionella* spp. in water systems, and measures taken to reduce microbial contamination of water systems during 2003 and 2004.

Statistical Analysis

Data were entered into Access version 2002 (Microsoft, Redmond, WA, USA) and analyzed by using SAS for Windows version 9.0 (SAS Institute, Cary, NC, USA). We conducted building- and site-specific analyses of the prevalence of *Legionella* colonization. A building was considered colonized at a timepoint if *Legionella* spp. were cultured from any site. We considered a point-of-use outlet colonized if *Legionella* spp. were cultured from either a water sample or biofilm swab. Wilcoxon rank sum test was used to analyze differences in the proportions of positive sites or concentrations of *Legionella*. We also calculated adjusted prevalence ratios (PRs) and 95% confidence intervals (CIs) or p values by using PROC GENMOD (SAS Institute) for the clustered nature of sites within buildings. Preconversion and postconversion sampling rounds were considered repeated measures. Multivariable models investigated associations between *Legionella* colonization and water measurements or building characteristics.

Results

Effects of Conversion to Monochloramine on Water Distribution System

The conversion to monochloramine provided higher concentrations of total chlorine (which includes both free chlorine and monochloramine) and lower concentrations of trihalomethane compounds, the principal disinfection byproducts in treated water entering the distribution system (Table 1). The conversion to monochloramine also resulted in an ≈10-fold increase in total chlorine concentrations measured in building hot water systems. Average temperature and pH measured in building water samples did not change significantly.

Table 1. Characteristics of treated water in the city distribution system or in water heaters in 53 sampled buildings, San Francisco, California, stratified by year

Measurement (unit)*	2003, mean (range)	2004,† mean (range)	Reference value‡
Treated water			
Total chlorine, ppm	0.60 (0.01–2.20)	1.97 (0.15–3.40)	4.00
Total trihalomethanes, ppb	65.3 (16.0–143.0)	34.8 (11.0–46.0)	80.0
Total haloacetic acids, ppb	19.5 (6.0–55.0)	20.3 (3.0–33.0)	60.0
Total organic carbon, ppb	2.8 (2.4–3.3)	2.9 (2.6–3.1)	NA
pH	9.0 (7.4–9.9)	8.8 (7.5–10.5)	NA
Lead, ppb§	6.7§	11.5	15.0
Copper, ppb§	120§	90	1,300
Water sampled from water heaters in 53 buildings¶			
Total chlorine, ppm	0.13 (0–0.86)	1.10 (0–2.20)	NA
Temperature, °C	44.9 (17.8–87.8)	45.1 (17.8–79.4)	NA
pH	8.9 (7.5–10.5)	8.7 (7.5–10.4)	NA

*ppm, parts per million; ppb, parts per billion.
†Measurements were taken from March through December 2004, after conversion to chloramine for residual disinfection.
‡Maximum allowable levels for compliance with Environmental Protection Agency standards. NA, not applicable.
§Measurements for lead and copper are the 90th percentile for samples collected from point of use. Prechloramine samples were collected during 2001.
¶Water heater samples were collected on 3 occasions over a 10-month period. Mean and range were calculated for 159 samples.

Environmental Sampling

Prospective *Legionella* testing was performed in 53 buildings, including 24 public and 29 commercial buildings. When chlorine was the residual disinfectant in municipal drinking water, *Legionella* spp. were cultured from building water systems on 96 (60%) of 159 occasions, and 37 (70%) of 53 buildings were colonized with *Legionella* spp. in ≥1 of the 3 sampling rounds (Table 2). After conversion to monochloramine, *Legionella* spp. were found on 7 (4%) of 159 occasions in 5 (9%) of 53 buildings. These 5 buildings had been colonized at multiple sites before disinfection with monochloramine. Conversion to monochloramine resulted in a 93% reduction in the prevalence of *Legionella* colonization in building water systems (PR 0.07, 95% CI 0.03–0.16). Colonized water systems were no more likely than *Legionella*-free systems to include hot water storage tanks or material other than copper for hot water plumbing, although sample size limited building-level analyses. *Legionella* spp. were recovered from 12 (60%) of 20 buildings for which engineers reported maintaining water systems according to standard practices, such as maintaining backflow prevention and flushing outlets after interruption of water service, versus 18 (75%) of 24 buildings for which no standard maintenance of water systems was reported (p = 0.28).

A total of 364 (13%) of 2,822 water and biofilm samples yielded *Legionella* spp: 352 (25%) of 1,405 samples collected before conversion and 12 (<1%) of 1,417 samples collected after conversion to monochloramine. Five *Legionella* species and 7 serogroups of *L. pneumophila* were identified (Figure). *L. pneumophila* serogroup 1 accounted for >60% of all *Legionella* organisms. The same species of *Legionella* and serogroups of *L. pneumophila* were repeatedly cultured from individual sites (Figure).

Legionella spp. were cultured from 46 (15%) of 316 water samples from building water heaters: 45 (29%) of 157 samples collected before conversion versus 1 (<1%) of 159 after conversion to monochloramine (p<0.001). When we controlled for water heater temperature, building height, and interruptions in water service, monochloramine use decreased the prevalence of *Legionella* colonization in water heaters by 96% (Table 3). Colonization of water heaters was more prevalent in buildings with >10 stories and in which water service had been interrupted in the past 3 months. Water temperatures ≥50°C were associated with the lowest prevalence of colonization, and *Legionella* spp. were not detected when the temperature exceeded 60°C (140°F). Over the 2-year study period, temperatures of water in building water heaters were ≥50°C at 88 (28%) of 318 sampling timepoints. In buildings in which engineers reported familiarity with industry guidelines for controlling *Legionella* growth in water systems, water heater temperatures were ≥50°C on 26 (21%) of 125 occasions versus 46 (32%) of 144 occasions in buildings in which engineers were not familiar with industry guidelines (p = 0.04).

At point-of-use outlets, *Legionella* spp. were cultured from 247 (20%) of 1,252 water samples and 70 (6%) of 1,254 biofilm swab samples. Combining culture results from the water samples and biofilm swabs from each site, *Legionella* spp. were cultured from 246 (39%) of 624 paired samples before conversion to monochloramine versus 9 (1%) of 622 paired samples after conversion (p<0.001). Median concentrations of *Legionella* spp. at colonized outlets were significantly lower after conversion to monochloramine (Table 2). *Legionella* were cultured from both the water and biofilm samples on 59 (24%) of 246 occasions before conversion versus 2 (22%) of 9 occasions after conversion. The same *Legionella* species and

Table 2. Prevalence of *Legionella* colonization and concentrations in hot water systems in buildings in San Francisco, California, by residual disinfectant and sampling interval*

	Chlorine (2003)			Chloramine (2004)			
Water source	Jan–May	Jun–Jul	Oct–Dec	Mar–May	Jun–Aug	Sep–Dec	p value†
Building water systems (n = 53)							
No. colonized with *Legionella* spp. (%)	27 (51)	34 (64)	35 (66)	5 (9)	0	2 (4)	<0.001
Median no. *Legionella*-positive samples in colonized buildings (range)‡	3 (1–9)	3 (1–8)	4 (1–8)	1 (1–4)	–	1 (1–2)	0.007
Water heaters (n = 53)							
No. colonized with *Legionella* spp. (%)	12 (23)	16 (30)	17 (33)	1 (2)	0	0	<0.001
Median *Legionella* concentration in colonized water heaters, CFU/mL (range)‡	200 (40–400)	40 (10–500)	20 (10–5,000)	10	–	–	0.17
Point-of-use outlets (n = 212)							
No. colonized with *Legionella* spp. (%)	75 (37)	85 (41)	86 (41)	7 (3)	0	2 (1)	<0.001
Median *Legionella* concentration in colonized sites, CFU/mL (range)‡	2.5 (0.05–25.0)	1.5 (0.05–25.0)	1.0 (0.05–25.0)	0.05 (0.05–0.40)	–	0.18 (0.10–0.25)	<0.001

*Percentages calculated based on number of samples included in analyses (see Methods).
†For Wilcoxon rank sum test comparing number of sites colonized with *Legionella* spp. before and after the conversion to chloramine disinfectant.
‡Includes only *Legionella*-positive sites.

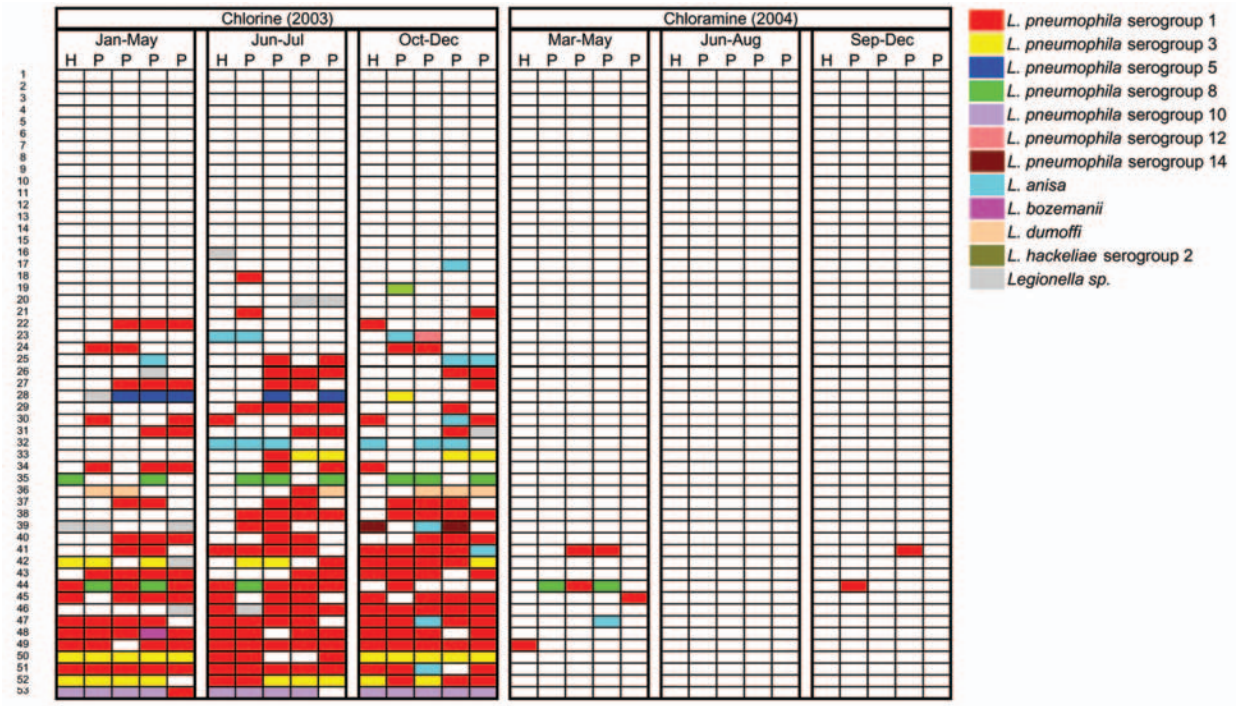

Figure. *Legionella* colonization of water heaters and point-of-use outlets sampled during 6 rounds of environmental sampling in buildings, San Francisco, California (in rows), by residual disinfectant and sampling interval. *Legionella* species or serogroups of *Legionella pneumophila* are represented with different colors. Each row represents a single building and each cell represents the results of *Legionella* culture for a site within the building. H, water heater; P, point-of-use outlet.

serogroup was cultured from biofilm swabs and water samples on 56 (92%) of 61 occasions when both were positive. When we controlled for *Legionella* spp. in the sampled water heater, water temperature at the point of use, building height, and interruptions in water service, monochloramine use decreased the prevalence of *Legionella* colonization at point-of-use outlets by 96% (Table 4). *Legionella* colonization at point-of-use outlets was independently associated with *Legionella* spp. in the sampled water heater, building height, and interruptions in water service. *Legionella* spp. were cultured only from point-of-use outlets and not from water heater samples on 72 (61%) of 118 occasions when building water systems were colonized, including 6 (86%) of 7 occasions after monochloramine conversion.

Amebae at sampled sites were associated with *Legionella* spp. colonization only when chlorine was used for residual disinfection. *Legionella* spp. were cultured from 61 (36%) of 169 samples in which amebae were present versus 291 (24%) of 1,236 samples without amebae ($p = 0.01$). After conversion to monochloramine, *Legionella* were found in 1 (1%) of 78 samples containing amebae and 8 (1%) of 866 samples without amebae ($p = 0.75$). During disinfection with chlorine, *Legionella* concentration was higher in samples containing amebae (median 9.0 CFU/mL, range 0.1–25.0) compared with those without amebae (median 1.5 CFU/mL, range 0.05–25.0, $p<0.001$). The prevalence of amebae decreased from 169 (12%) of 1,405 samples when chlorine was the residual disinfectant to 78 (8%) of 944 samples collected in the first 2 rounds after conversion to monochloramine ($p = 0.006$). Results of ameba cultures from the final round of sampling were discarded after amebae were found in negative control water samples.

Surveillance for Legionnaires' Disease

Active, population-based surveillance for *Legionella* infections identified 1 confirmed case of Legionnaires' disease in a San Francisco resident in November 2004, who traveled to Mexico within 2–10 days of symptom onset. Infection control departments in 7 (70%) of 10 hospitals in San Francisco, including the 3 largest hospitals, reported no hospitalized patients meeting the definition of a probable or confirmed case of Legionnaires' disease (*20*) during the study period. Review of case report forms from the national passive surveillance system did not identify any cases of Legionnaires' disease in persons with history of travel to San Francisco during the incubation period of their illness.

No environmental testing for *Legionella* spp. in hospital water systems was conducted during 2003 and 2004 in the San Francisco hospitals that responded to the survey.

Table 3. Factors associated with *Legionella* colonization of water heaters in sampled buildings, San Francisco, California

Factor	No. samples	% *Legionella* colonization	Adjusted prevalence ratio (95% CI)*	p value
Residual disinfectant				
Chlorine	157	29	Referent	
Chloramine	159	1	0.04 (0.01–0.21)	<0.001
Water heater temperature, °C				
<30	29	21	Referent	
30–39	68	24	0.43 (0.27–0.70)	0.001
40–49	131	18	0.27 (0.16–0.46)	0.001
≥50	88	1	0.09 (0.05–0.18)	0.001
Building height (stories)				
3–10	215	11	Referent	
>10	101	24	2.96 (1.51–5.79)	0.002
Disruption in service in last 3 mo				
Yes	39	26	2.26 (1.31–3.88)	0.003
No	277	13	Referent	

*Prevalence ratios and 95% confidence intervals (CIs) were adjusted for repeated sampling and for effects of all other variables.

Two hospitals added supplemental chlorine to their water system to prevent microbial contamination before monochloramine conversion; supplemental chlorination was discontinued after monochloramine was added to municipal drinking water.

Discussion

This is the largest study to prospectively evaluate the effect of monochloramine disinfection on *Legionella* colonization in a water distribution system. *Legionella* spp. were prevalent and stable in building water systems over 3 rounds of sampling when chlorine was used for residual disinfection of drinking water. Monochloramine disinfection of the water supply reduced *Legionella* colonization in hot water systems. Our findings suggest that monochloramine in drinking water provides better control of *Legionella* growth in building plumbing systems than chlorine. This study supports the biologic plausibility of decreased risk of nosocomial outbreaks of Legionnaires' disease associated with chloraminated water compared with chlorinated water (*14*,*15*).

The conversion from chlorine to monochloramine for residual disinfection resulted in lower concentrations of trihalomethane compounds in drinking water, which met the objectives of the municipal water supplier. Increased stability of monochloramine resulted in higher disinfectant concentrations in potable hot water systems because chlorine dissipates rapidly at higher temperatures. Higher concentrations of disinfectant and the ability of monochloramine to penetrate biofilms were likely responsible for the effect on *Legionella* spp. In model systems, monochloramine eliminates 99.9% of biofilm-associated *Legionella* spp. (*21*), and *Legionella* spp. are cleared rapidly after addition of monochloramine (*22*). Although amebae in model systems

Table 4. Factors associated with *Legionella* colonization at point-of-use outlets in sampled buildings, San Francisco, California

Factor	No. samples	% *Legionella* colonization	Adjusted prevalence ratio (95% CI)*	p value
Residual disinfectant				
Chlorine	617	39	Referent	
Chloramine	627	1	0.04 (0.02–0.09)	<0.001
Water heater colonized with *Legionella* spp.				
Yes	182	74	1.74 (1.26–2.40)	<0.001
No	1,062	11	Referent	
Temperature of water at point-of-use outlet, °C				
<30	23	13	Referent	
30–39	268	24	0.90 (0.39–2.08)	0.8
40–49	786	22	0.85 (0.35–2.05)	0.7
≥50	175	10	0.75 (0.29–1.90)	0.5
Building height (stories)				
3–10	855	15	Referent	
>10	397	31	1.85 (1.46–2.35)	<0.001
Disruption in service in last 3 mo				
Yes	142	23	1.34 (0.99–1.81)	0.06
No	1,109	20	Referent	

*Prevalence ratios and 95% confidence intervals (CIs) were adjusted for repeated sampling and correlations between samples taken from the same building and for the effects of all other variables. Twenty-eight observations were missing.

protect *Legionella* spp. from the short-term effects of monochloramine (*21*), we found no evidence of this protective effect in the buildings we sampled.

The results of this study are relevant for strategies to control Legionnaires' disease in hospitals. Several strategies are currently used by hospitals to control *Legionella* growth in water systems and prevent nosocomial transmission of Legionnaires' disease (*23*). Thermal eradication (superheating water followed by flushing point-of-use outlets) and hyperchlorination were among the earliest methods effective at controlling *Legionella* growth (*23*,*24*). However, superheating increases the risk of scalding injuries and hyperchlorination is associated with increased corrosion of plumbing. Copper-silver ionization has also been used with mixed success (*25*–*27*). Monochloramine use for drinking water disinfection has been associated with lower prevalence of *Legionella* spp. in plumbing systems of hospitals (*13*). Our study demonstrated that *Legionella* colonization in a plumbing system was effectively eliminated by monochloramine. Hospitals or other facilities colonized with *Legionella* spp. might control *Legionella* growth and prevent disease transmission by adding monochloramine to their potable water system. The potential use of supplemental monochloramine in hospitals to prevent nosocomial Legionnaires' disease needs to be evaluated.

The results of our study are striking considering that we observed few cases of Legionnaires' disease despite evidence that *Legionella* spp. colonized most of the San Francisco buildings tested before use of monochloramine. Some cases of Legionnaires' disease may have gone undetected because patients with community-acquired pneumonia are increasingly treated empirically with antimicrobial drugs without microbiologic confirmation (*28*). Although we sampled 4 point-of-use outlets in each building, exposures to aerosols produced by these outlets may have been minimal. Persons exposed to any *Legionella*-containing aerosols may have been at low risk for Legionnaires' disease. Alternatively, the *Legionella* organisms present, even though some were *L. pneumophila* serogroup 1, might lack virulence factors needed to cause human disease (*29*).

Routine maintenance programs for plumbing systems were not effective in preventing colonization with *Legionella* spp., which is consistent with a previous study of hospital water systems (*30*). However, our findings suggest that existing guidelines were not fully implemented in the buildings sampled. Although nearly half of building engineers reported knowledge of industry guidelines for preventing *Legionella* colonization of potable water systems, only 13% of sampled water heaters were set at the recommended temperature of ≥60°C (140°F) (*16*). *Legionella* spp. were not found in water heaters set at the recommended temperature. Maintaining the recommended temperatures in water heaters could help prevent *Legionella* growth in hot water systems. Investigations of legionellosis outbreaks have consistently demonstrated that temperatures of 25°C to 42°C facilitate the growth and amplification of *Legionella* spp. to high concentrations (*1*).

The repeated measurement of *Legionella* colonization at the same sites over time represents a strength of this study. Colonization was stable during the first 3 sampling rounds and no seasonal effect on the prevalence of colonization was observed before conversion to monochloramine. Collection of samples at multiple point-of-use outlets in each building, in addition to water heater samples, increased detection of colonization within buildings. In an outbreak setting, widespread sampling, including sampling of sites that served as likely exposures for cases, is an important step in identifying possible sources of transmission. Filter concentration of water samples from point-of-use outlets increased the yield of positive cultures and provided additional information about the distribution of *Legionella* spp.

This study was not designed to analyze effects of conversion from chlorine to monochloramine on outcomes other than *Legionella* colonization in building water systems. Few data exist on the health effects of ingestion of monochloramine despite a long history of its use in water disinfection (*31*). Since monochloramine eliminates *Legionella* spp., other organisms may colonize water distribution systems (*32*). Our findings may be specific to characteristics of the water or distribution system in San Francisco, although they are consistent with results of a similar study in Pinellas County, Florida (*33*). Because monochloramine was added continuously to the municipal water supply after conversion and concentrations were maintained within specified ranges, effects on *Legionella* spp. at different monochloramine concentrations may vary.

Monochloramine disinfection of municipal water supplies is the only community-based intervention associated with reduced risk of Legionnaire's disease (*14*,*15*). Control of Legionnaires' disease is unlikely to be a major factor in a water utility's decision to convert to monochloramine for residual disinfection. However, if water suppliers increasingly convert to monochloramine to reduce concentrations of disinfection byproducts, control of the growth of *Legionella* spp. in potable water systems may be an additional health benefit.

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Atypical Enteropathogenic *Escherichia coli* Infection and Prolonged Diarrhea in Children

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Some clinical isolates of enteropathogenic *Escherichia coli* (EPEC) lack bundle-forming pili and are termed atypical EPEC. The aim of this study was to determine if atypical EPEC are pathogens by comparing the clinical features of patients infected with atypical EPEC with those of children infected with other causative agents of diarrhea. Fecal samples obtained from children attending the Royal Children's Hospital in Melbourne for investigation of diarrhea were examined for adenovirus, rotavirus, *Campylobacter* spp., *Salmonella* spp., protozoa, and pathogenic *E. coli*. Clinical data were obtained by using a standardized pro forma and analyzed separately. Patients infected with atypical EPEC experienced mild, nondehydrating, and noninflammatory diarrhea that was not particularly associated with fever, vomiting, or abdominal pain. However, the duration of diarrhea in patients infected with atypical EPEC was significantly longer than that caused by the other species or where no pathogens were identified. Infection with atypical EPEC is associated with prolonged diarrhea.

The varieties of *Escherichia coli* that cause diarrhea are classified into pathogenic groups (pathotypes) according to their virulence determinants (*1*,*2*). The specific nature of these virulence determinants imbues each pathotype with the capacity to cause clinical syndromes with distinctive epidemiologic and pathologic characteristics (*2*). For example, enterotoxigenic *E. coli* causes watery diarrhea in children in developing countries and in travelers to those countries, whereas enterohemorrhagic *E. coli* (EHEC) may cause hemorrhagic colitis and the hemolytic uremic syndrome because of the production of Shiga toxins. Enteropathogenic *E. coli* (EPEC) shares several key virulence determinants with the most common varieties of EHEC but does not produce Shiga toxins nor cause hemorrhagic colitis or hemolytic uremic syndrome. Instead, it causes nonspecific gastroenteritis, especially in children in developing countries (*3*,*4*). EPEC also differs from other pathotypes of *E. coli* in that it typically carries an EPEC adherence factor plasmid (pEAF). This plasmid encodes 1) bundle-forming pili (Bfp), which promote bacterial adherence to epithelial cells and are an essential virulence determinant (*5*), and 2) a transcriptional activator, Per, that upregulates genes within a chromosomal pathogenicity island, termed the locus for enterocyte effacement (*6*,*7*). This pathogenicity island encodes a number of essential virulence proteins, including the surface protein intimin (the product of the *eae* gene), which is required to produce the attaching-effacing lesions that are a key feature of EPEC-induced pathology. A subset of EPEC, known as atypical EPEC, do not carry pEAF and hence do not produce Bfp or Per (*4*). Accordingly, their role in disease is controversial. Recently, we and others investigated the causes of community-acquired gastroenteritis in Melbourne (*8*,*9*). Among the infectious agents that were sought in these studies was atypical EPEC, which emerged as the single most frequent pathogen in the study population (*9*).

To determine if atypical EPEC are also responsible for diarrhea in hospitalized children, we undertook a comprehensive microbiologic study of patients with diarrhea at the Royal Children's Hospital in Melbourne.

Patients and Methods

Patients

Patients were children with diarrhea attending the Royal Children's Hospital, Melbourne, between March 1 and August 31, 2003. They were considered for inclusion

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in the study when an obviously loose stool sample from a child <14 years of age was received at the Diagnostic Microbiology Laboratory for investigation. After their caregivers, attending physicians, and medical records had been consulted, patients were considered eligible for inclusion in the study if, during the current illness, they had passed ≥3 loose stools within a day or had experienced loose stools plus vomiting, abdominal pain, or rectal bleeding. Patients with chronic gastrointestinal disorders, such as inflammatory bowel or celiac disease, were excluded, as were those with cystic fibrosis, leukemia, and other immunosuppressive disorders. Repeat samples and samples from children who had received antimicrobial agents within the preceding week were also excluded.

Clinical data were obtained in accordance with a standardized pro forma and were analyzed before the results of the laboratory findings were known. Data collected included age; gender; date of onset of illness; symptoms and clinical signs, including characteristics of stools, abdominal pain, vomiting (number per day and duration), fever, abdominal tenderness, largest number of bowel movements in a 24-hour period preceding the sample collection, and extent of dehydration. Duration of diarrhea was estimated from the passage of the first loose stool to the patient's last appearance in the ward or 1 day after discharge. Patients with temperatures of ≥38°C, taken by tympanic thermometer, were considered febrile. Severity of illness was estimated by using the 20-point scale developed by Ruuska and Vesikari (*10*).

Laboratory Methods

All stool specimens were the first specimen obtained from a patient on a hospital visit, and specimens were investigated within 4 hours of collection. Specimens were examined macroscopically for color and consistency and by light microscopy for leukocytes, erythrocytes, and parasitic forms (amebas, cysts, and ova) by using a saline-and-iodine wet preparation and a modified Ziehl-Neelsen stain for oocysts of *Cryptosporidium* spp. (*11*). Samples were tested by enzyme immunoassay for enteric adenoviruses and rotaviruses and cultured for *E. coli*, *Salmonella*, *Shigella*, *Yersinia*, and *Campylobacter* spp. (*12*).

To reduce the cost of the investigation, diarrheogenic strains of *E. coli* were sought only during the first 11 weeks of the study, from March 1 to May 15. Bacteria were isolated from fecal samples by direct plating on MacConkey agar (Oxoid Ltd., Basingstoke, UK). After overnight incubation at 37°C, a sterile cotton swab was used to transfer the entire growth from each plate into Luria broth containing 30% (vol/vol) glycerol, which was then frozen at –70°C until required. *E. coli* pathotypes were identified by polymerase chain reaction (PCR) and confirmed by Southern hybridization (*9*). Briefly, template DNA for use in PCR was prepared from bacteria isolated from MacConkey agar plates and grown in 2.5 mL MacConkey broth with shaking at 37°C overnight. Bacteria from this culture were washed in phosphate-buffered saline, resuspended in sterile distilled water, and heated for 10 min at 100°C. Samples were then placed on ice for 5 min and recentrifuged for 5 min at 16,000 × *g*. Aliquots of the supernatant were pipetted into sterile tubes, stored at –20°C for <1 week, and then diluted 1 in 10 in distilled water before being added to the PCR mix. PCR amplifications were performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with the PCR primers and conditions described previously (*9*). Genes identified by these primers and their association with each pathotype of diarrheogenic *E. coli* are listed in Table 1. PCR for the *lacZ* gene, which is present in almost all wildtype strains of *E. coli*, was included as a control to ensure that negative PCR results were not due to the absence of viable bacteria in the sample or the presence of inhibitors in the reaction mixture. Samples that were PCR negative for *lacZ* were excluded from further analysis. At the conclusion of the PCR, 10 μL of the reaction mixture underwent electrophoresis on 2.5% 96-well format agarose gels (Electro-fast; Abgene, Epsom, UK). Gels were stained with ethidium bromide, visualized on a UV transilluminator, and photographed. A portion of the PCR product was retained for Southern blotting, which was performed by using capillary transfer of separated DNA fragments onto positively charged nylon membranes (Roche Diagnostics Ltd., Lewes, UK). Digoxigenin-labeled DNA probes were prepared from the control strains of diarrheogenic *E. coli* listed in Table 1 and used as described (*9*). PCR- and probe-positive bacteria were assigned to a pathotype according to the criteria in Table 1. Equivocal or ambiguous assays were repeated, and if still unclear, were excluded from further analysis. Atypical EPEC strains were isolated in pure culture from the original sample and then serotyped by using hyperimmune rabbit antisera to O-antigens O1 through O181 (*18*). These strains were also subjected to PCR to determine the intimin subtype and to investigate the presence of selected virulence-associated genes by using the PCR primers and conditions described previously (*9*).

Statistical Analysis

Statistical analysis of quantitative and qualitative data was performed by using InStat, Version 3.05 (GraphPad Software Inc., San Diego, CA, USA). A 2-tailed p value of <0.05 indicated statistical significance. For the analysis of clinical features associated with infection, patients whose stools yielded >1 pathogen were excluded.

Table 1. Classification of pathogenic *Escherichia coli* according to amplicon(s) generated by polymerase chain reaction (PCR) for virulence-associated determinants

Interpretation†	Gene or virulence-associated determinant*										Control strain (reference)
	pCVD432	*aggA*	*bfpA*	*eae*	*ipaC*	*stlA*	*ltA*	*ehxA*	*stx1*	*stx2*	
EAEC‡	+	+	–	–	–	–	–	–	–	–	O42 (*13*)
Typical EPEC	–	–	+	+	–	–	–	–	–	–	E2348/69 (*14*)
Atypical EPEC	–	–	–	+	–	–	–	–	–	–	E128012 (*14*)
EIEC	–	–	–	–	+	–	–	–	–	–	223/83 (*15*)
ETEC‡	–	–	–	–	–	+	+	–	–	–	H10407 (*16*)
EHEC§	–	–	–	+	–	–	–	+	+	+	EDL933 (*17*)
STEC not EHEC¶	–	–	–	–	–	–	–	–	+	+	

*Factors specified by virulence genes: *aggA*, aggregative fimbria, AAF/I; *bfpA*, bundle-forming pilus; *eae*, intimin; *ipaC*, invasion plasmid antigen; *stlA*, heat-stable enterotoxin; *ltA*, heat-labile enterotoxin; *ehxA*, EHEC hemolysin; *stx*, Shiga toxin.
†EAEC, enteroaggregative *E. coli*; EPEC, enteropathogenic *E. coli*; EIEC, enteroinvasive *E. coli*; ETEC, enterotoxigenic *E. coli*; STEC, Shiga toxin–producing *E. coli*; EHEC, enterohemorrhagic *E. coli*.
‡Either positive by polymerase chain reaction.
§Either *eae* or *ehxA*, and either *stx1* or *stx2* positive.
¶Either s*tx1* or *stx2* positive.

Results

Frequency of Viral, Parasitic, and Bacterial Pathogens

After exclusion of repeat samples and samples from patients >14 years of age or with cystic fibrosis, chronic inflammatory bowel disease, leukemia, or a history of recent antimicrobial drug usage, 303 of 972 consecutive fecal samples remained for analysis. Of these, 134 were from the first period of the study, March 1–May 15, when diarrheogenic *E. coli* were sought together with other enteropathogens, and 169 were from the period May 16–August 31, when *E. coli* were not sought.

The frequency of bacterial, viral, and parasitic pathogens identified during the 2 phases of the study are shown in Table 2. During the first period, a putative etiologic agent was identified in 88 (66%) of 134 children. Diarrheogenic *E. coli* were found in 42 (31%) of these children, followed by enteric adenovirus (10%), *Salmonella* sp. (10%), *Campylobacter* spp. (9%), *Giardia* sp. (6%), rotavirus (4%), and *Cryptosporidium* sp. (2%). Of the 42 *E. coli* isolates, 30 (71%) were EPEC; 6 (14%) were Shiga toxin–producing *E. coli* (STEC), of which 3 were EHEC; 4 (10%) were enteroaggregative *E. coli* (EAEC), 1 (2%) was enterotoxigenic *E. coli*; and 1 (2%) was enteroinvasive *E. coli*. Nine children (7%) were infected with >1 pathogen, including 2 concurrently infected with EPEC and adenovirus or EPEC and rotavirus, and 1 each with EPEC and *Giardia* sp.; STEC and *Campylobacter* sp.; STEC and *Giardia* sp.; EAEC and *Campylobacter* sp., and EAEC and rotavirus.

All EPEC isolates were atypical EPEC (i.e., PCR negative for *bfpA*). Determination of the O:H serotype and intimin subtype of 29 of the 30 EPEC strains (1 was not viable) indicated that they were highly heterogeneous (Table 3). Although 3 strains (R41, R151, and R446) were O-nontypable:H34, intimin-α2; and 2 (R89 and R104) were O153:H7, intimin β, these isolates were neither temporally nor geographically related to each other and showed some differences in their carriage of accessory virulence-related factors (data not shown). Two other isolates (R250 and R436) were O33:H6 but had different intimin types. Ten isolates were O-serogroups that were classified as nontypable because they did not react with any of the available O-typing sera (O1–O181), and 2 isolates could not be serotyped because they were rough. Only 1 isolate (R404) belonged to an *E. coli* serotype, O128:H2, that is commonly associated with EPEC (*4*).

During the second period of the study, when *E. coli* was not sought, putative pathogens were identified in 99 (58.6%) of 169 children; rotavirus was the most frequent (33.7%), followed by *Campylobacter* (11.8%), adenovirus (7.7%), *Salmonella* (5.3%), *Giardia* (1.8%), and *Cryptosporidium* (0.6%) spp. Four patients were infected with >1 pathogen: 3 concurrently infected with rotavirus

Table 2. Frequency of diarrhea-associated pathogens detected during the course of this study

Pathogen	1st period (Mar 1–May 15), n = 134 (%)	2nd period (May 16–Aug 31), n = 169 (%)
Diarrheogenic *Escherichia coli* (all)	42 (31.3)	Not done
Campylobacter spp.	12 (9.0)	20 (11.8)
Salmonella spp.	14 (10.4)	9 (5.3)
Adenovirus	14 (10.4)	13 (7.7)
Rotavirus	5 (3.7)	57 (33.7)
Giardia sp.	8 (6.0)	3 (1.8)
Cryptosporidium sp.	2 (1.5)	1 (0.6)
>1 pathogen	9 (6.7)	4 (2.4)
No pathogens identified	46 (34.3)	70 (41.4)

Table 3. Characteristics of atypical EPEC identified during this study*

Strain no.	Serotype	Intimin type
R41	ONT:H34	α2
R69	NT	θ
R89	O153:H7	β
R104	O153:H7	β
R151	ONT:H34	α2
R154	O71:H6	α1
R175	O128:H21	κ
R176	ONT:H8	ι
R182	OR:H40	θ
R215	O117:H2	ε/η
R218	ONT:R	α2
R219	O51:H49	α1
R227	O170/172:H49	θ
R228	O88:H-	κ
R249	O145:H34	ι
R250	O33:H6	ζ
R261	O2:H45	κ
R278	ONT:H19	υ
R281	O49:H10	κ
R380	ONT:H31	ζ
R392	ONT:H6	α2
R394	O5/71:H31	θ
R396	O98:H8	ι
R404	O128:H2	β
R420	ONT:H21	θ
R436	O33:H6	β
R446	ONT:H34	α2
R447	O28:H45	ζ
R457	ONT:H–	β

*EPEC, enteropathogenic *Escherichia coli*; H-, nonmotile; NT, nontypable (O1-O181; H1-H56); R, rough

and adenovirus and 1 with adenovirus and *Salmonella* sp. *Shigella* and *Yersinia* spp. were not identified during either period of the study. The frequency of rotavirus infection during the second phase of the study was significantly greater than during the first phase (odds ratio [OR] 13.13; 95% confidence interval [CI] 5.08–33.91, $p<10^{-6}$, 2-tailed Fisher exact test), confirming the well-known association of rotavirus with winter diarrhea (*12*). The frequency of patients in whom no pathogens were identified was not significantly different between the 2 study periods (OR 0.74, 95% CI 0.46–1.18, p>0.2, 2-tailed Fisher exact test), despite the omission of tests for diarrheogenic *E. coli* during the second period. This finding suggests that *E. coli* did not account for a large number of cases during the second period of the study and accords with our previous observations that diarrhea due to *E. coli* is relatively less frequent during winter (*9*,*19*).

Comparison of Clinical and Laboratory Findings

The clinical and laboratory features of patients infected with different pathogens were compared (Table 4). Patients infected with >1 pathogen were excluded from this analysis, as were those infected with *Giardia* or *Cryptosporidium* spp. or diarrheogenic *E. coli* other than EPEC because their numbers were too small for the results to be meaningful. For this analysis, only those patients in whom no pathogens were identified from the first study period were considered because of the possibility that some of those studied during the second period were infected with diarrheogenic *E. coli*.

Patients infected with EPEC were of a similar age (median 16.9 months) to those from whom no pathogens were isolated (median age 11.6 months). Of the various groups of patients defined according to the cause of diarrhea, only those infected with *Campylobacter* spp. (median age 34.2 months) differed significantly in age from those with EPEC (p = 0.0002, Mann-Whitney U test). Eighteen (72%) of 25 children monoinfected with EPEC were boys compared with 20 (44%) of 46 children in whom no pathogens were identified (OR = 3.34, 95% CI 1.17–9.55, p = 0.03, 2-tailed Fisher exact test), and with 49 (47%) of 104 children enrolled in phase 1 of the study who were not infected with EPEC (OR = 2.89, 95% CI 1.11–7.5, p = 0.03).

Patients infected with rotavirus or adenovirus were significantly more likely to have a history of vomiting than those with no pathogen identified or those infected with EPEC, *Salmonella*, or *Campylobacter* spp. The frequency of vomiting in patients infected with EPEC and in those with no pathogen identified was similar. Abdominal pain was reported significantly more frequently in patients infected with *Campylobacter* spp. than in those infected with EPEC (p = 0.007, 2-tailed Fisher exact test), adenovirus (p = 0.0005), rotavirus (p = 0.0005), or those in whom no pathogen was detected (p = 0.003).

The duration of diarrhea was significantly longer in patients with EPEC than in those infected with adenovirus (p = 0.002, 2-tailed Student *t* test), rotavirus (p = 0.0003), *Campylobacter* (p = 0.0003), *Salmonella* (p = 0.02), and those without an identifiable pathogen (p = 0.02). Moreover, persistent diarrhea (defined as diarrhea lasting >14 days) was significantly more common in patients infected with atypical EPEC than in those infected with adenovirus, rotavirus, *Campylobacter*, *Salmonella*, and those with no pathogen identified (Table 4). Persistent diarrhea also developed in 4 (36%) of 11 patients infected with *Giardia* sp. The frequency of persistent diarrhea associated with *Giardia* was significantly greater than that attributable to adenovirus (p = 0.03), rotavirus (p = 0.01), and *Camplyobacter*, but not *Salmonella* or atypical EPEC (p>0.1, 2-tailed Fisher exact test).

Fever was significantly more common in patients infected with rotavirus or *Salmonella* than in those infected with EPEC, adenovirus, or *Campylobacter*, and those with no

Table 4. Association between presumed etiologic agent and clinical and laboratory findings in children with diarrhea*

Clinical or laboratory parameter	Presumed etiologic agent					
	EPEC, n = 25	Adenovirus, n = 22	Rotavirus, n = 55	*Campylobacter*, n = 30	*Salmonella*, n = 22	NPI, n = 46
Age in months, median (interquartile range)	16.9 (11.4–28.2)	9.5 (4.4–19.5)†	15.2 (9.4–28.0)	34.2 (26.9–98.1)‡§	29.8 (9.3–90.9)¶	11.6 (3.1–52.7)
Sex, no. (% male)	18 (72)¶	14 (64)	30 (55)	17 (57)	10 (46)	20 (44)†
Vomiting, no. (%)	11 (44)	17 (77)†¶	49 (89)‡§	13 (43)	7 (32)	23 (50)
Abdominal pain, no. (%)	5 (20)	2 (9) ‡	10 (18)	17 (57)#**	10 (45)	10 (22)**
Days with diarrhea, mean (95% CI)	12.1 (7.5–16.7)¶	4.9 (3.6–6.2)‡	6.0 (4.9–7.1)‡	4.9 (3.9–5.9)‡	6.5 (4.2–8.6)†	6.3 (3.5–9.0)†
Diarrhea >14 days, no. (%)	12 (48)¶	1 (5)‡	3 (5)‡¶	1 (3)‡	2 (9)#	9 (20)†
Temperature ≥38°C, no. (%)	6 (24)	2 (9)	30 (55)†¶	7 (23)	17 (77)‡§	13 (28)
Dehydration ≥5%, no. (%)	1 (4)	2 (9)	35 (64)‡§	2 (7)	6 (27)†	6 (13)
Severity score, mean (95% CI)	9.0 (7.7–10.3)	8.6 (7.5–9.7)	13.5 (12.6–14.4)‡§	8.4 (7.5–9.3)	11.3 (9.8–12.8)#**	8.3 (7.4–9.2)
Fecal blood						
Macroscopic, no. (%)	0	0	0	4 (13)	3 (14)	1 (2)
Microscopic only, no. (%)	4 (16)	2 (9)	1 (2)	17 (57)¶§	9 (41)	9 (20)
Fecal leukocytes, no. (%)	5 (20)	4 (18)	10 (18)	23 (77)‡§	14 (64)¶#	10 (22)

*EPEC, Enteropathogenic *Escherichia coli*; NPI, no pathogen identified; CI, confidence interval.
†Significantly different from EPEC (Mann-Whitney U test, Fisher exact test, or Student *t* test, 2-tailed), p<0.05, >0.01.
‡Significantly different from EPEC (Mann-Whitney U test, Fisher exact test, or Student *t* test, 2-tailed), p<0.001.
§Significantly different from NPI (Mann-Whitney U test, Fisher exact test, or Student *t* test, 2-tailed), p<0.001.
¶Significantly different from NPI (Mann-Whitney U test, Fisher exact test, or Student *t* test, 2-tailed), p<0.05, >0.01.
#Significantly different from EPEC (Mann-Whitney U test, Fisher exact test, or Student *t* test, 2-tailed), p<0.01, >0.001.
**Significantly different from NPI (Mann-Whitney U test, Fisher exact test, or Student *t* test, 2 tailed, p<0.01, >0.001.

pathogen identified. Dehydration of ≥5% occurred significantly more often in patients infected with rotavirus than in those infected with EPEC, adenovirus, *Campylobacter*, *Salmonella*, and those without an identifiable pathogen.

The disease severity score, determined according to the criteria of Ruuska and Vesikari (*10*), was highest in patients infected with rotavirus followed by *Salmonella* sp. The mean severity scores in patients infected with EPEC, adenovirus, and *Campylobacter* sp. and those in whom no pathogen was found were similar. Stools from patients infected with *Campylobacter* or *Salmonella* spp. were more likely to contain frank blood, although the differences between patients infected with different etiologic agents were not significant (p>0.05, 2-tailed Fisher exact test). Erythrocytes were more commonly detected on microscopic examination in patients infected with *Campylobacter* or *Salmonella* spp. than in those infected with EPEC, adenovirus, rotavirus, or no identifiable pathogen, but the differences were significant with respect to *Campylobacter* spp. only. Fecal leukocytes were present significantly more often in patients infected with *Campylobacter* or *Salmonella* spp. than in those infected with EPEC, adenovirus, rotavirus, or those with no identifiable pathogen.

Discussion

The principal aims of this study were to compare the frequency of atypical EPEC with frequencies of established enteropathogens in children attending hospital with diarrhea and to determine the clinical and laboratory features associated with each pathogen. During the first part of the study (when pathogenic *E. coli* was sought), atypical EPEC was the predominant pathogen identified; it was found in 31% of 134 children compared with 10% for adenovirus, 10% for *Salmonella* sp., 9% for *Campylobacter* sp., and 4% for rotavirus. In the second period of the study, when EPEC was not sought, rotavirus predominated. In agreement with our findings from a community-based study in Melbourne and reports from investigators in Brazil, Norway, and elsewhere (*9*,*20*,*21*), the atypical EPEC strains obtained in this study were highly heterogeneous in terms of O:H serotype and intimin type, which indicates that the high frequency of atypical EPEC was not due to an outbreak caused by a limited number of strains. Also in agreement with our previous study, we observed that serotypes of EPEC associated with diarrhea differed from those listed by the World Health Organization as being characteristic of EPEC (*9*).

To determine whether atypical EPEC is a cause of diarrhea, we compared the clinical and laboratory findings of children who were infected with these bacteria with those who were infected with well-established pathogens and those in whom no pathogens were identified. The hypothesis underlying this investigation was that if atypical EPEC is not a pathogen, the symptoms, signs, and laboratory findings in patients infected with these bacteria would be

the same as those in patients in whom no pathogens were found. The results showed that diarrhea attributable to atypical EPEC was significantly more common in boys and that it persisted significantly longer than diarrhea in patients without an identifiable pathogen or in those infected with adenovirus, rotavirus, *Campylobacter* spp., or *Salmonella* sp. This study also showed that infection with atypical EPEC generally occurred in children <2 years of age, with 72% <24 months of age compared with 55% for the first study group as a whole (OR 3.0, 95% CI 1.17–7.85, p = 0.03, 2-tailed Fisher exact test). Infection with EPEC was associated with vomiting in ≈50% of patients, was generally not accompanied by fever, abdominal pain, or dehydration, and was not characterized by fecal blood or leukocytes, indicating that it was not inflammatory in nature. The reason for the higher frequency of atypical EPEC in boys is not known but confirms our unpublished observations from a community-based study, in which 55 isolates were obtained from 338 male patients, and 34 were obtained from 358 female patients (OR 1.85, 95% CI 1.17–2.92, p = 0.009, 2-tailed Fisher exact test).

The validity of the clinical and laboratory assessments performed in this study was indicated by the confirmation of the well-known associations of specific pathogens with particular parameters: younger age of children infected with EPEC and viruses than those infected with *Campylobacter* or *Salmonella* spp.; rotavirus and *Salmonella* infections with fever; rotavirus with dehydration and an overall greater severity of disease; *Campylobacter* sp. with fecal blood; and *Campylobacter* and *Salmonella* spp. with fecal leukocytes (*22*).

Persistent diarrhea (lasting more then 14 days) eventually develops in a substantial proportion of children with acute infectious gastroenteritis and may become chronic, leading to malabsorption, failure to thrive, and malnutrition (*23*). A wide range of infectious agents has been implicated in the cause of persistent diarrhea, including viruses, in particular rotavirus; protozoa, such as *Giardia* and *Cryptosporidium* spp., and bacteria, including *E. coli* (*23*,*24*). In most cases, however, laboratory investigation of children with persistent diarrhea fails to yield an identifiable cause. The findings of this study suggest that a number of these cases may be caused by infection with atypical EPEC, which is seldom sought in these patients.

Despite the persuasive evidence of a volunteer study and reports of outbreaks of diarrhea attributed to atypical EPEC (*25*,*26*), the role of atypical EPEC in disease is controversial. In several reports, however, from countries as diverse as Iran, Norway, Peru, Poland, South Africa, the United States, and the United Kingdom (*20*,*27*–*32*), as well as Australia (*9*,*33*), atypical EPEC strains have been identified in children with acute diarrheal disease. Atypical EPEC has also previously been reported in association with prolonged diarrhea (*34*). For example, Hill et al. (*35*) reported that of 26 children infected with EPEC requiring hospital admission for acute diarrhea, life-threatening, chronic symptoms developed in 6 (23%). Five of these 6 children were infected with EPEC of serogroups O114 or O128, which frequently do not produce Bfp (*14*,*36*). Notwithstanding these previous reports, however, the current study is the first to characterize the illness caused by atypical EPEC in a systematic way and to compare the features of atypical EPEC infection with those of other etiologic agents of diarrhea.

The reasons why persistent diarrhea develops more frequently in children infected with atypical EPEC than in those infected with adenovirus, rotavirus, *Campylobacter* or *Salmonella* spp. are not known. In a recent study, Mellmann et al. (*37*) found that only 12 (<9%) of 137 patients who were infected with *eae*-positive EHEC strains when investigated within 14 days of the onset of diarrhea remained culture-positive when retested 3–16 days later, compared with all 5 patients who were initially infected with *eae*-positive, *stx*-negative *E. coli* (i.e., atypical EPEC) (OR 110.4, 95% CI 5.8–2117.6, p<0.0001, 2-tailed Fisher exact test). These findings indicate that atypical EPEC may have an innate propensity to persist longer in the intestine than varieties of *E. coli* which cause diarrhea that is more transient in nature. EPEC adheres tightly to epithelial cells and disrupts normal cellular processes (*38*), and evidence suggests that atypical EPEC may retard apoptosis of intestinal epithelial cells (*39*), possibly because of the lack of Bfp (*40*). These features may favor prolonged intestinal colonization by atypical EPEC compared with other intestinal pathogens. Although disease due to atypical EPEC was mild and generally not associated with dehydration, its importance lies in its association with prolonged diarrhea, a major contributor to childhood illness, especially in developing countries. Our findings also suggest that interventions targeted towards atypical EPEC may be beneficial in managing children with prolonged diarrhea.

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Epidemic Spread of Lyme Borreliosis, Northeastern United States

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We examined the degree of host specialization of different strains of *Borrelia burgdorferi*, the tickborne pathogen that causes Lyme borreliosis in the northeastern United States. We first assessed the genetic population structures of *B. burgdorferi* in ticks obtained from different mammalian host species and in questing ticks sampled in a woodland ecosystem in Connecticut. By comparing the patterns found in our study with data from another cross-sectional study, we demonstrate that *B. burgdorferi* is a generalist microparasite and conclude that efficient cross-species transmission of *B. burgdorferi* is a key feature that has allowed the rapid spread of Lyme borreliosis across the northeastern United States.

The evolution of specialization remains a major problem in ecology and evolutionary biology; why some species are generalists and others are specialists is not resolved (*1*,*2*). Like all organisms, parasites have evolved to different levels of ecologic specialization (*3*–*5*). The level of host specialization of parasites is a key issue in infectious disease research because patterns of cross-species transmission affect parasite dispersal and can facilitate epidemics. West Nile virus is a recent example illustrating that the utilization of many highly mobile host species can enable a pathogen to disperse across an entire continent within a few years (*6*). Multihost parasites are usually considered to be generalists; however, this is not universally true, and several examples exist in which generalist parasites are structured into subpopulations that are host specialized (*7*). Theory predicts that natural selection favors host specialization if hosts are abundant and predictable, whereas generalist strategies evolve if hosts are erratic (*8*).

Borrelia burgdorferi, the spirochetal agent of Lyme borreliosis (LB) in the United States, is a tickborne zoonotic pathogen that infects an expansive range of vertebrate species, involving diverse mammalian and avian hosts (*9*–*14*). For this reason, it has been suggested *B. burgdorferi* is likely less specialized than the other genospecies that cause LB in Eurasia (*15*–*18*). Several loci of *B. burgdorferi* are polymorphic (*19*), and balancing selection seems to maintain the bacterium's diversity (*20*). Given the pronounced strain structure of this bacterial species, natural selection possibly has driven *B. burgdorferi* towards host specialization, and different spirochete strains exploit different sets of vertebrate hosts (*4*,*13*).

The issue of vertebrate host specialization of *B. burgdorferi* is of substantial public health importance. Since the reemergence of LB 3 decades ago, the disease has been spreading across the entire northeastern United States and beyond (*21*,*22*). A condition necessary for this dispersal has been the geographic expansion of its principal and generalist tick vector, *Ixodes scapularis*. This expansion is believed to be driven by large-scale reforestation and an explosive growth of deer populations (*21*). Deer, however, do not contribute directly to the dispersal of *B. burgdorferi* (*23*). Only hosts that can infect ticks affect spirochete migration. If *B. burgdorferi* were host specialized, the strains of this microparasite would migrate differentially, resulting in geographic structuring of this pathogen. Unrestricted cross-species transmission, in contrast, would generate a spatially uniform population structure of *B. burgdorferi* and substantially facilitate its dispersal. Information on the level of host specialization of this multihost pathogen is required to understand the patterns and mechanisms of the current spread of LB.

We examined the level of host specialization of *B. burgdorferi* in the northeastern United States by using a

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comparative approach. We first assessed the genetic population structures of *B. burgdorferi* in ticks obtained from different mammalian host species and in questing ticks sampled in a woodland ecosystem at Lake Gaillard, Branford, Connecticut. By comparing the patterns in our study with data from another cross-sectional study carried out in a similiar ecosystem in Millbrook, New York (*13*), we aimed to capture patterns of cross-species transmission and to identify the niche breadth of the various genotypes of *B. burgdorferi*.

Materials and Methods

Mammal Sampling

The fieldwork was carried out at Lake Gaillard (41°34′N, 72°77′W), Connecticut, as described previously (*24*). Mammals were captured alive at 2-week intervals from early June until late August in 2002 and until mid-September in 2003. All trapping and handling procedures were approved by the Yale University Institutional Animal Care and Utilization Committee (Study Protocol 07596). Small mammals were trapped for 23 days/nights (432 trap nights) using Sherman (Tallahassee, FL, USA) traps. In addition, Pitfall traps were set up for 14 days/nights (98 trap nights) in 2003. Medium-sized mammals were captured for 27 days/nights (820 trap nights) and 25 days/nights (724 trap nights) using Tomahawk (Tomahawk, WI, USA) traps no. 205 and no. 207, respectively. All captured mammals were housed over pans of water for 72 hours to recover engorged ticks. Ticks were allowed to molt to the next developmental stage, determined to species, and stored in 70% ethanol. Mammals were marked, sexed, and measured. Before handling, mammals were anesthetized with ketamine hydrochloride or a combination of ketamine hydrochloride and xylazine. After captivity, mammals were released at their original location.

Host-seeking Ticks

Questing *I. scapularis* nymphs were collected over the same period and in the same area where the mammals were captured by dragging the vegetation with 1–m^2 drag cloths. Collected ticks were preserved in 70% ethanol.

DNA Extraction and PCR

DNA was extracted from ticks according to a DNeasy Tissue Kit protocol (Qiagen, Valencia, CA, USA) as described previously (*25*). Ticks were screened for *B. burgdorferi* DNA by real-time Taqman polymerase chain reaction (PCR) targeting the 16S rDNA of *B. burgdorferi* (*24*). Positive samples were then subjected to a nested PCR amplifying a fragment of the *rrs* (16S)–*rrl* (23S) intergenic spacer of *B. burgdorferi* and sequenced (*19*).

Data Analysis

Infectivity of hosts to ticks was determined by identifying *B. burgdorferi* in molted nymphs derived from mammals. Since transovarial transmission of *B. burgdorferi* to larval *I. scapularis* has not been demonstrated, infections found in molted nymphs were assumed to be acquired from a host through feeding. A mammal, therefore, was considered infectious to ticks if $\geq$1 nymphs that had fed, as larva, on that mammal tested positive.

To evaluate the exposure of animals to infected nymphs for each host species, the attachment rate of nymphs per animal per day (RD_S) was computed for each capture time point as $RD_S = A/F$; A is the mean number of feeding ticks per host, and F is the average feeding time of *I. scapularis* nymphs which was conservatively assumed to be 5 days (*26*). The minimum attachment rate of nymphs per animal per season (RS_S) was computed as $RS_S = \Sigma\,(RD_S \times C)$; C is the number of days between capture points. The number of nymphs infected with a genotype encountered by a host per season (RSI_S) was calculated as $RSI_S = IP/N \times RS_S$; IP is the infection prevalence of a genotype in field-collected questing nymphs, and N is the number of nymphs tested. Since no data for May were obtained empirically, we extrapolated the data on nymphal infestation obtained at the end of the nymphal peak activity (i.e., end of June) and applied it to May. This provided a conservative estimate of the total number of infected nymphs a host encountered over the nymphal activity season.

Statistical Analysis

Differences in mean numbers of ticks per host were examined by using the nonparametric Kruskal-Wallis test. Logistic regression was used to estimate the infection prevalences in ticks or hosts and to compare them among host species. Presence of *B. burgdorferi* infection in a tick or host was the response variable in the model, and a dummy variable for host species was used as the predictor. The advantage of using logistic regression models for proportional data is that different coding systems can be applied to compare infection prevalence among various groupings of host species (e.g., mice versus other hosts). Additionally, logistic models can control for the fact that several ticks were collected from the same mammal and were not independent samples. In this analysis, a cross-sectional procedure (Stata xtlogit) was applied to control for the correlation among ticks collected from the same mammal (*27*). To test for a sample size effect on the number of genotypes found in a host species, a Spearman rank correlation was performed between the number of genotypes and the number of mammals sampled for each host species. The differences in genotype frequency distributions were estimated through exact nonparametric inference by the Fisher-Freeman-Halton test (Monte Carlo

testing). Pearson's χ^2 test was used to compare the proportions of ticks infected with different genotypes within and among host species. Data were analyzed with Stata, version 8, (Stata Corporation, College Station, TX, USA) and StataXact, version 6, (Cytel, San Diego, CA, USA).

Results

Mammal Trapping

Sampling over 2 years yielded 403 captures that included 222 individual mammals, representing 9 mammalian species of 6 families (*Muridae*, *Soricidae*, *Sciuridae*, *Mustelidae*, *Procyonidae*, and *Didelphiidae*) belonging to 4 orders (Rodentia, Insectivora, Carnivora, and Marsupialia). Six species (white-footed mouse, pine vole, eastern chipmunk, gray squirrel, Virginia opossum, and raccoon) accounted for 98% of all mammals caught (Table 1).

Tick Infestation

Altogether, 9,032 immature ticks were collected from 399 captured hosts. The most abundant tick species, *I. scapularis*, represented 99% (7,611 larvae and 1,373 nymphs) of all ticks examined. The additional 3 species, *I. texanus*, *Dermacentor variabilis*, and *Amblyomma maculatum*, comprised the remaining 1% and were omitted from further analysis. The mean numbers of *I. scapularis* ticks per host varied significantly among mammalian species for both larvae and nymphs (Table 1).

B. burgdorferi Prevalence in Host-derived Ticks

Of the nymphs sampled from 62 mammals as engorged larvae, 1,117 specimens were screened for presence of *B. burgdorferi*. The number of tested nymphs per host varied from 1 to 51, depending mainly on the number of engorged larvae recovered. *B. burgdorferi*–positive ticks were obtained from all 6 mammalian species.

Infection prevalence of *B. burgdorferi* in animals varied significantly among host species (logistic regression, χ^2 =14.15, p<0.01) (Table 2). Each of the 3 tested chipmunks produced ≥1 infected nymphs and, therefore, this species was excluded from the logistic regression model, since the presence of a zero category (noninfectious chipmunks) produced infinite odds ratios (OR), which precluded the estimation of the model. No significant differences were found between voles, squirrels, raccoons, and opossums. Hence, these species were pooled and compared with mice. The proportion of infectious mice was significantly higher than that of the pooled group of other host species (logistic regression, OR 13.42, 95% confidence interval (CI) 1.63–110.41, p<0.001).

Infection prevalences of *B. burgdorferi* in host-derived ticks also varied significantly among host species (logistic regression, χ^2 = 42.38, p<0.001) (Table 3). A considerably higher infection prevalence in ticks was observed for mice than for voles (logistic regression, OR 16.37, 95% CI 4.73–56.69, p<0.001). On the other hand, no significant differences in tick infection prevalence were found among raccoons, opossums, squirrels, and chipmunks. Therefore, data for these host species were pooled into 1 group. Infection prevalence in ticks from mice was significantly higher than in ticks from the pooled group (logistic regression, OR 47.89, 95% CI 14.97–153.23, p<0.001), as was infection prevalence in ticks from voles compared to the pooled group (logistic regression, OR 2.92, 95% CI 1.16–7.34, p<0.001).

Population Structure of *B. burgdorferi* in Host-derived Ticks

A total of 205 *B. burgdorferi* infections in nymphs obtained from mammals as engorged larvae could be sequenced successfully. The IGS alleles were assigned to previously identified multilocus genotypes (*19*), designated here as genotypes 1 to 9. A total of 8 genotypes was shown (Tables 2–4). The white-footed mouse was the only host species that transmitted all 8 genotypes to ticks. None of the genotypes was transmitted by all host species. However, genotypes 1–5 and 7 were found in ticks collected from as many as 5 host species belonging to 3 different

Table 1. Captured mammals and their infestation with *Ixodes scapularis* *

			Larvae†		Nymphs‡	
Host species	No. hosts	No. captures	N	Mean (SE)	N	Mean (SE)
White-footed mouse (*Peromyscus leucopus*)	132	283	2,548	9.0 (0.7)	414	1.5 (0.2)
Pine vole (*Microtus pinetorum*)	23	23	127	5.5 (2.3)	17	0.7 (0.2)
Eastern chipmunk (*Tamias striatus*)	3	8	106	13.3 (7.3)	145	18.1 (13.0)
Gray squirrel (*Sciurus carolinensis*)	14	22	117	5.3 (1.3)	321	14.6 (4.8)
Raccoon (*Procyon lotor*)	39	49	3,630	77.0 (14.5)	394	8.0 (1.1)
Virginia opossum (*Didelphis virginiana*)	7	14	1,083	77.4 (25.2)	82	5.9 (3.3)
Common shrew (*Sorex cinereus*)	1	1		NA		NA
Short-tailed shrew (*Blarina brevicauda*)	2	2		NA		NA
Stripped skunk (*Mephitis mephitis*)	1	1		NA		NA

*SE, standard error; NA, not analyzed.
†Kruskal-Wallis test, χ^2 = 33.61, degree of freedom (df) = 5; p<0.001.
‡Kruskal-Wallis test, χ^2 = 57.76, df = 5; p<0.001.

Table 2. Proportions of infectious hosts*

Host species	No. infectious/ tested hosts (%)	No. infectious hosts†								
		GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	MI
White-footed mouse	14/15 (93.3)	3	8	10	2	6	1	1	3	11
Pine vole	9/17 (52.9)	4	4	1	1	1		1		2
Eastern chipmunk	3/3 (100)		1			1			2	1
Gray squirrel	4/10 (40.0)				3				3	1
Raccoon	3/10 (30)	1	2			1		1		1
Virginia opossum	4/6 (66.7)	1		3	1	1				2
Total	37/62 (59.8)	9	15	14	7	10	1	3	8	18

*GT, genotype; MI, mixed infections.
†In some cases, the sum of genotype infections was greater than the number of infected ticks because of mixed infections.

orders (Rodentia, Carnivora, Marsupialia). Only mice were found to be infectious for genotype 6. No significant relationship was found between the number of genotypes and the number of sampled individuals of a host species (Spearman rank correlation, $r_s = 0.5$, $p>0.05$). The frequency distribution of transmitted genotypes differed significantly among host species (6×8 Fisher-Freeman-Halton test, Fisher statistic = 41.93; Monte Carlo p 0.05) (Table 2). The average number of genotypes per infectious host was 2.4 (standard error [SE] = 0.3) for mice, 1.7 (SE = 0.3) for opossums, 1.7 (SE = 0.7) for raccoons, 1.5 (SE = 0.5) for squirrels, 1.3 (SE = 0.2) for voles, and 1.3 (SE = 0.3) for chipmunks.

The frequency distributions of genotypes in host-derived ticks are shown in Table 3. In most of the ticks obtained from mice, genotype 2 was identified, followed by genotype 3. Most of the ticks that had fed on voles were found to carry genotype 1. On the other hand, genotypes 8 and 4 were the most frequently detected variants in ticks obtained from chipmunks and squirrels, respectively. Genotype 5 was the most common genotype found in ticks derived from raccoons, and genotype 3 was the most frequent in ticks obtained from opossums.

Transmissibility of each genotype from infectious mammals to ticks can be regarded as a fitness index of strains infecting hosts. The values varied significantly within and among host species as shown in Table 4.

B. burgdorferi in Field-collected Questing Nymphs and Exposure of Animals

A total of 178 field-collected questing nymphs were screened for *B. burgdorferi*. The overall infection prevalence was 39%. In this tick population, the same 8 genotypes as in nymphs derived from the animal pool were found. However, significant differences in the genotype frequency distribution between these 2 tick populations were observed (2×8 Fisher-Freeman-Halton, Fisher statistic = 19.93, Monte Carlo $p<0.01$) (Table 3). Questing nymphs were chosen to estimate the exposure of hosts to *B. burgdorferi* genotypes. The calculated values of exposure show that animals with higher nymphal burdens were frequently exposed to >1 infected nymph. This value was occasionally <1, reflecting the conservative estimation of exposure (Table 5).

Comparative Analysis of *B. burgdorferi* Population Structures

Only 1 other study has analyzed the population structures of *B. burgdorferi* in different vertebrate host species in the northeastern United States (*13*). As in our study, transmissible infections in hosts were determined through host-derived ticks. In contrast, the population structures of *B. burgdorferi* were measured at the outer surface protein *C* (*ospC*) locus. However, because the *ospC* locus and the IGS used in our study are linked (*19*), the population struc-

Table 3. *Borrelia burgdorferi* infections and frequency distributions of genotypes in ticks*

Host species	No. of infected/ tested ticks (%)	No. of ticks infected with different genotypes (%)†								
		GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8	ND
White-footed mouse	90/100 (90.0)	11 (11.0)	31 (31.0)	26 (26.0)	4 (4.0)	10 (10.0)	2 (2.0)	1 (1.0)	4 (4.0)	11 (11.0)
Pine vole	29/113 (25.7)	13 (11.5)	7 (6.2)	1 (0.9)	2 (1.8)	1 (0.9)		1 (0.9)		5 (4.4)
Eastern chipmunk	17/73 (23.3)		1 (1.4)			4 (5.5)			12(16.4)	
Gray squirrel	19/60 (31.7)				16 (26.7)				7 (11.7)	
Raccoon	38/500 (7.6)	5 (1.0)	5 (1.0)			15 (3.0)		8 (1.6)		5 (1.0)
Virginia opossum	27/271 (10.0)	1 (0.4)		13 (4.8)	3 (1.1)	1 (0.4)				5 (1.8)
Subtotal	220/1,117 (19.7)	30 (2.7)	44 (3.9)	40 (3.6)	25 (2.2)	31 (2.8)	2 (0.2)	10 (0.9)	23 (2.1)	25 (2.2)
Field-collected nymphs	69/178 (38.8)	8 (4.5)	33 (18.5)	17 (9.6)	6 (3.4)	5 (2.8)	2 (1.1)	1 (0.6)	3 (1.7)	

*GT, genotype; ND, not determined.
†In some cases, the sum of genotype infections was greater than the number of infected ticks because of mixed infections (data not shown).

Table 4. Transmissibility of *Borrelia burgdorferi* genotypes from infectious hosts to ticks*

Host species	No. infected/ tested ticks (%)								Pearson χ^2 (df)	p value
	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8		
White-footed mouse	11/21 (52)	31/56 (55)	26/65 (40)	4/14 (29)	10/42 (24)	2/7 (3)	1/7 (14)	4/21 (19)	18.33 (7)	<0.01
Pine vole	13/25 (52)	7/52 (14)	NC	NC	1/7 (14)		1/7 (14)		14.56 (3)	<0.01
Eastern chipmunk		1/14 (7)			4/14 (29)			12/59 (20)	2.12 (2)	>0.05
Gray squirrel				16/26 (62)				7/32 (22)	9.43 (1)	<0.01
Raccoon	5/50 (10)	5/99 (5)			15/49 (31)		8 /51 (16)		19.36 (3)	<0.001
Virginia opossum	1/50 (2)		13/126 (10)	3/27 (11)	1/50 (2)				6.51 (3)	>0.05
Pearson χ^2 (df)	45.17	46.94	22.97	15.20	15.16	NC	0.02	0.07		
p value	<0.001	<0.001	<0.001	0.001	<0.01	NC	>0.05	>0.05	6.51 (3)	>0.05

*GT, genotype; df, degrees of freedom;NC, not calculated.

tures found in both studies may be compared. Genotypes 1–8 were present in questing nymphs in each study, which indicates that the host populations were exposed to a similar spectrum of spirochete strains. Three rodent species, white-footed mice, chipmunks, and squirrels, were captured in both studies and used for comparison. The population structures of *B. burgdorferi* in each host species were different in the 2 data sets. However, analysis of the combined data set shows that, with the exception of genotypes 6 and 7 being missing in both squirrel populations, all 9 major genotypes that were prevalent in questing ticks were also found in the 3 rodent species (Table 6).

Discussion

We explored the question of whether, and to what level, *B. burgdorferi* is specialized to vertebrate host species. By analyzing 2 independent data sets obtained from cross-sectional field studies in the northeastern United States (New York and Connecticut), we show that most of the known genotypes of *B. burgdorferi* can, in principle, infect a range of different rodent hosts. Furthermore, our own data set indicates that several genotypes can infect as many as 5 host species. This suggests that cross-species transmission of *B. burgdorferi* among various mammalian species is common.

Several issues, however, need to be addressed before the level of host specificity of *B. burgdorferi* can be confidently compared with that of other microparasites. First, the level of host specificity is generally dependent on the spatial and temporal scale of observation (*1*,*28*). Our findings exemplify this notion, because the combined data sets of the 2 studies analyzed in this study shows a pattern of more relaxed host specificity than each of the data sets would suggest on its own. Second, the "niche breadth" of a parasite is influenced by the phylogenetic relationships of its hosts (*29*). If, for example, a parasite infects a given number of hosts belonging to different orders or classes, one would consider such a parasite to be less specialized than a parasite that exploits the same number of closely related species.

The analysis of the combined data sets obtained by the 2 field surveys compared in this study shows that mice, chipmunks, and squirrels (order Rodentia) are susceptible to most of the *B. burgdorferi* genotypes described in the United States. Therefore, the niche breadth of *B. burgdorferi* genotypes is not congruent with host species. Furthermore, genotypes 1–5 and 7 can, at least transiently, infect many additional, phylogenetically distant host species, covering as many as 3 orders. This indicates that the niche breadth of most *B. burgdorferi* genotypes in the United States is even wider than the taxonomic unit of order.

The issue of host specificity in *B. burgdorferi*, however, is more complicated. Experimental work has shown that some isolates of *B. burgdorferi* do not disseminate, or slowly disseminate, in mice (*30*). Slowly disseminating strains are less efficiently transmitted to ticks by mice (*31*). For this reason, it has previously been suggested that such strains may occupy nonrodent or even nonmammalian niches in nature, such as avian hosts (*31*). On the other

Table 5. Exposure of animals to infected nymphs*

Host species	No. infected nymphs per host†							
	GT1	GT2	GT3	GT4	GT5	GT6	GT7	GT8
White-footed mouse	2.2	9.7	5.7	1.1	1.7	0.29	0.4	0.4
Pine vole	0.8	2.2	0.7	0.3	0.2	0.3	0	0.5
Eastern chipmunk	9.1	45.5	29.6	4.6	9.1	0	2.3	0
Gray squirrel	12.6	58.0	34.3	6.3	10.4	1.5	2.4	2.3
Raccoon	6.5	25.4	12.2	3.2	3.5	2.0	0.6	2.9
Virginia opossum	9.0	29.1	9.0	4.5	2.2	4.5	0	6.7

*GT, genotype.
†For mice, raccoons and squirrels, calculated values are given as average numbers per season for years 2002 and 2003. For other species, average numbers are given per season for year where data were available.

Table 6. *Borrelia burgdorferi* genotypes transmitted by hosts and detected in field-collected ticks in 2 ecosystems

Host species	Locality*	Genotype (GT) (major *ospC* group)†								
		GT1 (A)	GT2 (K)	GT3 (B)	GT4 (N)	GT5 (D)	GT6 (M)	GT7 (I)	GT8 (U)	GT9 (E)
White-footed mouse	Millbrook	+	+	+	+	+	+	+	–	+
	Lake Gaillard	+	+	+	+	+	+	+	+	–
	Combined	+	+	+	+	+	+	+	+	+
Eastern chipmunk	Millbrook	+	+	+	+	+	+	+	+	+
	Lake Gaillard	–	+	–	–	+	–	–	+	–
	Combined	+	+	+	+	+	+	+	+	+
Gray squirrel	Millbrook	+	+	+	–	+	–	–	–	+
	Lake Gaillard	–	–	–	+	–	–	–	+	–
	Combined	+	+	+	+	+	–	–	+	+
Field-collected nymphs	Millbrook	+	+	+	+	+	+	+	+	+
	Lake Gaillard	+	+	+	+	+	+	+	+	–

*Millbrook data based on (*13*); Lake Galliard data based on our observation.
†+ indicates presence of genotype; – indicates absence of genotype.

hand, certain strains of *B. burgdorferi* can infect both rodent and avian hosts (*10*), which demonstrates that some strains of *B. burgdorferi* are extreme generalists. In view of all ecologic and experimental information available to date, we conclude that host specificity of *B. burgdorferi* ranges from generalism to specialism, depending on genetic background.

Several possible explanations exist for the discordance between the data sets from New York and Connecticut. First, differences in the local ecologic conditions could shape the local population structures of *B. burgdorferi* in hosts (*1*). Furthermore, the 2 data sets could represent snapshots of population structures that are spatially and temporally variable due to stochastic effects or other forces (*32*). In other words, the spirochete populations could be dynamic. In fact, strong evidence exists for this scenario, since pronounced temporal shifts in genotype frequency distribution of *B. burgdorferi* within 2 years have been observed in questing adult ticks (*33*). Considering that adult *I. scapularis* ticks have a history of taking only 2 blood meals in 2 years (*26*), the scale of this temporal variation is remarkable.

One of the most fundamental parameters in infectious disease biology is the time scale of infectivity relative to host lifetime, which affects the epidemic/endemic behavior of all microparasites (*34*). *B. burgdorferi* infections in mice are believed to be lifelong (*35*). The universality of this paradigm, however, has recently been challenged by experimental studies in white-footed mice, which found that the infectivity of some strains to ticks declines within a few weeks (*31*,*36*). This feature is crucial in 2 ways. First, it indicates that fitness of *B. burgdorferi* is a quantitative trait. This is corroborated by our study that provides ample evidence for fitness variation within and across diverse host species. Second, the finding of declining infectivity shows that the transmission kinetics of some *B. burgdorferi* strains is dynamic. Therefore, both intrinsic transmission dynamics of *B. burgdorferi* strains in hosts (*37*) and population fluctuations of the hosts (*38*) may result in population fluctuations of *B. burgdorferi*. Time series analyses of spirochete populations are required to clarify the scale of the spatiotemporal dynamics of *B. burgdorferi* (*32*).

We are beginning to understand key molecular processes that enable cross-species transmission of *B. burgdorferi* (*11*,*16*). Individual strains of *B. burgdorferi* have been found to contain large arrays of prophage-encoded outer surface proteins that differentially bind complement control factors of a wide range of vertebrate species, preventing the bacteria from being killed by innate immunity (*11*). That the repertoire of these prophage genes determines the host range of LB spirochetes has been hypothesized (*11*,*16*). *B. burgdorferi*, thus, is 1 of the very few examples of zoonotic pathogens for which a molecular mechanism of host-switch has been proposed (*39*).

OspA serotypes 2–8, which comprise the Eurasian genospecies *B. afzelii* and *B. garinii*, occupy distinct host niches, such as rodent versus avian hosts (*16*). Here we demonstrate that *B. burgdorferi* (OspA serotype 1) in the northeastern United States is much less specialized than *B. afzelii* (serotype 2) and *B. garinii* (serotypes 3–8), because the niche breadth of most of its genotypes covers a much larger range of phylogenetically distant hosts than any of the other OspA serotypes. The generalist strategy of *B. burgdorferi* is consistent with its uniform population structure across much of the northeastern United States (*33*). We may speculate that the generalist strategy of *B. burgdorferi* echoes adaptation to impoverished ecologic conditions in the past because of large-scale habitat destruction in the northeastern United States in the course of the post-Columbian settlement and during the industrial revolution (*8*). We conclude that cross-species transmission of *B. burgdorferi* is a key property that has allowed LB to spread rapidly across the northeastern United States. Our study emphasizes that accurate information on the degree of cross-species transmission is necessary to understand and predict the spread of zoonotic pathogens.

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Emerging Pediatric HIV Epidemic Related to Migration

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In 2002, Canada introduced routine, mandatory HIV antibody screening for all residency applicants, including selected children. We report screening results from January 2002 to February 2005. Thirty-six pediatric HIV cases were detected (14/100,000 applicants); 94% of infected children were eligible to arrive in Canada. Thirty-two of the affected children were from Africa, and maternal infection was the main risk factor. Only 4 (11%) of the children had received antiretroviral therapy. In countries of low HIV incidence, migration-related imported infection in children may be an emerging epidemic. The early identification of HIV-infected immigrant women permits intervention to prevent mother-to-child HIV transmission. Routine HIV testing as a component of the medical examination of immigrants has national and international health policy and programmatic implications.

In 2004, the Joint United Nations Programme on HIV/AIDS reported that the number of persons living with HIV and AIDS in the world was 39.4 million, 4.9 million were newly infected with HIV, and 3.1 million had died because of HIV/AIDS (*1*). Within these numbers are an estimated 2.2 million children <16 years of age who are living with HIV/AIDS. Globally in 2003, ≈640,000 new HIV pediatric infections and 510,000 pediatric deaths occurred. Most pediatric HIV/AIDS occurs in the developing world because of mother-to-child transmission, but pediatric HIV/AIDS is also a concern in Western nations, where several strategies have been developed and implemented to prevent pediatric HIV infection and subsequent AIDS-related disease.

A link between HIV infection in hyperendemic zones of the developing world and pediatric HIV infections in Western countries is largely undocumented (*2*). Irregular migrants (those who arrive by smuggling or trafficking) and those seeking asylum in Europe represent a separate epidemiologic pattern of HIV/AIDS introduction (*3*). For regions without extensive immigration assessment programs, or where most international arrivals for permanent residency are seeking asylum or are arriving through other irregular means, migration-associated HIV/AIDS prevalence and the pediatric HIV/AIDS epidemic are emerging policy and programmatic issues. Injection drug use and sexual exploitation, particularly for women, are 2 potential risks associated with illegal immigration status that increase HIV exposure, with the potential consequence of mother-to-child viral transmission (*4*).

Prearrival immigration medical screening has been used to identify conditions such as tuberculosis, syphilis, and HIV/AIDS that could affect admission to receiving nations. Many immigrant-receiving nations in the industrialized world now have national policies designed to better address the needs of vulnerable, foreign-born migrants or to facilitate the immigration process for preferred applicants. Nations who either have existing medical screening programs or who are planning such programs are likely to identify persons with HIV/AIDS. Decisions on screening immigrants for HIV infection not only have direct implications for admissibility programs but also affect the need for culturally and linguistically appropriate clinical and public health services.

The 2002–2003 annual report of the Ministerial Council on HIV/AIDS in Canada "estimated that 70% of all maternal HIV transmissions to children in Canada have occurred among women of African and Caribbean origin" (*5*). From November 1985 to June 2004, the Public Health Agency of Canada reported notification of 56,523 positive HIV test results (*6*). From 1984 to 2002, it also reported 420 HIV infections in 1,584 children born to HIV-positive mothers (*7*).

Apart from the potential for perinatal HIV exposure, other pediatric risk factors for nonmaternal HIV acquisition in the industrialized world, such as blood transfusion, tattooing, or illicit drug use, are rarely encountered or documented. Strategies designed to reduce mother-to-child

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HIV transmission and pediatric HIV infection include HIV screening programs for pregnant women (*8*), risk behavior counseling, recommendations for antiretroviral treatment to prevent mother-to-child-transmission or to treat a newborn exposed to HIV at birth, conception control, and birthing methods. These strategies have been relatively successful in reducing pediatric HIV infections in most industrialized nations but have been less effective in developing nations (*9–11*).

The role of population mobility between hyperendemic HIV transmission zones and countries of lower prevalence is emerging as a contributing factor in risk for pediatric HIV infection between HIV high-prevalence and low-prevalence regions. This article describes the results of the first 3 years of a medical screening program for HIV antibodies in selected children who were applicants for residency in Canada. The results of this study confirm that mobile population dynamics between countries and regions and demographic changes in pediatric HIV/AIDS epidemiology are directly related and have consequences for immigration and health policy at domestic and international levels.

Methods

Population

Mandatory, routine serologic HIV testing of immigrants in Canada began in January 2002 as a component of the Canadian immigration medical examination (*12,13*). While routine testing was not conducted on applicants <15 years of age, those identified as being at risk in the pediatric migrant populations were evaluated for HIV infection. An enzyme-linked immunoassay (EIA) HIV screening test for HIV-1 and HIV-2 antibodies was required for all children <15 years of age who had received blood or blood products, had an HIV-positive mother, or in the judgment of the assessing medical practitioner, was noted to have any other identified HIV infection risk factor. Potential adoptees were also screened for HIV antibodies until a policy decision changed this requirement in late 2004. Reflecting national and international legal processes of adoption, international adoptees brought to Canada by Canadian citizens or permanent residents of Canada are considered part of the existing family, and although they are required to apply for Canadian citizenship, they are exempted from most immigration processes (including the medical examination to determine inadmissibility).

The HIV-tested migrant groups included children of applicants for permanent residence (immigrants and refugees) and those who filed refugee or asylum claims in Canada. Immigration medical screening, including routine HIV testing for those ≥15 years of age is also required for some other persons arriving in Canada, including visitors staying >6 months from certain locations (e.g., tourists, students, and seasonal workers). Children of persons in this group could also be referred for HIV testing if risk factors were noted during the process (*14*).

Medical Assessment and HIV Testing Protocols

The guardians of all pediatric applicants were counseled and consented to HIV testing. Venous blood was collected and transported to an approved local testing facility. HIV antibody was tested by EIA. Immunofluorescence antibody testing and Western blot (or other approved manufacturer's EIA kit) testing on another blood sample were used as confirmatory tests on positive or indeterminate HIV antibody test results.

Data Management and Protection of Personal Information

Immigration medical screening data were provided by Citizenship and Immigration Canada. Personal data were protected according to national guidelines on information privacy (*15,16*). Age was calculated from the date of birth and application date on the immigration file. The 5 immigration categories used in the analysis, reflecting current Canadian classifications, were economic, family, refugee (abroad), refugee claimant (in Canada), and other (*17*). The "other" category includes temporary resident applicants, such as visitors, workers, and students.

Currently, Canadian immigration policy reflects 3 major goals. New immigrants are selected on the basis of 1 of these 3 basic principles: reunification of families, economic ability to become successfully established in Canada, or humanitarian reasons based on displacement or persecution. Specific components of each of these major immigration selection groups are further described by legislation and regulation (*18*).

Refugee claimants, known as asylum seekers in Europe, differ from refugees whose convention-defined status has been determined before arrival (*19–21*). Refugee claimants arrive through uncontrolled and irregular means and complete the immigration medical assessment processes entirely within Canada as part of the procedures for determining their refugee status. During the past decade, the number of refugee claimants arriving in Canada has varied from 23,000 to 43,000 claimants per year (*22*). Since the implementation of the Safe Third Country Agreement between Canada and the United States on December 29, 2004, the number of refugee claimants has declined by ≈25% (*23*).

Results

From January 2002 to February 2005, a total of 1,307,718 persons underwent a Canadian immigration medical assessment (*24*), including 256,970 applicants

(124,195 female, 132,775 male) <15 years of age. Thirty-six new HIV-positive diagnoses were made in the pediatric applicant population; 18 were boys and 18 were girls. Twelve HIV-positive applicants were <1 year of age at diagnosis, 7 were 2–4 years, 7 were 5–7 years, 2 were 8–10 years, and 8 were 11–14 years. Median age for girls was 6 years (range from infancy to 14 years) and for boys, 4 years (range from infancy to 12 years). All HIV-infected children except 4 (2 from Europe, 2 from Asia/Pacific Islands) identified a country in Africa as either their parents' home or their country of birth. Twenty-seven (75%) of the 36 HIV-infected children were tested because of known maternal HIV positivity, 6 children (17%) were tested because of adoption, 2 (6%) were tested because of an HIV-positive sibling, and 1 (3%) was detected because of concurrent treatment for tuberculosis. Two girls (ages 6 and 12 years) and 2 boys (ages 6 and 11 years) (11%) had documentation of having received highly active antiretroviral therapy (HAART). Two of the 4 received HAART in North America, 1 in the United Kingdom, and 1 in Ethiopia. By applicant category, 2 of the children receiving HAART were family class, 1 was a refugee claimant, and 1 was an economic applicant.

HIV-positive children by immigration category and year of testing are shown in the Table. Refugees represented 26 (72%) of the 36 HIV diagnoses, family class represented 7 (19%), and economic, refugee claimant, and temporary resident applicants represented 1 case each (3%). Only 2 HIV-infected children were processed in the immigration medical office responsible for North America, which indicates their application originated in Canada or the United States.

Thirty-three (92%) of the 36 HIV pediatric cases occurred in populations deemed to be eligible for admission to Canada, despite medical status, on the basis of these application categories (26 in the refugee class and 7 in the family class). Of those children found to be HIV positive in the study period, 24 (66%) had arrived in Canada.

Discussion

In the context of all immigration applicants, 36 HIV-infected children were identified. During the study, 256,970 applicants <15 years of age underwent medical examinations. This number represents a crude ratio of 14/100,000 pediatric applicants from January 2002 through February 2005. A ratio per tested pediatric applicant cannot be determined because negative serologic test results are not recorded for this age group, and no estimate of the tested population size is possible because of variations in medical examiner and adjudicator practices during the study. All but 4 of the pediatric HIV -positive applicants were originally from Africa (89%), which reflects the relationship between population flow and the global epidemiologic features of HIV infection and disease (*25*). In contrast, domestically reported pediatric HIV cases are rare in Canada (0.02/100,000 general population).

Applicants in the refugee (26 cases) and family (7 cases) categories accounted for 33 (91%) of the 36 HIV diagnoses in this study period. The refugee and family categories are exempted from provisions that can render applicants inadmissible because of medical reasons. Consequently, the 33 children in these groups were all medically eligible to be admitted to Canada. When those children are combined with the HIV-positive pediatric refugee claimant who was in Canada when tested, 34 (94%) of the 36 HIV-infected children detected during the first 3 years of the mandatory immigration screening program were medically admissible or had already arrived in Canada.

In the 2004 World Health Organization report, Canadian public health officials estimated an overall HIV prevalence ratio in Canada of 3–5 cases per 10,000 pregnant women on the basis of pregnancy HIV screening results (*26*). Fecundity data in Canada from 1986 to 2001 show that 1,197,300 children were born to Canadian-born women and 337,700 children were born to immigrant women (1,535,000 total newborns) (*27*). If the estimated HIV prevalence rate for pregnant women in Canada is applied to the average of 102,333 newborn children per year, an estimated 92–154 singleton births occur to at-risk pregnant women.

The actual number of documented mother-to-child HIV exposures reported in Canada has been less than that figure. From 1984 to 2002, national statistics in Canada show that 1,584 infants (an average of 88 per year) were perinatally exposed to HIV. Of these infants, 420 have been reported as HIV infected. An additional 120 children had unconfirmed HIV status, including those with indeterminate serologic status, those who died, or those who were lost to follow-up. As a possible reflection of the effectiveness of the national perinatal HIV screening, mother-to-child transmission–prevention programs, and other factors, <4% of at-risk pregnancies in HIV-positive mothers resulted in viral transmission to their newborns in 2001 and 2002, with only 12 pediatric HIV cases reported in those 2 years (0.02 cases/100,000 population per year).

As indicated in this study of immigrant applicants, more HIV-infected children are detected through selective immigration medical screening in migrant pediatric populations arriving or arrived in Canada. Growing global population mobility and immigration could more than double the annual domestic pediatric HIV/AIDS caseload in Canada.

Despite the success of industrialized nations' domestic mother-to-child transmission prevention programs, the

Table. Immigration applicants and HIV-positive pediatric applicants by immigration category and year

Immigration applicants	2002	2003	2004	2005*	Total	% by category
Total applicants	347,438	430,259	455,553	74,468	1,307,718	
Total applicants <15 years of age	68,734	81,540	92,055	14,641	256,970	
Immigration category of HIV-positive pediatric applicants						
Economic	0	1	0	0	1	3
Family class	1	4	1	1	7	19
Refugees	3	6	12	5	26	72
Refugee claimants	1	0	0	0	1	3
Other (temporary residents)	0	1	0	0	1	3
Total	5	12	13	6	36	100

*January and February 2005 only.

potential for HIV infection in children still exists in Western society. For several reasons, including education, language, culture, and fear of personal and social reprisals, foreign-born migrant women may have limited access to healthcare services or delay medical care. They may not be able to fully access prenatal care or HIV screening programs that could benefit them and their unborn children (*28*,*29*). Pregnant migrants and migrating women of childbearing potential may be a source for pediatric HIV cases in migrant-receiving nations.

The required medical examination for persons applying for residence from abroad can precede their arrival in Canada by up to 12 months, which raises the possibility of new maternal HIV infection, the new conception of a child, and new birth to HIV-infected mothers. The immigration medical examination and processing represents a time-limited opportunity to detect at-risk pregnancies to provide treatment to prevent mother-to-child transmission of HIV. The medical assessment, including HIV antibody testing, may identify several groups of foreign-born women at risk for transmitting HIV to their children, including women with defined risk factors who are pregnant or may become pregnant during the immigration application process, while in Canada or abroad.

An immigration application provides an opportunity to inform and educate all applicants, particularly women of childbearing potential, of the benefits of HIV screening in pregnancy. Even women who test negative for HIV antibodies during the immigration medical examination should be retested if they become pregnant, whether they are still abroad or have arrived in Canada. The medical examination is also an opportunity to identify resources for HIV screening in pregnancy for these women, separate from the immigration process, and the local access points for maternal management and, if needed, antiretroviral treatment to reduce the risk for perinatal HIV transmission. Three of the 4 children who received HAART in this study did so in Western countries. None of the remaining 32 HIV-infected children, all of whom were in developing nations, had any indication of receiving HAART for either prevention or treatment.

Opportunities for active, programmatic intervention to protect the health of pregnant women and reduce the risk for HIV infection and transmission during pregnancy exist for both refugees and refugee claimants on the basis of their category of application. By definition, refugees have already had their status determined and have already come under the jurisdiction of a national or international authority, such as the United Nations High Commission for Refugees. Programs on health promotion and HIV infection and disease prevention in pregnant women and women of childbearing potential can be part of the international protection offered to this vulnerable population (*30*). In this study, 72% of all pediatric HIV infections were in the refugee category.

By definition, refugee claimants and asylum seekers make their applications from within the host country. In Canada, refugee claimants are provided access to healthcare services while their claim is determined. In spite of the challenges of providing culturally and linguistically accessible healthcare programs for foreign-born, migrant women, this situation offers an opportunity to educate and test pregnant applicants. In this study, one 6-year-old refugee claimant with HIV, who traveled from Africa, received HAART in Canada.

Other risk groups include women known to be HIV positive and their children with risks for exposure to HIV infection, such as breastfeeding; children with risk factors for HIV infection other than birth to an HIV-positive mother; and foreign-born children being adopted. Failure to recognize and use these opportunities may have implications for maternal and pediatric HIV infection in migrant populations.

HIV antibody testing in immigrants is primarily a part of the administrative process of determining the medical status of migrants in accordance with immigration legislation (*31*,*32*). As such, it may not be directly linked to HIV/AIDS clinical management programs. Routine immigration testing for diseases such as HIV/AIDS or tuberculosis provides an opportunity to identify groups at increased risk that may benefit from specific health promotion and disease prevention programs (*33*).

Given the emerging patterns of global HIV/AIDS epidemiology and current immigration patterns, similar situations could be observed over time in other locations. A proportional shift in total and pediatric HIV cases related to foreign-born migrant arrivals in immigrant-receiving nations, where cases in children are less common, can be anticipated. Migrants, and the communities of newly arrived persons that they tend to gravitate to, often have health needs that may differ substantially from those of the host population. The shifting demographics of pediatric HIV infection in Canada can thus be expected to influence local aspects of healthcare planning and delivery. The multicultural aspects of HIV infection in immigrants will affect case management by local public health authorities, social services, clinical pediatric HIV/AIDS services, and other health and social service providers. Existing programs, designed in the context of domestically acquired infections, may not have considered either the size of the population or the culturally and linguistically diverse characteristics of immigrant, HIV-infected children. Other unanticipated program effects, including policy expectations from the international community on the medical, cultural, and social aspects of pediatric HIV/AIDS and immigration should receive greater attention (*34*,*35*).

One of the potential consequences of this shared knowledge related to pediatric HIV and AIDS for domestic program development could be the integration of immigration medical programs and public health programs to prevent mother-to-child transmission of HIV overseas. Only 4 (11%) of 36 of HIV-infected children received specific antiretroviral therapy, and none of the 12 HIV-positive children who were <1 year of age, nor their mothers, were known to have received perinatal HAART to prevent HIV transmission (*36*,*37*). Programs and strategies designed to mitigate some infectious disease risks in migrant populations before arrival have already been used in some situations (*38*); similar rationales could be evaluated for HIV infection in those already involved in immigration formalities.

The analysis and opinions expressed in this article are those of the authors and are not to be attributed to Citizenship and Immigration Canada or the government of Canada, and they do not necessarily reflect or represent the position of any government department, agency, university, or professional society to which the authors may belong or have belonged.

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Lineage 1 and 2 Strains of Encephalitic West Nile Virus, Central Europe

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Two different West Nile virus (WNV) strains caused lethal encephalitis in a flock of geese and a goshawk in southeastern Hungary in 2003 and 2004, respectively. During the outbreak in geese, 14 confirmed human cases of WNV encephalitis and meningitis were reported in the same area. Sequencing of complete genomes of both WNV strains and phylogenetic analyses showed that the goose-derived strain exhibits closest genetic relationship to strains isolated in 1998 in Israel and to the strain that emerged in 1999 in the United States. WNV derived from the goshawk showed the highest identity to WNV strains of lineage 2 isolated in central Africa. The same strain reemerged in 2005 in the same location, which suggests that the virus may have overwintered in Europe. The emergence of an exotic WNV strain in Hungary emphasizes the role of migrating birds in introducing new viruses to Europe.

Geographically, West Nile virus (WNV) is the most widespread member of the Japanese encephalitis virus (JEV) complex within the genus *Flavivirus* and the family *Flaviviridae*. The first strain (B 956) was isolated from a human patient in the West Nile district of Uganda in 1937; later the virus was also detected in several mosquito species, horses, humans, and other hosts in Africa, Europe, Asia, and Australia (where it has been named Kunjin virus) (*1–3*). WNV was introduced into the United States in 1999, and it spread quickly over large parts of North America and reached Mexico (*4–7*). The clinical impact of WNV varies in different regions. In the Old World, WNV causes relatively mild infections with influenzalike symptoms or no apparent disease (*2*); encephalitis and fatalities in the human population, horses, or poultry are spasmodic (*3,8,9*). In the New World, WNV exhibits increased virulence among the local wild bird populations and causes more frequent severe central nervous system symptoms and deaths in humans and horses (*6,10*). Although exactly how WNV was introduced into New York is unclear, phylogenetic comparison of the viral nucleic acid sequences has shown a close relationship between the American WNV isolates and strains isolated from encephalitic geese and storks in Israel in 1998 (*11–13*). Experimental infections of rodents indicated that the neurovirulence of WNV correlates with its genotype, and the North American strains are highly neurovirulent for mice (*14*).

WNV shows relatively high levels of sequence diversity. Comprehensive studies on the phylogenetic relatedness of WNV strains show that they form at least 2 main lineages (*15–17*). Lineage 1 is composed of WNV strains from different geographic regions, and it is subdivided into at least 3 clades. Clade A contains strains from Europe, Africa, the Middle East, and America; clade B represents the Australian (Kunjin) strains; and clade C contains Indian WNV isolates. Lineage 2 contains the B 956 prototype strain and other strains isolated so far exclusively in sub-Saharan Africa and Madagascar. In addition to the 2 major WNV lineages, we recently proposed 2 lineages for viruses that exhibited considerable genetic differences to the known WNV lineages: lineage 3 consists of a virus strain isolated from *Culex pipiens* mosquitoes at the Czech Republic/Austria border (named Rabensburg virus), and lineage 4 consists of a unique virus isolated in the Caucasus. These 2 viruses, however, may also be considered independent flaviviruses within the JEV complex (*18*).

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WNV has been known to be present in central Europe for a long time. Seroprevalence in humans was reported in several countries, including Hungary, and WNV strains were isolated from mosquitoes, humans, migrating birds, and rodents during the last 30 years (*3*). Until 2003, however, WNV infections in Hungary have never been associated with clinical symptoms, although a severe outbreak of West Nile encephalitis in humans was reported in 1996 and 1997 in neighboring Romania.

In late summer 2003, an outbreak of encephalitis emerged in a Hungarian goose flock, resulting in a 14% death rate among 6-week-old geese (*Anser anser domesticus*). Based on histopathologic alterations, serologic investigations, and nucleic acid detection by reverse transcription–polymerase chain reaction (RT-PCR), WNV was diagnosed as the cause of the disease (*19*). Chronologically and geographically related to the outbreak in geese, a serologically confirmed WNV outbreak was also observed in humans, which involved 14 cases of mild encephalitis and meningitis (*20*).

One year later, in August 2004, a goshawk (*Accipiter gentilis*) fledgling showed central nervous system symptoms and died in a national park in southeastern Hungary. When histopathologic methods and RT-PCR were used, WNV antigen and nucleic acid were detected in the organs of the bird. Furthermore, the virus was isolated after injection of suckling mice. Here we report the sequencing and phylogenetic results of these 2 encephalitic WNV strains that emerged recently in central Europe.

Materials and Methods

Brain specimens from one 6-week-old goose, which died during the encephalitis outbreak in a Hungarian goose flock, and brain samples from a goshawk, which also died from encephalitis, were used for WNV nucleic acid determination. The brain samples were homogenized in ceramic mortars by using sterile quartz sand, and the homogenates were suspended in RNase-free distilled water. Samples were stored at –80°C until nucleic acid extraction was performed.

Viral RNA was extracted from 140 μL of brain homogenates by using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First, a universal JEV-group specific oligonucleotide primer pair designed on the nonstructural protein 5 (NS5) and 3′-untranslated regions (UTR) of WNV (forward primer: 5′-GARTGGATGACVACRGAAGACATGCT-3′ and reverse primer: 5′-GGGGTCTCCTCTAACCTCTAGTCCTT-3′; [*21*]) was applied on the RNA extracts in a continuous RT-PCR system employing the QIAGEN OneStep RT-PCR Kit (Qiagen). Each 25-μL reaction mixture contained 5 μL of 5× buffer (final $MgCl_2$ concentration 2.5 mmol/L), 0.4 mmol/L of each deoxynucleoside triphosphate, 10 U RNasin RNase Inhibitor (Promega, Madison, WI, USA), 20 pmol of the genomic and reverse primers, 1 μL enzyme mix (containing Omniscript and Sensiscript Reverse Transcriptases and HotStarTaq DNA polymerase) and 2.5 μL template RNA. Reverse transcription was carried out at 50°C for 30 min, followed by a denaturation step at 95°C for 15 min. Thereafter, the cDNA was amplified in 40 cycles of heat denaturation at 94°C for 40 s, primer annealing at 57°C for 50 s, and DNA extension at 72°C for 1 min, and the reaction was completed by a final extension for 7 min at 72°C. Reactions were performed in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler (Wellesley, MA, USA) and in a Hybaid PCR Sprint thermocycler (Thermo Electron Corporation, Waltham, MA, USA).

After RT-PCR, 10 μL of the amplicons was subjected to electrophoresis in a 1.2% Tris acetate-EDTA-agarose gel at 5 V/cm for 80 min. The gel was stained with ethidium bromide; bands were visualized under UV light and photographed with a Kodak DS Electrophoresis Documentation and Analysis System using the Kodak Digital Science 1D software program (Eastman Kodak Company, Rochester, NY, USA). Product sizes were determined with reference to a 100-bp DNA ladder (Promega).

Where clear PCR products of the previously calculated sizes were observed, the fragments were excised from the gel, and DNA was extracted by using the QIAquick Gel Extraction Kit (Qiagen). Fluorescence-based direct sequencing was performed in both directions on PCR products. Sequencing of PCR products was carried out with the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit (Perkin-Elmer), according to the manufacturer's instructions, and an ABI Prism 310 genetic analyzer (Perkin-Elmer) automated sequencing system. Nucleotide sequences were identified by Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/blast) search against gene bank databases. Based on the sequence information obtained from the amplification products, complete WNV sequences that exhibited the highest nucleotide identities with the Hungarian genotypes were selected from the GenBank database to design primers that amplify overlapping RT-PCR products covering the entire genome of the strains. Oligonucleotide primers were designed with the help of the Primer Designer 4 for Windows 95 (Scientific and Educational Software, Version 4.10; Microsoft, Redmond, WA, USA) and were synthesized by GibcoBRL Life Technologies, Ltd. (Paisley, Scotland, UK). Detailed information on all primers is available as an online appendix (http://www.cdc.gov/ncidod/EID/vol12no04/05-1379_app.htm). PCR amplification products were directly sequenced in both directions; the sequences were compiled and aligned to complete genome sequences of selected representatives of

WNV lineages 1a, 1b, 2, and putative lineages 3 and 4 (listed in Table). Phylogenetic analysis was performed by using the modified neighbor-joining method (ClustalX; [*22*]), and trees were constructed to demonstrate the relationship between the Hungarian WNVs and other WNV strains (Figure).

The nucleotide sequences of the Hungarian WNV strains goose-Hungary/03 (Hu03) and goshawk-Hungary/04 (Hu04) were submitted to the GenBank database. They are available under accession numbers DQ118127 and DQ116961, respectively.

Results

In this study, the complete genome sequences of WNV strains derived from a 6-week-old goose, which died in 2003 during an outbreak of encephalitis in a Hungarian goose flock (strain goose-Hungary/03), and from a goshawk, which also died from encephalitis in the same region 1 year later (strain goshawk-Hungary/04), were determined, aligned, and phylogenetically analyzed. The genome of the goose-Hungary/03 strain is composed of 10,969 nucleotides (nt) and contains 1 open reading frame between nucleotide positions 97 and 10,398, coding for a 3,433 amino acid (aa)–long putative polyprotein precursor. The complete genomic sequence of the virus was subjected to a BLAST search against gene bank databases. The highest identity rates (98% at the nucleotide and 99% at the amino acid level) were found with WNV strains isolated in 1998 in Israel and in 1999 in the United States. In addition, phylogenetic analysis was performed to indicate the relationships between the Hungarian goose–derived WNV strain and selected representatives of WNV clades and clusters. The resulting phylogenetic tree (Figure) confirmed the results of the BLAST search, i.e., the Hungarian goose–derived WNV strain is clustering close to the previously mentioned WNV strains isolated in the United States and Israel, which belong to lineage 1a of WNV. Other European WNV strains (isolated in Italy, France, and Romania) are more distant to the Hungarian strain; they form a separate cluster consisting of a Romanian/Russian and a French/Italian subcluster.

The complete nucleotide sequence of the goshawk-Hungary/04 WNV strain is composed of 11,028 nt and contains 1 open reading frame between nucleotide positions 97 and 10,401, coding for a 3,434-aa putative polyprotein precursor. In BLAST search, the strain showed the highest (96% nt and 99% aa) identity to the WNV prototype strain B 956. Consequently, as the phylogram also indicates (Figure), this virus belongs to lineage 2 of WNV. Alignments of the available partial sequences from the E protein coding regions of other representatives of this cluster showed even higher identities (97%–98% nt and 100% aa) with WNV strains isolated in central Africa in 1972 (AnB3507, AF001563) and in 1983 (HB83P55, AF001557), respectively (*15*).

More recently (in early August 2005), additional lethal cases of encephalitis occurred in birds of prey in the same place in which the goshawk died of West Nile encephalitis

Table. West Nile virus strains included in the phylogenetic analysis

Name	Code	Accession no.	Isolation			Lineage, clade
			Year	Host	Origin	
WNV HNY1999	NY99a	AF202541	1999	Human	New York, USA	1a
WNV NY99flamingo38299	NY99b	AF196835	1999	Flamingo	New York, USA	1a
WNV IS98STD	Is98	AF481864	1998	Stork	Israel	1a
WNV goose-Hungary/03	Hu03	DQ118127	2003	Goose	Hungary	1a
WNV Italy1998Equine	It98	AF404757	1998	Horse	Italy	1a
WNV RO9750	Ro96	AF260969	1996	*Culex pipiens*	Romania	1a
WNV VLG4	Rus99a	AF317203	1999	Human	Volgograd, Russia	1a
WNV LEIV-Vlg99-27889	Rus99b	AY277252	1999	Human	Volgograd, Russia	1a
WNV PaH001	Tu97	AY268133	1997	Human	Tunisia	1a
WNV PaAn001	Fr00	AY268132	2000	Horse	France	1a
WNV Eg 101	Eg51	AF260968	1951	Human	Egypt	1a
WNV Chin-01	Chin01	AY490240	1950s	?	Russia	1a
WNV Kunjin MRM61C	Kunjin	D00246	1960	*Cx. annulirostris*	Australia	1b
WNV Sarafend	Sarafend	AY688948		Laboratory strain		2
WNV B956 (WNFCG)	Ug37	NC_001563	1937	Human	Uganda	2
WNV goshawk-Hungary/04	Hu04	DQ116961	2004	Goshawk	Hungary	2
Rabensburg virus (97-103)	RabV	AY765264	1997	*Cx. pipiens*	Czech R.	3?
WNV LEIV-Krnd88-190	Rus98	AY277251	1998	*Dermacentor marginatus*	Caucasus, Russia (Georgia?)	4?

in 2004, involving up to a total of 3 goshawks and 2 sparrow hawks (*A. nisus*); 2 of the goshawks and 1 sparrow hawk died. Preliminary investigations detected WNV-specific nucleic acid in the brains of the birds. The partial nucleotide sequence of the 2005 virus (1,000 bp at the NS5′–3′-UTR regions) showed 99.9% identity with the goshawk-Hungary/04 strain (only 1 substitution at nucleotide position 9,376 [g→a] has been observed, which did not influence the putative amino acid sequence). Additional observation of the outbreak and investigations of the cases are in progress.

Discussion

The primary aim of our investigations was to show the genetic relatedness of the WNV strains detected in Hungary in the last 2 years and to estimate their clinical and epidemiologic impact. The phylogenetic analysis emphasizes the close genetic relationship of the goose-Hungary/03 strain with a WNV strain isolated in Israel in 1998 and the WNV strain introduced in New York in 1999, since the 3 WNVs form 1 single cluster within clade 1a of lineage 1. These strains caused outbreaks in birds, humans, and horses. Previous European WNV isolates exhibited lower identity values, e.g., the strain that was responsible for the Romanian outbreak(s) in 1996 and 1997 showed only 96% nt identity with the Hungarian goose-2003 strain, and in the phylogenetic tree the other European isolates form a separate cluster consisting of 2 subclusters (Figure). The earliest representatives of the Israel/USA/goose-Hungary/03 cluster were reported by Malkinson et al. (*23*) from ill and dead white storks (*Ciconia ciconia*) in Israel in 1998. These storks, however, had hatched in central Europe, and during their autumn migration southwards, strong winds had blown them off course, from their usual route to Africa, to southern Israel. Malkinson et al. suspected that these birds introduced the neurovirulent genotype of WNV to Israel from their hatching place. The wetlands of southeastern Hungary are foraging and nesting habitats for storks and many other wild bird species, and the goose farm, where the WNV outbreak occurred in 2003, is located in this region. These facts, together with the close phylogenetic relatedness of the Israeli/US/Hungarian WNV strains, strongly support the theory that storks carried the neurovirulent WNV strain from central Europe (that is, from Hungary) to Israel, which sheds new light on the introduction of WNV to New York. This virus could have originated in Israel (which is the generally accepted although not proven theory) or central Europe. In both cases, however, the virus seems to have its true origin in Europe. In a recent publication, Lvov et al. suggested that WNV could have been introduced into New York by ships traveling from Black Sea ports (*24*).

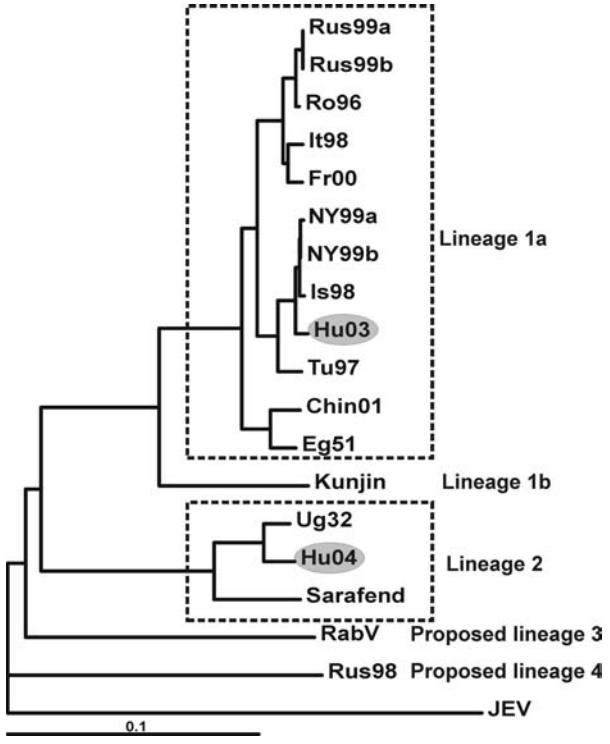

Figure. Phylogenetic tree based on the complete nucleotide sequences of selected West Nile virus strains demonstrating the genetic relatedness of these strains (abbreviations are listed in Table.) Boxes indicate different lineages and clades. The Hungarian strains reported in this article are highlighted with gray background). RabV, Rabensburg virus; JEV, Japanese encephalitis virus. Scale bar depicts degree of relatedness.

When a WNV infection was detected in 2004 in a goshawk fledgling, which died from encephalitis in the same region of Hungary in which the outbreak in geese and humans occurred during the previous year, we anticipated a WNV strain more or less identical to the genotype detected there in 2003. The genomic sequence of this strain was not closely related to the sequence of the WNV strain detected in geese in the year before, however, but belonged to the group of central African lineage 2 WNV strains. A closely related strain from this cluster (ArB3573, AF001565, and AF458349) was identified as a neuroinvasive strain of WNV in a mouse model (*14*). To our knowledge, this report is the first on the emergence of a lineage 2 WNV strain outside Africa. Migratory birds that had overwintered in central Africa probably introduced this exotic strain to the wetlands of Hungary. On the other hand, as the goshawk is not a migratory species, and infection occurred in August, the African WNV strain must have already successfully adapted to local mosquito vectors. Consequently, this neurotropic, exotic WNV strain may become a resident pathogen in Europe with all the possible public health consequences.

Our results indicate that the WNV strains that emerged in 2 consecutive years and caused avian deaths in Hungary are epidemiologically unrelated. Genetically distinct WNV strains are circulating simultaneously yet independently in local birds and thus most likely also in local mosquito populations within the same region. They cause sporadic cases of encephalitis and also raise the possibility of spreading to other European countries or even to other continents, as happened in 1999 with another WNV strain, which resulted in a public health catastrophe in America.

In addition to the above 2 novel WNVs, we recently characterized another novel flavivirus of so far unknown human pathogenicity named Rabensburg virus, which has been isolated from *Culex pipiens* mosquitoes in 1997 and 1999 at the Czech Republic–Austria border, only a few hundred kilometers from the region where the Hungarian WNVs emerged. After the entire genome was sequenced, Rabensburg virus turned out to represent either a new (third) lineage of WNV or a novel flavivirus of the JEV group (*18*). Thus, several distinct WNV strains seem to circulate in central Europe. In 2001 another flavivirus of the JEV group, Usutu virus, which has never previously been observed outside Africa, emerged in Austria and resulted in deaths in several species of birds, especially Eurasian blackbirds (*Turdus merula*) (*21*). This virus became a resident pathogen in Austria and continues to disperse and cause deaths in blackbirds and other species of birds (*25*,*26*).

The snowy winter and rainy spring of 2005 resulted in serious floods in the area in which the Hungarian WNV strains were identified. Since the floodplains and polders were under water, the conditions for mosquito development were ideal. The summer was also very rainy, which resulted in more floods in the region and continuous mosquito gradation. The most recent data imply that the lineage 2 WNV strain may have overwintered in Hungary, causing several clinical cases of encephalitis in *Accipiter* species in 2005 as well.

The routine diagnostic techniques in most of the European public health and veterinary laboratories are designed to detect lineage 1 WNV strains. In a recent PCR external quality assurance multicenter test, <40% of the involved laboratories could detect lineage 2 WNV strains (Matthias Niedrig, pers. comm.). Therefore, a major goal of this article is to increase the scientific and public awareness of this potential public health threat for Europe and, perhaps, America. Furthermore, comprehensive investigations on the occurrence, ecology, and epidemiology of the different WNV strains circulating in central Europe, as well as the development of monitoring and surveillance programs, must be of highest priority. One may also speculate on environmental factors, such as climate change or global warming, that may have enhanced the recent emergence of viruses, which had previously been restricted to Africa, in new habitats and continents. Improved observation, reporting, and detection methods have also contributed to the apparent increasing emergence of these viruses.

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ANOTHER DIMENSION

Bedside Manners

Christopher Wiseman

How little the dying seem to need–
A drink perhaps, a little food,
A smile, a hand to hold, medication,
A change of clothes, an unspoken
Understanding about what's happening.
You think it would be more, much more,
Something more difficult for us
To help with in this great disruption,
But perhaps it's because as the huge shape
Rears up higher and darker each hour
They are anxious that we should see it too
And try to show us with a hand-squeeze.

We panic to do more for them,
And especially when it's your father,
And his eyes are far away, and your tears
Are all down your face and clothes,
And he doesn't see them now, but smiles
Perhaps, just perhaps because you're there.
How little he needs. Just love. More Love.

From In John Updike's Room: New and Selected Poems, by Christopher Wiseman.

Human *Trypanosoma cruzi* Infection and Seropositivity in Dogs, Mexico

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We used 5 diagnostic tests in a cross-sectional investigation of the prevalence of *Trypanosoma cruzi* in Tejupilco municipality, State of Mexico, Mexico. Our findings showed a substantial prevalence of immunoglobulin G (IgG) and IgM antibodies to *T. cruzi* in human (n = 293, IgG 2.05%, IgM 5.5%, both 7.1%) and dog (n = 114, IgG 15.8%, IgM 11.4%, both 21%) populations. We also found antibodies to *T. cruzi* (n = 80, IgG 10%, IgM 15%, both 17.5%) in dogs from Toluca, an area previously considered free of *T. cruzi*. Our data demonstrate the need for active epidemiologic surveillance programs in these regions. A direct correlation (r^2 = 0.955) of seropositivity between humans and dogs suggests that seroanalysis in dogs may help identify the human prevalence of *T. cruzi* infection in these areas.

Trypanosoma cruzi, which causes Chagas disease, affects ≈17.4 million people in the Western Hemisphere (*1*). The first case of human infection with *T. cruzi* in Mexico was reported in 1936 (*2*). A national serosurvey from 1987 to 1989 reported a seroprevalence of 1.6% (»1.6 million people) and widespread *T. cruzi* infection in the inhabitants of 23 of the 32 provinces of Mexico (*3,4*). A similar prevalence of *T. cruzi*–specific antibodies (1.5%) was observed in national blood bank repositories (*5*). Rural Mexican villages were confirmed as endemic zones for *T. cruzi*. Other investigators reported ≤20% seropositivity in inhabitants of rural areas south of the Tropic of Cancer (*4,6,7*).

In 1992, the State of Mexico was documented to be free of *T. cruzi* (n = 2,800 seropositive, <0.2%) (*3*). Another survey of 3,300 blood donors in Mexico City identified a seropositive rate of 0.3% (*8*), and many of these donors had no history of traveling to disease-endemic areas. Other studies from 1998 to 2000 reported acute cases of *T. cruzi* infection and seropositivity among inhabitants of the State of Mexico (*9* and C. Guzman-Bracho, unpub. data).

Circulation of *T. cruzi* is maintained by the interaction of bloodsucking triatomines with humans and reservoir animal hosts (*1*). Of the 31 triatomine species identified in Mexico, *Triatoma barberi*, *Triatoma dimidiata*, and *Triatoma pallidipennis* have the highest vectorial activity in central and southern Mexico (*4,10,11*). An entomologic survey in the spring of 2001 documented widespread distribution of *T. pallidipennis* in the southern part of the State of Mexico (infestation index 9.9%, density index 2.7%–3.0%) and suggested that active transmission of *T. cruzi* may occur (*12*).

Dogs are considered important in the dynamics of *T. cruzi* infection of triatomines and transmission within human dwellings (*1,13,14*). Seropositive domestic and stray dogs have been found in some states of Mexico (*15–17*). However, the prevalence of *T. cruzi* in dogs and the role of these reservoir animals in parasite transmission in the State of Mexico have not been determined.

In this study, we report the seroprevalence of *T. cruzi* among persons and dogs in the villages in the southern part of the State of Mexico and discuss the potential diagnostic meaning of seropositivity in dogs for identifying seroprevalence in humans. We also present data suggesting the likelihood of *T. cruzi* transmission in Toluca. Our observations emphasize that relevant health agencies need to conduct active epidemiologic surveillance programs and implement vector control strategies in the State of Mexico.

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Materials and Methods

Parasites

T. cruzi epimastigotes were cultivated as previously described (*18*). Epimastigote (Mexican isolates) antigen extract was used for the serologic tests conducted at the Instituto de Diagnostico y Referencia Epidemiologicos Secretaría de Salud (InDRE) Mexico City. Trypomastigotes (SylvioX10/4) were propagated in monolayers of C2C12 cells (*19*) and used in the studies at the University of Texas Medical Branch (UTMB) in Galveston.

Study Area, Population, and Sample Collection

This study was conducted in southern villages of the Tejupilco municipality, State of Mexico (Figure 1). The area has seasonal climate variations (dry season from November through May and rainy season from June through October). The population is primarily indigenous, and the main occupations are agriculture and livestock production. Migration occurs among the men to cities in Mexico and to northern border regions near the United States.

For the human serosurvey (N = 356), we selected 5 villages (altitude range 1,090–1,730 m) where triatomine infestation was reported by the Instituto de Salud del Estado de Mexico (ISEM) in >50% of the households. For comparison, we also selected La Comunidad village (altitude 2,500 m) in the same area. Since this study focused on evaluating active *T. cruzi* transmission, most test samples (>94%) were from children (age range 2–15 years) with a sex distribution consistent with the regional and national census. Sample randomization was controlled by using EpiInfo version 3.3.2 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Oral informed consent was obtained from adults and parents of minors enrolled in the study. Trained ISEM personnel performed venipuncture to obtain blood samples. The study was reviewed and approved by the human subjects committees at ISEM and UTMB.

Dog serum samples were collected in Toluca and the villages selected for human screening. Toluca, the capital of the State of Mexico (altitude 2,680 m, average temperature 15°C, range 5°C–24°C) is considered free of vectorial *T. cruzi* transmission because triatomines (with or without *T. cruzi*) have never been documented in the area, and triatomines are believed not to proliferate at altitudes >2,500 m (*3,4*). Serum samples from 20 healthy dogs from an animal clinic in Hamburg, Germany, were used as negative controls. All animal experiments were reviewed and approved by the animal welfare committee at ISEM.

Serologic Analysis

At UTMB, human and dog serum samples were screened for antibodies to *T. cruzi* by enzyme-linked immunosorbent assay (ELISA) as previously described (*20,21*). All samples and controls were assayed in triplicate in at least 2 independent experiments. Seropositive samples were confirmed by immunofluorescence flow cytometry (IFC) (*21*), and data were expressed as the relative percentage of positively fluorescent parasites.

Figure 1. Study site in Mexico. A) Country of Mexico. B) State of Mexico. C) Southern part of the State of Mexico. Shown are the municipalities and villages in the State of Mexico where epidemiologic serosurveys were conducted.

At InDRE, serum samples were analyzed for immunoglobulin G (IgG) antibodies to *T. cruzi* by ELISA, an indirect hemagglutination (IHA) test, and an indirect immunofluorescence (IIF) assay. For the ELISA, 96-well, flat-bottomed plates were UV irradiated, incubated for 1 h at 37°C with epimastigote antigen extract, and blocked with 50 µL Tris-buffered saline, 0.1% Tween 20, and 5% nonfat dry milk. Plates were incubated at 37°C with 50 µL of each test serum sample (1:50 dilution) for 2 h, horseradish peroxidase–conjugated IgG (1:50 dilution) for 1 h, and substrate (o-phenylenediamine) for 20 min. The reaction was stopped by adding 2 N H_2SO_4, and the optical density (OD) was read at 490 nm (*22*). The IHA and IIF assays were performed with 4-fold serial dilutions of serum samples (range 1:8–1:128) (*5*), and samples were considered seropositive when a strong signal was obtained at a dilution $\geq$1:16. Epimastigote antigen extract was not used to determine IgM seropositivity for *T. cruzi* in this study because it has shown limited sensitivity (*23,24*).

Statistical Analysis

Significance ($p<0.05$) was determined with the Student *t* test and validated with the Fisher exact test. The level of agreement for serologic data from 5 tests conducted at UTMB and InDRE was assessed as previously described (*25*).

Results

Standardization of Serologic Assays

Results of the trypomastigote-based ELISAs for IgG and IgM antibodies in positive and negative samples are shown in Figure 2. Variations in reactivity of negative and positive sera in different experiments and the same experiment ranged from 3% to 12%. The highest signal-to-noise ratios between positive and negative controls from humans (IgG 4.0, 6.5, 9.1 and IgM 3.6, 5.5, 11.3) were obtained at dilutions of 1:80, 1:160, and 1:320, respectively (Figure 2A). Thirty-five serum samples and pooled negative samples (1:100 dilution) were analyzed by ELISA (Figure 2A). Mean cutoff OD values were 0.194 for IgG and 0.270 for IgM.

The highest signal-to-noise ratios by ELISA between positive and negative controls from dogs (IgG 4.3, 7.9, 8.8 and IgM 2.4, 3.0, 3.5) were obtained at dilutions of 1:80, 1:160, and 1:320, respectively (Figure 2B). Cutoff OD values of 0.288 for IgG and 0.219 for IgM were obtained with serum samples from uninfected dogs in Mexico and Germany.

All serum samples were analyzed at a 1:100 dilution by ELISA and IFC. The highest signal-to-noise ratio for detection of antibody to trypomastigote surface antigens by IFC was obtained with 5×10^5 parasites/reaction, which was also reported in other studies (*21*,*26*). Positive and negative control peaks were distinguishable: >90% of trypomastigotes incubated with negative sera (1:100 dilution) had a fluorescence intensity (LFI) <10 and 50%–98% of trypomastigotes incubated with positive sera (1:100 dilution) had an LFI of 10^2–10^3 (Figure 3).

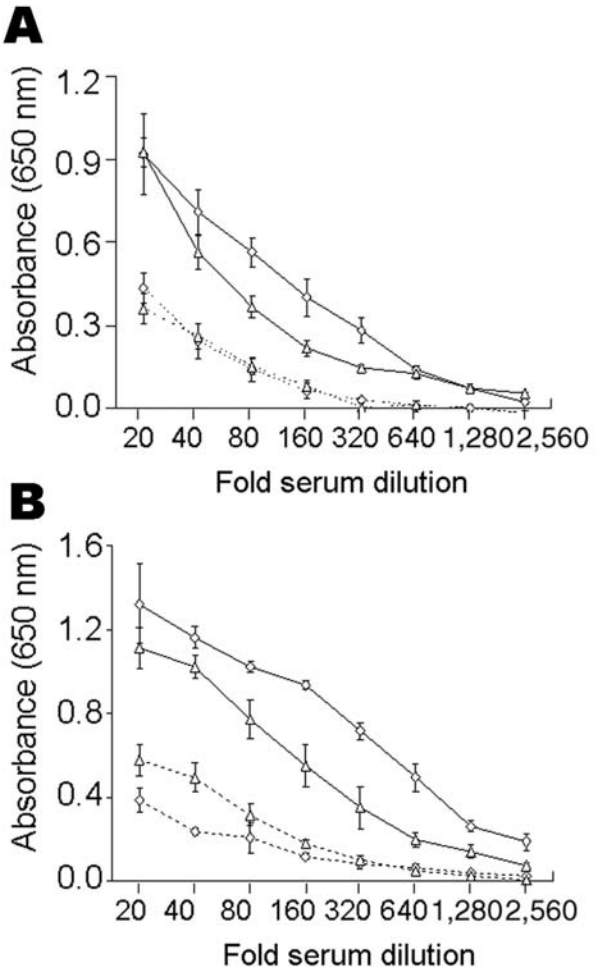

Figure 2. Serum titration curves of enzyme-linked immunosorbent assays comparing absorbance values for serial dilutions of pooled positive (solid lines) and negative (dashed lines) control sera from humans (A) and dogs (B). Absorbance values for immunoglobulin G (IgG) and IgM antibodies to *Trypanosoma cruzi* are represented by triangles and diamonds, respectively.

Seroprevalence of *T. cruzi*–specific Antibodies in Humans

At UTMB, we identified 9 seropositive persons (mean seroprevalence 3.1%, range 0%–21%) from the villages of Tejupilco municipality. The mean OD value, after subtracting for background, for the seropositive population was 0.213, and the highest value was 0.419. IgG seropositivity was similarly distributed among men and women (55% vs. 45%). All seropositive samples identified by ELISA were positive for *T. cruzi*–specific IgG by IFC. Approximately 73% of the trypomastigotes (range 62%–91%) incubated with positive serum samples had an LFI of 10 to 500. Trypomastigotes (>98%) incubated with negative serum samples had an LFI <10 (Figure 3). The results of ELISA and IFC for detection of *T. cruzi*–specific antibodies showed 100% agreement. *T. cruzi*–specific IgG was also detected by ELISA, IIF, and IHA in a blind study at InDRE. This study identified 7 seropositive human patients, of whom 6 were positive by UTMB tests (Table 1). The maximal percentage seropositivity was identified in El Carmen Ixtapan and El Puerto del Salitre, which are located at low altitudes (Table 2). All 63 persons tested in La Comunidad were seronegative (Table 2).

Our data showed that 16 (5.5%) of 293 persons in Tejupilco were seropositive for IgM antibodies to *T. cruzi* (Table 2). The prevalence of IgM antibodies was higher in female than in male patients (64% vs. 36%). All serum

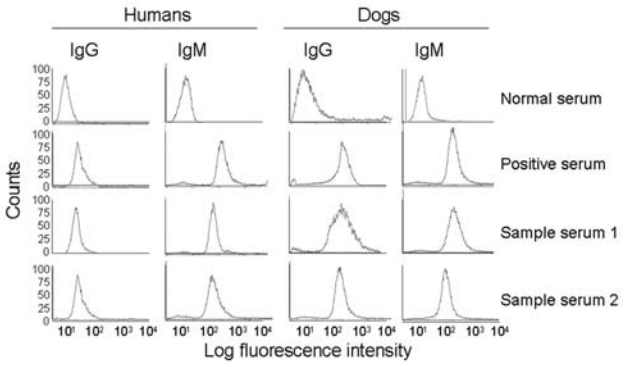

Figure 3. Detection of antibodies to *Trypanosoma cruzi* by immunofluorescence flow cytometry. Fluorescein isothiocyanate fluorescence intensities for *T. cruzi*–specific immunoglobulin G (IgG) and IgM antibodies in human and dog serum samples are shown. Background staining with normal serum, positive staining with chronic serum, and representative staining with 2 of the test serum samples are shown.

samples positive by ELISA for IgM antibodies were also positive by IFC (50%–93% of the parasites with an LFI of 10^2–10^3) (Figure 3). The overall prevalence of *T. cruzi*–specific antibodies (IgG and IgM) in persons in Tejupilco was 7.1% (21/293) (Table 2).

Seroprevalence of *T. cruzi*–specific Antibodies in Dogs

We used dog serum samples from Chiapas, where *T. cruzi* infection and transmission were reportedly endemic (*7*), as positive controls. These dogs had a seropositivity of 39.3% for IgG and 14.3% for IgM (Table 3 and Figure 4B). Dogs in Tejupilco had antibodies to *T. cruzi* (IgG 15.8%, IgM 11.4%, IgG and IgM 21.0%) (Table 3). A total of 6.1% of the dogs from Tejupilco were positive for both IgG and IgM (Figure 4C), and no sex-related differences in prevalence of parasite-specific antibodies were observed. IgG seropositivity increased with age, with the highest seroprevalence in dogs 3–6 years of age. All samples seropositive by ELISA were seropositive by IFC. A total of 57% to 94% of the parasites showed IgG-specific staining (LFI 10^2–10^4), and 86%–98% showed IgM-specific staining (LFI 100 to 4×10^3) (Figure 3). Samples seropositive for IgG were confirmed by IHA (data not shown). None of the serum samples from dogs in northern villages (Apaxco, Hueypoxtla, Jaltenco, and Nextlalpan) in the State of Mexico or the German veterinary clinic (Figure 4A) had *T. cruzi*–specific antibodies. This result demonstrated the specificity and sensitivity of the assays used. Pairwise linear analysis showed a positive correlation of IgG seropositivity in dogs and humans in study area ($r^2 = 0.955$). Parasite-specific antibodies (IgG 10%, IgM 15%, IgG and IgM 17.5%) were detected in dogs from Toluca (Table 3 and Figure 4D), a region previously considered free of *T. cruzi* infection.

Discussion

We detected *T. cruzi*–specific IgG and IgM in 7.1% of persons and 21.0% of dogs in Tejupilco. In addition, we observed an IgG and IgM seroprevalence of 17.5% in dogs in Toluca, which was previously reported to be free of *T. cruzi* infection. Epimastigote and trypomastigote antigens and 5 different tests were used to determine IgG seropositivity in selected areas. Nine of 293 IgG-positive patients were identified by trypomastigote-based tests, of whom 6 were also identified by ≥2 of the epimastigote-based tests at InDRE. Low IgG seropositivity in the InDRE survey might have occurred because epitopes shared by epimastigote and trypomastigote forms are intracellular antigens, whereas the IgG antibodies are specific for trypomastigote surface antigens (*27*). Alternatively, low seropositivity may be attributed to different parasite strains used for serologic tests at the 2 institutes. A positive correlation between IgG seropositivity in humans and dogs ($r^2 = 0.955$) implies that dogs help identify or monitor seroprevalence in these populations.

Serologic analysis has been reported to be limited in acute infections with *T. cruzi* (*23*,*24*). With trypomastigote antigens, we detected an IgM seroprevalence of 5.5% in humans, thus demonstrating that a robust *T. cruzi*–specific IgM response is mounted by humans. In addition, most of the IgM-positive persons were IgG negative. These data,

Table 1. Prevalence of immunoglobulin G (IgG) antibodies to *Trypanosoma cruzi* in persons in southern area of the State of Mexico*†

No. positive test results‡	Total screened		Tejupilco		Temascaltepec	
	No. (%)	% of population	No. (%)	% of population	No. (%)	% of population
0	321 (90.1)		268 (91.5)		55 (87.31)	
1	22 (6.2)		14 (4.78)		6 (9.52)	
2	7 (2.0)	98.3	5 (1.7)	97.95	2 (3.17)	100
3	**1 (0.28)**		**1 (0.35)**		0	
4	**2 (0.56)**		**2 (0.68)**		0	
5	**3 (0.85)**	**1.7**	**3 (1.02)**	**2.05**	0	0.0
Total	356 (100)		293 (100)		63 (100)	

*Samples positive in ≥3 tests were considered seropositive (shown in **boldface**).
†All except 2 participants seropositive for IgG antibodies by ≥2 tests were 5–18 y of age.
‡Level of agreement for serologic data for 5 tests: concordance level 98.2; κ coefficient 0.618; 95% confidence interval 0.622–0.714.

Table 2. Prevalence of antibodies to *Trypanosoma cruzi* in persons in southern area of the State of Mexico*

Municipality	Village	Altitude (m)	Seropositivity†, no. (%)			
			No. screened	IgG positive	IgM positive‡	IgG and IgM positive
Tejupilco	El Carmen Ixtapan	1,091	16	1 (6.3)	3 (18.7)	4 (25.0)
	El Puerto del Salitre	1,268	29	3 (10.3)	ND	3 (10.3)
	Zacatepec	1,311	200	2 (1.0)	10 (5.0)	11 (5.5)
	Rio Grande	1,554	3	0	ND	0
	Tenería	1,730	45	0	3 (6.6)	3 (6.6)
Subtotal			293	6 (2.05)	16 (5.5)	21 (7.1)
Temascaltepec	La Cominidad	2,500	63	0	1 (1.6)	1 (1.6)

*IgG, immunoglobulin G.
†p<0.001 for IgG, IgM, and IgG plus IgM seropositivity.
‡All IgM-seropositive persons were 4–13 y of age. ND, not determined.

along with the observation of high IgM seropositivity (11.4%) in dogs from the same area, suggest the occurrence of acute *T. cruzi* infection in rural villages in the State of Mexico. This view is supported by Wickner et al., who reported polymerase chain reaction–based detection of *T. cruzi* in blood samples from patients with acute infection identified as IgM seropositive by ELISA (agreement 94%) (*28*).

Our study identified seropositive patients (7.1%) and high seroprevalence (21.0%) in dogs from southern villages in the State of Mexico, which has a low altitude (<1,700 m) and warm temperatures. Nearly all inhabitants screened in the study had dogs that lived near their owners in small quarters, and we observed a correlation between seropositivity in dogs and humans in these communities.

Previous reports showed infestation with *T. barberi* and *T. pallidipennis* at low altitudes (<2,000 m) in all areas of Mexico (*4*) and inside and around houses in the southern part of the State of Mexico (*12*). Dogs provide frequent blood meals for *T. barberi* and *T. pallidipennis* and may acquire *T. cruzi* infection by ingesting infected triatomines. We surmise that the active transmission of *T. cruzi* occurs in the southern part of the State of Mexico, and the presence of *T. cruzi* in dogs and insect vectors can help determine the prevalence of *T. cruzi* infection in humans. Thus, low altitudes and warm temperatures may sustain vectorial activity and *T. cruzi* transmission in southern Mexico. Several observations support our hypothesis. First, dogs maintain parasitemia long after infection (*29*) and are the preferred source of blood meals for *Triatoma infestans* (*30*). Second, the prevalence rate of infective *T. infestans* in a household increases with the number of infected dogs in the vicinity (*13*). In accordance with the increase in infected insects, the seroprevalence of infected adults doubled in households with 1 to 2 infected dogs (*14*). Third, *T. cruzi*–specific antibodies have been identified in humans (4%) and dogs (10%) in rural villages of Puebla, Mexico, where active vectorial transmission, shown by a high dispersion area index (55%) and colonization index (40%), was also noted (*17*).

We observed a high seroprevalence of IgG and IgM antibodies (17.5%) in dogs from Toluca. Additional studies would determine whether changes in behavior and localization of triatomines at higher altitudes may lead to *T. cruzi* infection in dogs in Toluca. Alternatively, a high rate of migration from endemic to nonendemic zones exists in Mexico. At institutional blood banks, ≈40% of donors reported to be permanent residents of Mexico City were born in other states of Mexico (C. Guzman-Bracho, unpub. data). These immigrants bring their domestic animals with them, and thus may inadvertently contribute to the spread of *T. cruzi* infection. Our detection of *T. cruzi* in dogs from Toluca suggests that this city and others in Mexico located at high altitudes may not be free of *T. cruzi* infection.

Table 3. Prevalence of antibodies to *Trypanosoma cruzi* in dogs in the southern area of the State of Mexico

Municipality	Village	No. screened	Seropositivity,* no. (%)		
			IgG positive†	IgM positive‡	IgG and IgM positive
Tejupilco	El Carmen Ixtapan	16	5 (31.3)	0	5 (31.3)
	Rincon del Carmen	42	10 (23.8)	13 (30.9)	16 (38.0)
	Rio Grande	24	1 (4.2)	0	1 (4.2)
	Tejupilco	10	1 (10.0)	0	1 (10.0)
	Zacatapec	22	1 (4.5)	0	1 (4.5)
Subtotal		114	18 (15.8)	13 (11.4)	24 (21.0)
Toluca		80	8 (10.0)	12 (15.0)	14 (17.5)
Northern area§		24	0	0	0
Chiapas		28	11 (39.3)	4 (14.3)	12 (42.8)

*IgG, immunoglobulin G. p<0.001 for IgG, IgM, and IgG plus IgM seropositivity.
†IgG-seropositive dogs were 8 mo to 6 y of age; 85% were ≥2 y of age.
‡IgM-seropositive dogs were 4 months to 6 years of age; a similar distribution was observed in all age groups.
§Northern villages of Apaxco, Hueypoxita, Jaltenco, and Nextlalpan were included in this group.

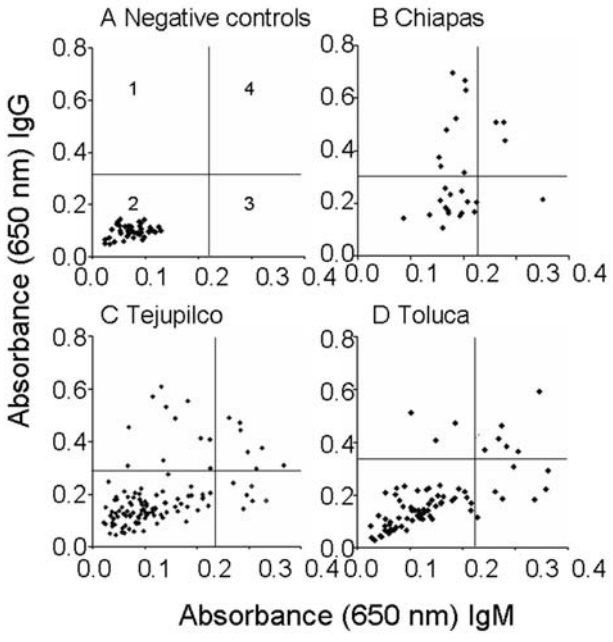

Figure 4. Distribution of immunoglobulin G (IgG) and IgM antibodies to *Trypanosoma cruzi* in dogs. An enzyme-linked immunosorbent assay was used to detect antibodies in dogs in Tejupilco (C) and Toluca (D) in the State of Mexico. Negative controls are shown in A. Seroanalysis of dogs from Chiapas, a *T. cruzi*–endemic zone, is shown in B. The quadrants in A indicate the following: 1, IgG positive; 2, IgG and IgM negative; 3, IgM positive; 4, IgG and IgM positive.

The seroprevalence of 21.0% (IgG and IgM) in dogs and the observed vectorial activity in these areas suggest that dogs may be domestic reservoir hosts and help maintain human transmission of *T. cruzi*. Our observations emphasize the importance of active epidemiologic surveillance programs throughout Mexico and implementation of sound vector control strategies in disease-endemic areas.

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etymologia

measles

[mēˈzəlz]

Highly contagious disease caused by a virus of the genus *Morbillivirus*, marked by an eruption of distinct, red, circular spots. From the Middle Dutch *masel*, "blemish." References to the disease date back to at least 700 AD, but the first recorded scientific description of measles was in the 10th century AD by the Persian physician Ibn Razi, who described it as "more dreaded than smallpox." Prior to 1963, when the first measles vaccine was licensed, 3–4 million cases and 450 deaths occurred in the United States every year. Measles remains a primary cause of death in developing nations, where vitamin A deficiency is common. According to the World Health Organization, measles is the leading cause of vaccine-preventable death in children; it is responsible for ≈850,000 deaths each year.

Sources: Merriam-Webster's collegiate dictionary. 11th ed. Springfield (MA): Merriam-Webster Incorporated; 2003; Centers for Disease Control and Prevention. Measles history. Available from http://www.cdc.gov/nip/diseases/measles/history.htm; and World Health Organization. Measles mortality reduction and regional elimination. Available from http://www.who.int/vaccines-documents/DocsPDF01/www573.pdf

Contrasting Pediatric and Adult Methicillin-resistant *Staphylococcus aureus* Isolates

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We performed a prospective study of all inpatient and outpatient methicillin-resistant *Staphylococcus aureus* (MRSA) isolates identified at the University of Chicago Hospitals from November 2003 through November 2004. Differences in resistance to non–β-lactam antimicrobial drugs were determined after stratification of the 578 MRSA isolates into 4 groups by patient age (pediatric vs. adult) and onset location (community vs. hospital). Non–β-lactam resistance was significantly greater among the 288 adult than the 177 pediatric community-associated isolates for erythromycin (93.2 vs. 87.0%, p = 0.03), clindamycin (51.8 vs. 7.3%, p<0.001), ciprofloxacin (62.1 vs. 10.7%, p<0.001), gentamicin (11.1 vs. 1.1%, p<0.001), and tetracycline (19.9 vs. 6.4%, p<0.001). In contrast, hospital-associated MRSA isolates from children and adults had similar rates of non–β-lactam antimicrobial drug resistance. In our region, clindamycin is an appropriate empiric therapy of community-associated MRSA infection in children but should be used with caution in adults.

Colonization by and infection with methicillin-resistant *Staphylococcus aureus* (MRSA) in children and adults who have little or no contact with the healthcare system, phenomena almost unknown before the mid-1990s, have been reported with increasing frequency (*1–34*). In some cities and rural areas, rates of these community-associated MRSA (CA-MRSA) infections, particularly in skin and soft tissue, are increasing rapidly (*1–4*). Many CA-MRSA infections have been attributed to isolates that are distinct from hospital-associated MRSA (HA-MRSA) strains.

The definition of CA-MRSA has not been standardized. No single definition would likely suffice for all purposes. Attempts to define CA-MRSA have focused upon a variety of criteria: temporal (i.e., the MRSA isolate from a specimen is submitted ≤72 hours after hospital admission), host-risk-factor profile (i.e., the patient with the MRSA isolate lacked certain exposure parameters relevant to healthcare facilities, variously defined), antimicrobial drug susceptibility of the MRSA isolates (e.g., susceptibility to clindamycin), and certain molecular aspects of the isolates (e.g., SCC*mec* type or pulsed-field gel electrophoresis type). Which definition is most important in the choice of empiric antimicrobial drug treatment is not clear. However, a clinician confronted with a putative *S. aureus* infection needs guidance.

The epidemiology of the emerging CA-MRSA strains, including their origins, reservoirs, modes of dissemination, and effective approaches to their control, is not completely understood. We previously noted that CA-MRSA isolates often lacked resistance to multiple non–β-lactam antimicrobial drugs when compared with HA-MRSA strains (*5,6*). This phenomenon has been documented in adults (*4,7*) and children (*8–10*). More recently, we anecdotally observed that children had MRSA infections caused by strains susceptible to non–β-lactam antimicrobial drugs more often than adults. Few data exist that compare antimicrobial drug resistance profiles of adult and pediatric MRSA infection isolates stratified by site of onset (hospital vs. community). Accordingly, using the temporal definition of CA-MRSA, we examined the hypothesis that these 2 groups may be infected by MRSA strains with different rates of resistance to non–β-lactam antimicrobial drugs when the isolates had onset in the community. Therefore, we compared several criteria for the temporal definition of CA-MRSA to assess the impact of changing these criteria on our findings. This article summarizes our study.

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Materials and Methods

The University of Chicago Hospitals (UCH) is a tertiary-care medical center on the south side of Chicago with 577 inpatient beds and 29,500 annual admissions. It includes an outpatient care facility with 379,000 annual visits. UCH serves an inner city population and draws tertiary referrals from the surrounding region.

The UCH Clinical Microbiology Laboratories prospectively identified all MRSA isolates collected at the medical center from November 7, 2003, to November 7, 2004, from inpatients and outpatients. *S. aureus* isolates were identified by gram stain, growth on BBL mannitol salt fermentation agar (Beckton, Dickinson and Company, Sparks, MD, USA), positive catalase test results, and a positive Staphaurex Plus test (Remel Europe Ltd., Dartford, UK) result.

The Clinical Microbiology Laboratories determined the susceptibility profile of each isolate by using the Vitek 2 system (bioMérieux Vitek, Inc., Durham, NC, USA) for methicillin, erythromycin, clindamycin, ciprofloxacin, rifampin, gentamicin, tetracycline, and vancomycin. From November 7, 2003, to July 2004, any isolate with an MIC of vancomycin >2 μg/mL reported by the Vitek system was retested for vancomycin susceptibility by the Etest Strip (AB Biodisk, Solna, Sweden). After July 2004, the Etest was performed for these isolates only if growth occurred on a vancomycin agar screen plate. For isolates that tested resistant to erythromycin but susceptible to clindamycin, a D-test to detect inducible resistance to clindamycin was performed, as was a Kirby-Bauer disk-diffusion test for susceptibility to trimethoprim-sulfamethoxazole (TMP-SMX), both according to Clinical and Laboratory Standards Institute (CLSI) guidelines (*35*).

For each MRSA specimen, patient information was collected from the laboratory information system and the electronic medical record, including the date of specimen procurement, age and sex of the patient, location of specimen procurement (inpatient, outpatient, emergency department), and antimicrobial drug susceptibility profile. For inpatient isolates, the number of days from admission to culture procurement was determined by subtracting the admission date from the procurement date.

We defined CA-MRSA to include all isolates cultured from outpatients and isolates from hospitalized patients obtained within 72 hours of admission. We also examined the impact of changing the 72-hour criterion in the definition to 24 and 48 hours. With each change in definition, the effect on the percentage of MRSA infections considered to be community associated was assessed.

The bacteriologic and patient information was compiled in an electronic database designed for the study by using Access software (Microsoft, Redmond, WA, USA). Only the first isolate from each patient collected during the surveillance was included in the database. The study was approved by the institutional review board of the Biological Sciences Division of the University of Chicago. Data were analyzed with Stata, version 8.0 (Stata Corp, College Station, TX, USA). Comparisons between groups were performed by the χ^2 test or the Fisher exact test. All hypotheses were 2-tailed and were considered significant if $p<0.05$.

Results

The UCH Clinical Microbiology Laboratories identified 1,149 MRSA-positive cultures from 578 patients. Of these, 201 (34.7%) were from children ≤18 years of age and 377 (65.3%) were from adults ≥19 years of age. Of the adult MRSA isolates, 27.9% were cultured from outpatients; of the pediatric MRSA isolates, 57.2% were cultured from outpatients. The median age of pediatric patients was 3 years (16 days to 18 years), with a median of 6 years among outpatients and 2 years among inpatients. For adults, the median age was 56 years (range 19–100), with a median of 51 years among outpatients and 60 years among inpatients.

Using the 72-hour definition, 64.3% of adult and 72.1% of pediatric inpatient isolates were CA-MRSA ($p = 0.2$) (Table 1). Restricting the definition of CA-MRSA to the 48-hour definition, 58.5% of adult and 69.8% of pediatric inpatient isolates were CA-MRSA ($p = 0.06$). Fifty-four percent and 65.1%, respectively, of adult and pediatric inpatient isolates met the criteria for the 24-hour definition of CA-MRSA ($p = 0.07$). Thus, by using the 24-, 48-, or 72-hour criteria for CA-MRSA, most of the pediatric and adult inpatient isolates would be considered community-associated.

Combining the isolates cultured in the emergency department with those cultured in clinics, 105 outpatient isolates were obtained from adults and 115 from children. Adult isolates were substantially more likely than pediatric isolates to be resistant to ciprofloxacin, clindamycin, gentamicin, and tetracycline. No difference was seen in resistance rates for erythromycin or rifampin (Table 2).

A small percentage of the temporally defined CA-MRSA isolates (i.e., those either from outpatients or cultured <72 hours after admission from inpatients) was resistant only to β-lactam antimicrobial drugs; this resistance pattern was more common among pediatric (9.6%) than adult (3.6%) isolates ($p = 0.001$) (Table 3). Among the temporally defined CA-MRSA isolates, those from adults were more likely than those from children to be resistant to most of the non–β-lactam antimicrobial drugs. The rate of resistance to erythromycin was high among both the pediatric and adult isolates, although significantly higher among the adult isolates. The rate of resistance to clindamycin, ciprofloxacin, gentamicin, and tetracycline was much higher for adult than for pediatric CA-MRSA

Table 1. Inpatient adult and pediatric methicillin-resistant *Staphylococcus aureus* isolates by procurement time after admission*

Time after admission, h	Adult inpatient isolates (%), n = 272	Pediatric inpatient isolates (%), n = 86	p value†
≤24	147 (54.0)	56 (65.1)	0.07
≤48	159 (58.5)	60 (69.8)	0.06
≤72	175 (64.3)	62 (72.1)	0.18

*Values are number (%).
†p value comparing adult and pediatric isolates for each category of time after admission, χ^2 test.

isolates (Table 3). There was no significant difference for rifampin, TMP-SMX, or vancomycin.

The D-test for inducible clindamycin resistance was performed on 97% (112/116) of adult and 89% (125/142) of pediatric CA-MRSA isolates that were resistant to erythromycin and susceptible to clindamycin. Among those tested, adult (20.5%, 23/112) isolates were more likely than pediatric (15.2%, 19/125) isolates to have a positive D-test result (Table 3).

In contrast to the differences we found among CA-MRSA isolates from adults and children, however, the hospital-associated MRSA isolates (>72 hours after admission) had similar rates of antimicrobial drug resistance (Table 3). For example, clindamycin resistance occurred in 74.2% of adult and 75.0% of pediatric HA-MRSA isolates (p = 0.9). Only the rates of ciprofloxacin (p = 0.004) and gentamicin (p = 0.01) resistance were substantially different when the HA-MRSA isolates were compared (Table 3).

We defined a multidrug-resistant (MDR) MRSA isolate as being resistant to ≥3 of the non–β-lactam antimicrobial drugs tested. When the temporal definition (<72 hours after admission) was used, adult CA-MRSA isolates were more likely than pediatric CA-MRSA isolates (52.3% vs. 6.4%, p<0.0001) to be MDR (Table 3). HA-MRSA isolates from both children and adults were more often MDR, but the rates did not differ substantially (76% vs. 66.7%, p = 0.4) (Table 3).

Discussion

When community association or onset was defined by using the temporal criterion of procuring isolates ≤72 hours after admission, most adult and most pediatric MRSA isolates in our study would be considered to have a community origin. A narrower procurement definition, e.g., ≤24 hours or ≤48 hours, would have led to the same conclusion.

MRSA isolates from children's specimens compared with isolates from adults' specimens obtained ≤72 hours after admission were more likely to be resistant to only β-lactams and more likely to be susceptible to clindamycin, ciprofloxacin, and gentamicin. These differences suggest that there may be distinct CA-MRSA isolates colonizing and infecting children and adults in the population served by our medical center or, less likely, that there may be a reservoir of CA-MRSA that affects children and adults differently.

Children may have unique risk factors for MRSA colonization in the community related to their environment, such as daycare centers (*11*,*12*), schools (*13*), or recreational facilities, and may have different behavioral habits than most adults. Alternatively, children may be different from adults as hosts, and they may encounter novel MRSA strains either by different colonization of the skin or by some undefined difference in host defense. Antimicrobial drugs used to treat infections among children may differ from those used among adults; perhaps distinct MRSA strains colonizing children result from differential antimicrobial drug selection pressures in the community.

Table 2. Percentage of methicillin-resistant *Staphylococcus aureus* isolates resistant to non–β-lactam antimicrobial drugs among pediatric and adult patients, stratified by hospital- and community-associated designation

Antimicrobial drug	Adult outpatient isolates (n = 105)	Pediatric outpatient isolates (n = 115)	p value*
Ciprofloxacin	55.2	9.6	<0.001
Clindamycin			
Resistant†	37.1	6.1	<0.001
D-test positive‡	20.4	14.8	0.39
Erythromycin	91.4	88.7	0.16
Gentamicin	8.6	1.7	0.03
Rifampin	1.9	0	0.23
Tetracycline§	13.3	3.6	0.01
TMP-SMX¶	0	0	NA
Vancomycin	0	0	NA

*p value compares resistance to indicated antimicrobial drugs or positive test result among adult vs. pediatric isolates by χ^2 test or Fisher exact test. NA, not applicable.
†Figures in this row represent Vitek testing results for clindamycin.
‡54 (97.1%) and 88 (93.9%) of the adult and pediatric isolates, respectively, that were erythromycin resistant and clindamycin susceptible by Vitek were evaluated by D-testing.
§Tetracycline susceptibility was not tested for 5 pediatric outpatient isolates and 2 adult outpatient isolates.
¶TMP-SMX, trimethoprim-sulfamethoxazole. Only 42 adult and 27 pediatric isolates that were erythromycin resistant and clindamycin susceptible were tested.

Table 3. Percentage of methicillin-resistant *Staphylococcus aureus* (MRSA) resistant to non–β-lactam antimicrobial drugs among pediatric and adult patients, stratified by hospital- and by community-associated designation

In addition to methicillin,* % resistant to	Adult community-associated, % (n = 280)	Pediatric community-associated, % (n = 177)	p value†	Adult hospital-associated, % (n = 97)	Pediatric hospital-associated, % (n = 24)	p value‡
No other antimicrobial drugs§	3.6	9.6	0.001	3.1	4.2	0.99
Ciprofloxacin	62.1	10.7	<0.001	87.6	62.5	0.004
Clindamycin						
Resistant¶	51.8	7.3	<0.001	74.2	75.0	0.94
D-test positive#	20.5	15.2	0.29	50	0	0.48
Erythromycin	93.2	87.0	0.03	93.8	95.8	0.99
Gentamicin	11.1	1.1	<0.001	14.4	37.5	0.01
Rifampin	1.8	0	0.16	1.0	0	0.99
Tetracycline**	19.9	6.4	<0.001	13.5	8.3	0.73
TMP-SMX††	0	0	NA	0	0	NA
Vancomycin	0	0	NA	0	0	NA
≥3 non–β-lactam antimicrobial drugs	52.3	6.4	<0.001	76.0	66.7	0.35

*Methicillin resistance inferred from oxacillin resistance test.
†p value compares community-associated (CA) adult and pediatric isolates for resistance to each antimicrobial drug or test, χ^2 or Fisher exact test. NA, not applicable.
‡p value compares hospital-associated (HA) MRSA adult and pediatric isolates for resistance to each antimicrobial drug or test, χ^2 or Fisher exact test.
§Includes erythromycin, clindamycin, ciprofloxacin, gentamicin, rifampin, and tetracycline and does not include D-test–positive results.
¶Data in this row represent single-agent Vitek testing results for clindamycin.
#112 (97%) of the adult CA-MRSA isolates, 125 (89%) of the pediatric CA-MRSA isolates, 18 (95%) of the adult HA-MRSA isolates, and 2 (67%) of the pediatric HA-MRSA isolates that were erythromycin resistant and clindamycin susceptible by Vitek were evaluated by D-testing.
**Nine isolates not tested for susceptibility to tetracycline were omitted.
††TMP-SMX, trimethoprim-sulfamethoxazole. Only 100 adult CA-MRSA, 121 pediatric CA-MRSA, 15 adult HA-MRSA, and 2 pediatric HA-MRSA isolates that were clindamycin susceptible and erythromycin resistant were tested for TMP-SMX susceptibility.

Our findings have obvious implications for empiric therapy of CA-MRSA infections and MRSA control measures. In the population served by UCH, for children with suspected community-onset MRSA infections, clindamycin is an appropriate first-line empiric antimicrobial drug, at least for those who are not critically ill (*8,10*). In adults, by contrast, clindamycin would be much less suitable in this role, as most temporally defined CA-MRSA isolates are clindamycin-resistant. Therefore, among adults at our institution, clindamycin can be considered only after susceptibility testing results have been obtained from a culture specimen.

CA-MRSA isolates from children have a low rate of clindamycin resistance. Studies of children with CA-MRSA infections or colonization in many cities, including Houston, Corpus Christi, Memphis, Nashville, Louisville, Providence, and Chicago, have demonstrated high rates of clindamycin susceptibility among these isolates, ranging from 67%–100% (*1,8,10,14–16*). The 2 studies with the lowest susceptibility rates were conducted in Providence from 1997 to 2001 with a 74% rate (*8*), and Louisville from 1999 to 2001 with a 67% rate (*16*). These lower rates may have been due to geographic variation or were a window on an earlier phase in the CA-MRSA epidemic.

A possible confounder in calculating clindamycin resistance rates is the 2004 CLSI guideline stating that each isolate that initially tests susceptible to clindamycin and resistant to erythromycin should be tested for inducible clindamycin resistance by the D-test; if the test result is positive, that isolate should be considered resistant (*35*). Published data from before 2004 may not have included D-test results in reporting clindamycin resistance rates among MRSA isolates. However, the impact of the new CLSI guideline does not change the conclusions derived from our data, although the rate of clindamycin resistance among all pediatric MRSA isolates in our sample would increase from 15.4% to 24.9% when the new guideline was considered.

We have continued to use clindamycin for initial empiric therapy of mild or moderately ill pediatric patients likely to have a CA-MRSA infection. We take this approach because better alternative therapy is not available. The results of TMP-SMX therapy for CA-MRSA have been reported for relatively few patients (*36*); therapy was frequently unsuccessful despite in vitro susceptibility of the infecting MRSA strains. Tetracyclines are not suitable for young children, and linezolid is prohibitively expensive. Our policy is to abandon clindamycin when susceptibility testing or D-test results suggest the possibility of treatment failure, usually 2–4 days into the treatment course or, as mentioned, for severe illness when CA-MRSA is suspected. To date, this approach has been suitable.

The pattern of clindamycin resistance among adult CA-MRSA isolates has been more complex. At UCH, more than half of the CA-MRSA isolates from adults were resistant to clindamycin. Similarly, at Northwestern

Memorial Hospital, also in Chicago, from 1998 to 1999, few MRSA isolates were clindamycin susceptible among those that were collected <72 hours after admission from adults with no known hospitalizations in the previous 2 years (*37*).

In contrast, more recent studies of CA-MRSA infections and MRSA colonization isolates have demonstrated low rates of clindamycin and other non–β-lactam resistance among MRSA isolates from young and urban poor adults. For example, 9 of 67 patients with MRSA infections in a military-beneficiary population in Texas from 1999 to 2001 had no risk factors for HA-MRSA. These patients all had onset of infection in the community and had an isolate that was susceptible to clindamycin. Moreover, they were younger than the patients reported to have HA-MRSA infections (*4*). In San Francisco in 1999–2000, among asymptomatic, homeless and marginally housed adults in the community, 23 MRSA isolates were identified from nasal swabs of which only 2 (8.7%) were resistant to clindamycin (*17*). In a sample of 45 colonizing and infecting MRSA isolates collected from healthy young adults (18–44 years of age) training at a military facility in Texas, 22% were resistant to clindamycin (*7*).

Why these disparities exist in the rates of clindamycin susceptibility among CA-MRSA isolates obtained from adults is unclear. Possible explanations include regional variation or characteristics of the patient populations sampled. The 2 studies with high clindamycin resistance rates were both conducted in Chicago at tertiary medical centers. Community-based surveys among adults, in contrast, have demonstrated a high rate of clindamycin susceptibility, perhaps reflecting an exposure to a community MRSA isolate pool similar to the pool affecting pediatric populations in many urban centers.

Unlike clindamycin, erythromycin resistance has been common among both pediatric and adult CA-MRSA isolates. In hospitalized children with MRSA infections at UCH during 1988–1990 and 1993–1995, 74.3% carried isolates resistant to erythromycin (*5*). Our current rate, 88.1% in pediatric isolates, is slightly higher. Erythromycin resistance has also been common among CA-MRSA isolates colonizing and infecting children at many urban centers from 1990 to 2002, with rates from 52% to 100% (*8*,*10*,*14*,*16*). In adults, recent studies have shown similarly high rates of erythromycin resistance, 60.9%–80%, among CA-MRSA strains in selected populations, including soldiers (*7*), inmates in jail (*38*), and homeless and impoverished urban adults (*17*).

Analysis of antimicrobial resistance patterns among MRSA isolates obtained >72 hours after admission produced a sharp contrast with the CA isolates. Children and adults in this HA group were infected by MRSA strains with similar rates of resistance to non–β-lactam antimicrobial drugs. This finding suggests that adults and children face a common source of antimicrobial drug selection pressure and a common reservoir of MRSA isolates in our hospital, but not in the community.

Before this study, few data have been available to compare the resistance patterns of MRSA isolates collected in 1 medical center or region stratified by both age group (children vs. adults) and venue of onset (community vs. hospital). One previous analysis conducted from 1988 to 1997 at another medical center in Chicago compared adult and pediatric MRSA isolates and also found that MRSA strains susceptible to clindamycin were more common among pediatric than adult isolates; the isolates were not stratified by hospital or community origin (*18*). In contrast, among CA-MRSA isolates collected during 2000 from 12 Minnesota clinical microbiology laboratories serving both inpatients and outpatients, no differences in the rate of clindamycin susceptibility were found in isolates from children and adults (*19*). These investigators used a risk–factor-based definition for CA-MRSA, while we used a temporal definition. Other possible explanations for the discrepancy include a secular change in the 4 years separating the studies or demographic differences in the study populations.

Our study has certain limitations. It was conducted in 1 center and in 1 city, and it is unknown whether our data are representative of the CA-MRSA epidemic in other areas. Our study also assumes that adults and children seeking care in all areas of our medical center had an approximately equal chance of having a culture performed. We did not evaluate whether an assessment of risk factors for HA-MRSA would have determined the likelihood of resistance to non–β-lactam antimicrobial drugs.

With the recognition of CA-MRSA as a distinct epidemiologic phenomenon and the accompanying upsurge in both the *S. aureus* disease burden and methicillin resistance rates at many centers, considerable controversy has surrounded the definition most appropriate for CA-MRSA. Whether the best definition should be based on temporal, molecular, antimicrobial drug susceptibility, or host risk-factor criteria will not be resolved by this study and will continue to vary according to the issue being addressed. Despite these uncertainties, our data, using the temporal definition, have practical value in the initial antimicrobial drug management of patients with suspected CA-MRSA infections.

Understanding the complex epidemiology of CA-MRSA infection is critical to the development of control policies and treatment guidelines in areas with a high colonization and disease prevalence. Our data demonstrate the complexity of the contrasting definitions of CA-MRSA. While neither a time criterion alone nor a time criterion in addition to an antimicrobial drug susceptibility

profile is adequate to define CA-MRSA, our data provide valuable guidance for clinical practice.

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Identifying Influenza Viruses with Resequencing Microarrays

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Identification of genetic variations of influenza viruses is essential for epidemic and pandemic outbreak surveillance and determination of vaccine strain selection. In this study, we combined a random amplification strategy with high-density resequencing microarray technology to demonstrate simultaneous detection and sequence-based typing of 25 geographically distributed human influenza virus strains collected in 2004 and 2005. In addition to identification, this method provided primary sequence information, which suggested that distinct lineages of influenza viruses co-circulated during the 2004–2005 season, and simultaneously identified and typed all component strains of the trivalent FluMist intranasal vaccine. The results demonstrate a novel, timely, and unbiased method for the molecular epidemiologic surveillance of influenza viruses.

Influenza viruses are a major cause of respiratory infections in humans and result in substantial illness, death, and economic problems throughout the world. Along with regular seasonal epidemic outbreaks caused by common circulating strains, novel strains emerge sporadically because of reassortment in the segmented influenza RNA genome and have resulted in devastating influenza pandemics (*1*–*3*). Since mutations and reassortments are often determinants for infectious potential, antiviral drug susceptibility, and viral escape from vaccine-elicited immunity, continually surveying the genetic composition (i.e., primary sequence) of circulating and emerging variants is necessary. These needs have become increasingly relevant recently because the World Health Organization (WHO) has reported 85 human deaths caused by avian A/H5N1 influenza viruses throughout Asia since 2003 and raised concerns about the potential for another influenza pandemic (*4*).

Automated Sanger/electrophoresis-based sequencing technology has been used as the standard platform for DNA and genome sequencing. Although conventional sequencing produces accurate data, the requirement for knowledge of template sequences and the inability to quickly process multiple targets hinder its practical application in epidemiologic and diagnostic investigations. As an alterative, high-density oligonucleotide resequencing microarrays represent a promising new technology that has been used to rapidly and accurately identify nucleotide sequence variants (*5*–*7*) from viral, bacterial, and eukaryotic genomes (*8*–*13*). Use of resequencing microarrays to detect single nucleotide polymorphisms and generate primary sequences enables identification of genetic variants and provides valuable epidemiologic information that is critical for outbreak surveillance. In most cases, however, this technology has relied on specific amplification of a limited number of target sequences before hybridization, thus restricting throughput and limiting final identification to strains that retain primer-targeted sequences.

In an attempt to adapt resequencing microarray technology to surveillance and diagnostics, we developed the respiratory pathogen microarray (RPM) version 1 for detection and sequence typing of 20 common respiratory and 6 category A biothreat pathogens known to cause febrile respiratory illness (*14*). A large portion of RPM version 1 is focused on a subset of tiled sequences corresponding to partial fragments from the hemagglutinin (HA), neuraminidase (NA), and matrix (M) genes for detection of influenza A and B viruses. In this study, we demonstrate unbiased determination of viral subtype and lineage by generation of primary sequence using random nucleic acid amplification and resequencing microarray technology.

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Methods

RPM Version 1 Design

Each tiled prototype sequence was selected to have an intermediate level of sequence homology across a group of microbial or viral strains, which allowed for efficient hybridization and unique identification of most or all subtypes of targeted pathogenic species. For each relevant base of a given prototype sequence, the array contains eight 25mer probes (4 sense and 4 antisense). Two of 8 probes represent perfect matches, while the others correspond to possible mismatches at the central (13th) position of the 25mers. The prototype regions targeting influenza viruses were composed of partial sequences from HA genes of influenza A virus subtypes (H1, H3, and H5) and influenza B virus, NA genes of influenza A virus subtypes (N1 and N2) and influenza B virus, and the M genes of influenza A virus (full-length M1 and partial M2) and influenza B virus (Table 1). Both HA and NA regions encompassed a sufficient number of polymorphic sites to define subtypes. These regions were combined with prototype sequences for 22 other pathogens and tiled on 12.8-μm chips (Affymetrix Inc., Santa Clara, CA, USA), which contain ≈240 K 25mer probes and have the capacity to resolve 30,000 nucleotides. The design and content of RPM version 1 array have been previously described (*14*).

Sample Collection and Nucleic Acid Isolation

The influenza clinical specimens used in this study were collected through the Department of Defense Global Emerging Infections System during the 2004–2005 influenza season. Influenza throat swab specimens were collected in accordance with the case criteria previously described (*15*). Throat swabs were obtained within the first 72 h of the onset of symptoms, placed in viral transport medium (MicroTest M4, Remel Inc., Lenexa, KS, USA), and delivered by commercial carrier to the Air Force Institute for Operational Health in Brooks City Base, San Antonio, Texas, for culturing and molecular characterization. Specimens were passaged once through primary rhesus monkey kidney tissue culture (BioWhittaker, Walkersville, MD, USA). Cultures were tested for influenza A or B viruses by using the centrifugation-enhanced shell-vial technique with monoclonal antibody detection as previously described (*16*). Cultures testing positive for influenza A or B viruses were confirmed by using reverse transcription–polymerase chain reaction (RT-PCR) analysis with previously reported protocols (*16*,*17*). Total nucleic acids were extracted from 90-μL cultured samples or aliquots of live trivalent nasally administered influenza vaccine (FluMist 2004/05, MedImmune, Inc., Gaithersburg, MD, USA) by using the MasterPure DNA purification kit (Epicentre Technologies, Madison, WI, USA) and dissolved in 30 μL of nuclease-free water.

Random RT-PCR

Total RNA amplification from cultured samples using a random (RT-PCR) protocol was performed as previously described (*18*) with minor modifications. Briefly, 4 μL of total nucleic acids were reverse transcribed with 40 pmol of primer D (5′-GTTTCCCAGTAGGTCTCNNNN NNNNN-3′) and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a 20-μL volume at 42°C for 1 h, followed by heat denaturation at 70°C for 15 min. Aliquots (10 μL) of the RT reaction products were then amplified with the TaqPlus Long PCR System (Stratagene, La Jolla, CA, USA) for 35 cycles in a 50-μL volume consisting of 5 μL of 10× low salt buffer, 5 μL (25 mmol/L) $MgCl_2$, 2 μL (10 mmol/L) dNTP mix, 1 μL primer E (100 μmol/L) (5′-GTTTCCCAGTAGGTCTC-3′), and 0.5 μL TaqPlus Long polymerase (5 U/μL). Each cycle consisted of 94°C for 30 s, 40°C for 30 s, 50°C for 30 s, and 72°C for 2 min. This was followed by a final extension at 72°C for 7 min. After amplification, the PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and eluted in 45 μL of EB buffer (10 mmol/L Tris, pH 8.5).

RPM Version 1 Hybridization and Processing

Purified DNA amplicons were adjusted to 2 μg in 35 μL of EB buffer, mixed with 15.1 μL of fragmentation cocktail buffer (5 μL NEB buffer 4, 5 μL 10 mmol/L Tris, pH 7.8, and 0.1 μL GeneChip fragmentation reagent [3 U/μL], Affymetrix Inc.), and incubated for 10 min at 37°C

Table 1. Influenza sequences tiled on the respiratory pathogen microarray version 1

Gene	Prototype	GenBank accession no.	Tiled region	Length (bp)
A/HA1	A/New Caledonia/20/99 (H1N1)	AJ344014	110–808	699
A/HA3	A/Denmark/59/03 (H3N2)	AY531939	120–913	794
A/HA5	A/Hong Kong/486/97 (H5N1)	AF102671	1106–1629	524
A/NA1	A/Chile/1/83 (H1N1)	X15281	4–1363	1,360
A/NA2	A/Panama/2007/99 (H3N2)	AJ457937	1–1446	1,446
A/M	A/NWS/33 (H1N1)	L25814	1–923	923
B/HA	B/Yamanashi/166/98	AF100355	269–952	684
B/NA	B/Yamagata/16/88	AY1139081	1–896	896
B/M	B/Yamagata/16/88	AF100378	1–362	362

and 15 min at 95°C. The fragmented products were then biotin labeled with 1.5 μL of Biotin-N6-ddATP (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) and 1 μL of terminal transferase (20 U/μL) (New England Biolabs, Beverly, MA, USA) for 45 min at 37°C and 15 min at 95°C. RPM version 1 arrays were prehybridized with 200 μL of prehybridization buffer (10 mmol/L Tris, pH 7.8, and 0.01% Tween 20) for 15 min at 45°C. After the prehybridization step, 167.5 μL of hybridization cocktail master mix (3 mol/L tetramethylammonium chloride, 10 mmol/L Tris, pH 7.8, 0.01% Tween 20, 0.5 mg/mL bovine serum albumin, 0.1 mg/mL herring sperm DNA [Promega, Madison, WI, USA], 50 pmol/L Oligo B2 [Affymetrix Inc.]) and biotin-labeled DNA fragments were heated for 5 min at 95°C, equilibrated for 5 min at 45°C, and added to RPM version 1. All hybridizations were incubated for 16 h at 45°C in the GeneChip hybridization oven 640 at 60 revolutions per minute. The microarrays were then washed and stained with the GeneChip Fluidics Station 450 and scanned with the GeneChip Scanner 300 according to the GeneChip CustomSeq array protocol.

The hybridization intensities were analyzed with the GeneChip operating software to generate raw image files (.DAT) and simplified image files (.CEL) with intensities assigned to each of the corresponding probe positions. GeneChip DNA analysis software version 3.0 (GDAS), which implements the ABACUS algorithm (*7*), was used to produce an estimate of corrected base calls file (.CHP). Base calls generated from each tiled region of the array were then exported from GDAS as Federal Acquisition Streamlining Act (FASTA)–formatted sequences.

DNA Sequencing

Automated DNA sequencing was performed as previously described (*17*). HA nucleotide sequences for influenza strains used in this study are available at GenBank (accession nos. DQ265706–DG265730). The nucleotide sequences of primers used for amplification and sequencing are available upon request.

Sequence Analysis

DNA sequences generated from RPM version 1 were searched against the Influenza Sequence Database (http://www.flu.lanl.gov/) (*19*) by using the BLAST algorithm (*20*). Advanced options for blast*n* search were set as follows: –W (word size) 7, –r (reward for a nucleotide match) 1, –q (penalty for a nucleotide mismatch) –1. These parameters were chosen to maximize sensitivity and allow sequences with as many as 50% ambiguous calls to still produce full-length searches. Sequence alignments were performed with the ClustalX program (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/).

Results

Microarray Hybridization

To assess the performance of RPM version 1 with a real-world clinical isolate set, we tested 25 cultured strains collected from 4 continents during the 2004–2005 influenza season and previously diagnosed by culture and RT-PCR as influenza. One influenza subtype was identified in each tested sample based on the RPM version 1 hybridization profiles and sequence reads shown in Figure 1A–C and E. DNA fragments of HA1, NA1, and M genes randomly amplified from an H1N1 isolate specifically hybridized to their corresponding prototype regions on RPM version 1 (Figure 1A). Prototype regions of 1 influenza subtype exhibited no interference from other subtypes (Figure 1A–C), and prototype regions of other pathogens on RPM version 1 showed no cross-hybridization with any influenza virus segments (Figure 1D). Of the 25 isolates tested, we identified 12 A/H3N2, 12 influenza B, and 1 A/H1N1 (Table 2). The A/H1N1 and A/H3N2 subtypes effectively hybridized to the same prototype M sequence (derived from the A/NWS/33 H1N1 strain), confirming that M genes are conserved among different H/N subtypes of influenza A to allow the universal identification of influenza A subtypes with a single tiled prototype region. A computational hybridization simulation model we developed confirms this suggestion (A. Malanoski, unpub. data). Aside from the highly conserved matrix region, no cross-hybridization was observed between subtypes, which suggests that the more variable HA and NA tiles are subtype specific. The GDAS generated DNA sequences from 3 genes (HA, NA, and M) from each sample with 42%–92% of the prototype tiled sequences, resulting in unambiguous calls by the microarray (Table 2). To demonstrate the accuracy of microarray resequencing reads, the HA genes from all 25 samples were amplified by a specifically primed RT-PCR and subjected to conventional sequencing. The sequences produced by random amplification and RPM version 1 were identical to those identified by the conventional sequencing method with the exception of ambiguous base calls (Ns). That is, in cases where both methods assigned a base identity at a particular sequence position, those assignments were always identical (data not shown).

Sequence Analysis and Strain Identification

Microarray resequencing data and conventional sequencing data were searched by using the Influenza Sequence Database with the BLAST algorithm. Results for the highest bit scores were taken as strain identifications and are shown in Table 2.

Influenza A

Based on sequences of HA genes, which are routinely used for genetic and antigenic characterization, microarray strain identifications of all 13 influenza A isolates correlated with identifications from the conventional sequencing method. Although A/H3N2 isolates were sometimes matched with different specific strain sequences from the Influenza Sequence Database based on the top BLAST hits for each isolate, all were redundant representatives of the same A/Fujian/411/02 lineage identified by conventional sequencing. These results indicate that ambiguous calls (Ns) did not affect the accuracy of BLAST identification. At most, only 6 mismatches occurred between the actual sequence of each isolate and sequence of its top BLAST search hit (Table 2, column M1).

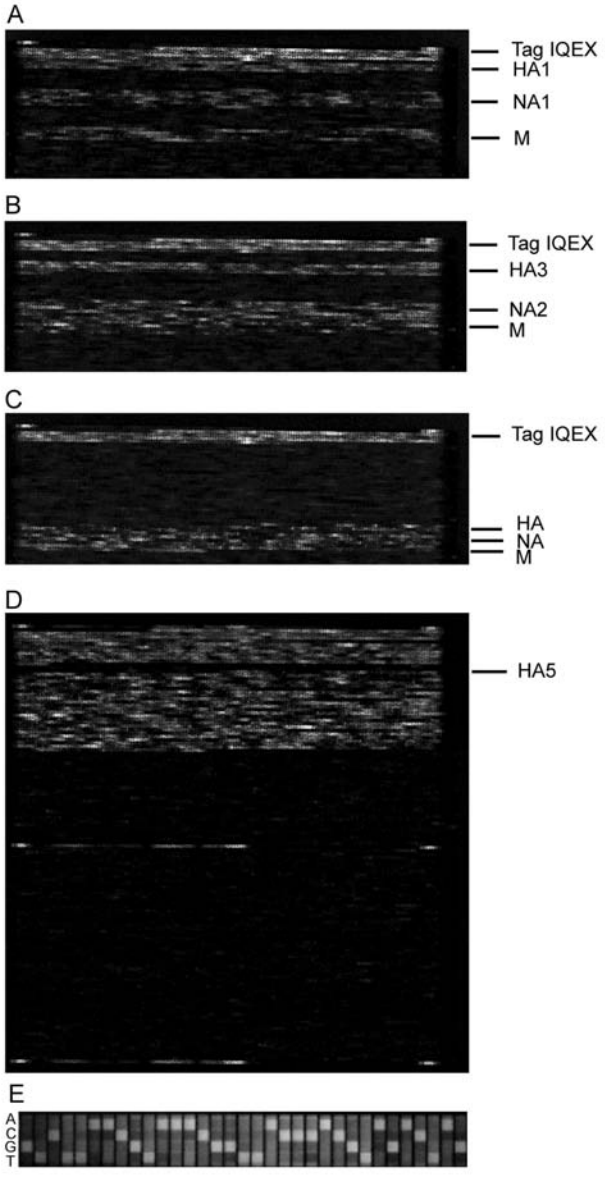

Figure 1. Hybridization images of the respiratory pathogen microarray (RPM) version 1 prototype regions for 3 influenza virus isolates and trivalent FluMist vaccine. A) A/H1N1, B) A/H3N2, C) influenza B, and D) trivalent FluMist vaccine. In A, B, and C, only the influenza-specific tiled prototype regions of RPM version 1 are shown. Hybridization-positive identifications are shown on the right. In D, the image of the entire RPM version when hybridized with FluMist vaccine is shown. The single influenza prototype region that was hybridization negative is denoted on the right. E) Magnification of a portion of profile B showing an example of the primary sequence data generated by the hybridization of randomly amplified targets to the RPM version 1 HA3 probe set. The primary sequence generated can be read from left to right. HA, hemagglutinin; NA, neuraminidase; IQEX, internal positive hybridization control (Affymetrix); M, matrix.

Alignment of the HA peptide sequences translated from RPM version 1–obtained DNA sequences for 12 A/H3N2 isolates (Figure 2) showed that they all shared signature Fujian-like lineage amino acid substitutions (threonine and histidine) at positions 155 and 156 (*17*). Serine (position 227), which is located within antibody binding site D, was also conserved in these isolates, distinguishing them from the A/California/7/04 strain, which has proline at this position (*17*). In addition, isolate A/Ecuador/1968/04 shared similar amino acids with those observed in the A/Fujian/411/02 strain at antigenic sites A (lysine, position 145) and B (serine, position 189). Because of a more limited collection of NA and M gene sequences in the Influenza Sequence Database, strain identifications based on these 2 genes could only place them into clade A strains of H3N2 influenza A viruses sampled from New York State, which caused the A/Fujian/411/2002-like epidemic of the 2003–2004 influenza season (data not shown) (*21*). Although the only tiled M sequence was adopted from an A/H1N1 strain (A/NW/33), M results generated from the H3N2 isolates were still clearly identifiable as belonging to the A/H3N2 subtype and more specifically to the Fujian-like strain. The A/England/400/05 isolate was the only isolate appropriately identified as A/H1N1, and all 3 sequences (HA, NA, and M) generated from RPM version 1 and conventional sequencing for this isolate matched A/New York/227/2003 (H1N1). This is an A/New Caledonia/20/99-like strain that has been consistently circulating globally since 1999 (*16*).

Influenza B

The 12 influenza B isolates were classified as belonging to 2 distinct subgroups based on BLAST searches of 3 genes generated from RPM version 1 analysis. The top BLAST hits for the RPM-obtained sequences of the HA gene identified subgroup 1 isolates as either B/Milano/66/04 or B/Texas/3/2002, both of which are B/Shanghai/361/2002-like strains and belong to the B/Yamagata/16/88 lineage. BLAST queries of conventional sequencing data yielded similar identifications for these isolates. Subgroup 2 isolates were identified as

Table 2. Influenza strain identification with respiratory pathogen microarray (RPM) version 1 versus conventional sequencing*

Sample name	Base call rate† (%)			Strain identification from HA	GenBank accession no.	M1‡	M2§
	HA	NA	M				
A/Colorado/360/05	84.4	72.7	63.2	A/Nepal/1679/2004 (H3N2)	AY945284	0	8
A/Qater/2039/05	88.4	74.0	68.5	A/Nepal/1727/2004 (H3N2)	AY945272	0	8
A/Guam/362/05	87.3	75.8	63.3	A/Nepal/1679/2004 (H3N2)	AY945264	2	10
A/Italy/384/05	83.3	69.6	63.5	A/Nepal/1727/2004 (H3N2)	AY945272	2	9
A/Turkey/2108/05	77.9	67.6	59.2	A/Nepal/1664/2004 (H3N2)	AY945265	2	12
A/Korea/298/05	82.7	70.5	61.7	A/Nepal/1727/2004 (H3N2)	AY945273	4	11
A/Japan/1337/05	87.5	76.7	67.4	A/Malaysia/2256/2004 (H3N2)	ISDN110616	4	14
A/Japan/1383/05	92.1	84.9	74.8	A/Malaysia/2256/2004 (H3N2)	ISDN110616	4	14
A/Ecuador/1968/04	87.7	75.2	58.3	A/New York/17/2003 (H3N2)	CY001053	0	4
A/Iraq/34/05	84.4	72.7	65.6	A/Christchurch/178/2004 (H3N2)	ISDN110530	1	9
A/Peru/166/05	86.9	79.0	65.9	A/Macau/103/2004 (H3N2)	ISDN64772	6	10
A/New York/2782/04	82.7	68.0	63.1	A/New York/391/2005 (H3N2)	CY002056	1	9
A/England/400/05	88.3	55.3	61.1	A/New York/227/2003 (H1N1)	CY002536	1	10
B/Peru/1324/04	75.2	83.4	89.4	B/Milano/66/04	AJ842082	1	25
B/Peru/1364/04	71.1	74.5	77.5	B/Milano/66/04	AJ842082	1	25
B/Colorado/2597/04	81.1	84.3	85.8	B/Texas/3/2002	AY139049	4	27
B/Japan/1905/05	76.2	76.5	76.6	B/Texas/3/2002	AY139049	2	25
B/Japan/1224/05	80.0	78.2	83.7	B/Texas/3/2002	AY139049	2	25
B/Alaska/1777/05	75.0	75.9	78.1	B/Texas/3/2002	AY139049	4	27
B/England/1716/05	80.5	81.4	85.2	B/Texas/3/2002	AY139049	2	25
B/England/2054 /05	81.1	80.1	78.7	B/Texas/3/2002	AY139049	1	24
B/Hawaii/1990/04	51.7	82.9	83.7	B/Tehran/80/02¶	AJ784042	4	68
B/Hawaii/1993/04	47.4	79.7	83.7	B/Tehran/80/02¶	AJ784042	4	69
B/Arizona/148/04	42.4	78.2	82.5	B/Tehran/80/02¶	AJ784042	6	69
B/Arizona/146/04	49.1	79.1	86.7	B/Tehran/80/02¶	AJ784042	6	69

*HA, hemagglutinin; NA, neuraminidase; M, matrix.
†No. of base calls generated from the RPM version 1 divided by the length of the tiled probe sequence.
‡No. of mismatches between the actual sequence and the sequence of the top BLAST search hit.
§No. of mismatches between the actual sequence and the tiled prototype probe sequence.
¶Influenza B group 2 isolates were identified as B/New York/1/2002 strain (accession no. AF532565) by the conventional sequencing method.

B/Tehran/80/02 by microarray and as B/New York/1/2002 by conventional sequencing. The query results of both methods were similar (different identification can be attributed to ambiguous base calls), and all isolates were members of the B/Victoria/2/87 lineage. This lineage is not covered by the 2004–2005 influenza B vaccine (L. Daum, pers. comm.). These results correspond to a Centers for Disease Control and Prevention (Atlanta, GA, USA) influenza activity report documenting that both of the identified influenza B lineages were reported worldwide and that the Yamagata lineage viruses predominated in the 2004–2005 influenza season (*22*).

Genotyping

RPM version 1 can differentiate a broad number of variants based on a single-tiled "prototype" probe region without relying on predetermined hybridization patterns (*9*). A number of nucleotide mismatches that distinguished tested isolates from tiled prototype probe sequences were identified in each sample (Table 2, column M2). Some were unique with respect to existing influenza database-recorded sequences. All of these polymorphisms were verified by conventional sequencing (Table 3). Analysis of HA sequences generated from 12 A/H3N2 isolates by RPM version 1 showed that 4 of these nucleotide variations are common to 11 of the samples, excluding the outlying A/Ecuador/1968/04 isolate. Two of these common base substitutions, 313 G→A and 352 A→C, are at the third nucleotide of their respective codons and represent synonymous mutations. Such mutations do not code for amino acid changes and are usually selectively neutral and much more likely to be shared by common ancestry than by parallel evolution. These facts strongly support phylogenetic grouping of these 11 strains (Figure 3). In contrast, 393 A→T and 483 G→A are nonsynonymous mutations and code for critical amino acid changes. Analysis of conventional sequencing data confirmed that these 2 positions are in the antigenic site B and that the affected amino acids were changed from tyrosine to phenylalanine and from serine to asparagine, respectively. These 2 substitutions are both characteristic features of the A/California/7/04 strain that distinguish this group at both sequence and antigenic

levels from other Fujian-like strains. The identified polymorphisms show that 11 of the 12 A/H3N2 isolates, although collected from 4 continents, are members of the same A/California/7/04 lineage, while the lone outlier, A/Ecuador/1968/04, is clearly identified as a member of the older A/Fujian/411/02 lineage. These observations demonstrate that RPM version 1 data can be effectively used for molecular epidemiologic tracking.

Nearly every isolate was shown to have unique base mutations, many of which resulted in amino acid substitutions. Identification of these mutations reaffirms common knowledge that genetic drift is a frequent event during circulation of influenza viruses and that the RPM version 1 gene chip is an effective tool for tracking unique genetic changes within influenza strains.

Detection of Multiple Targets

To test the capability of RPM version 1 to detect multiple pathogens with the random amplification protocol, we analyzed total nucleic acid isolated from trivalent FluMist

Table 3. Nucleotide differences among hemagglutinin (HA) influenza virus genes identified by respiratory pathogen microarray (RPM) version 1

		Mismatched nucleotides*											
Position†	TN‡	1	2	3	4	5	6	7	8	9	10	11	12
25	T												C
46	T		A										
61	A					T							
62§	G								A				
88	G										T		
189	G						A						
208	T										C		
233§	G												**A**
244	G												A
251§	G						A	A					
262	C								T				
274	G										A		
293§	A		G										
299	G	A	A	A	A	A	A	A	A	A	A	A	A
313§	G	**A**		**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
351§	A						G	G		G			
352	A	**C**		**C**	**C**	**C**	C	C	C	C		**C**	**C**
385	C				T								
393§	A	**T**		**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**
407	T					C							
429§	A				G								
434§	A						**G**	**G**					
446§	T												C
466	C								T				
469	C								T				
473§	G									A			
478	T						A	A					
479§	G						T	T					
483§	G	**A**		**A**	**A**	**A**	A	A	**A**	**A**	**A**	**A**	**A**
493	C			T									
511	A	**T**		**T**									
559	C										T		
564§	A												G
584§	G												A
571	A						G	G					
593§	G	A		A	**A**	**A**	A	A	A	A		A	
596§	T	C		C	C	C	C	C	C	C	C	C	C
602§	A		C										
646	T										C		
652	T			A									
698§	C											A	
734§	C							T					

*Mismatched nucleotides obtained from comparison between the tiled probe sequence and the conventional sequence. Mismatched nucleotides identified by the RPM version 1 are shown in **boldface**. 1–12 represent 12 influenza A/H3N2 isolates. 1, A/Colorado/360/05; 2, A/Ecuador/1968/04; 3, A/Guam/362/05; 4, A/Iraq/34/05; 5, A/Italy/384/05; 6, A/Japan/1337/05; 7, A/Japan/1383/05; 8, A/Korea/298/05; 9, A/New York/278/04; 10, A/Peru/166/05; 11, A/Qatar/2039/05; 12, A/Turkey/2108/05.
†HA3 tiled prototype sequence nucleotide position.
‡Tiled nucleotide.
§Potential nonsynonymous mutation position.

```
                     A        TH                      B           D
           138       *        **                      *           **            242
A/Colorado/360/05 ACKRRSXXXXFXXLNWLTHLKXKXPALXVXMXNNEKFXKXXXXGVXHXGXDXXQXXLXAQAXGRXTXSTKXSQQTVXXNIXSR
     A/Iraq/34/05 XCKRRSXXXXFXXLNWLTXXXXXYPALNVXMPNXEKFXXLYIXXVHHXGTDXXXIXLXAQAXGRITVSTKRSQQTVIXNIXSR
   A/Italy/384/05 ACKRRSXXXXFXXLNWLTHLKXXXPXXXVXMXNXEKFXXLYIXXVHHXGXDXXQXSLXXXAXGRXTXSTKRSQQTVXXNIGSR
 A/Turkey/2108/05 XCKRRSXXXXFXXLNWLTHLXXXXPALXVXMXNXEKFXXXXIXGXXHXGXXXDXXXXXAXAXGRXTXSTKRSQQTVXPXIGSX
   A/Korea/298/05 ACKRRSXXXXFXXLNWLTHLKXXXPALNVTMXNXEKFXKXXXXXXXXXXXXDXXQIXLXAQASGRXTXSTKRSQQTXIXNIGSR
 A/Ecuador1968/04 ACKRRXNKSFFXXLNWLTHLKXKXPALNVXXPNNEKFXKXYIXXVHHPGTDSXQISLXAQASGRXTXSTKRSQQTXXPNIGSR
    A/Guam/362/05 ACKRRSNXXFFXXLNWLTHLKXXYPALNVXMPXNEKFXKXYIWGVHHXGXXXXXXXLXAQAXGRXTXSTKRSXQTXIXNIGSR
    A/Peru/166/05 ACKRRSNKSXFXXLNWLTHLKXXXPALNVXMPXXEKFXKLYXXGVHHXGXXXXQISLXAQAXGXXTVSTKRSQQXVXPXIGSR
 A/Japan/1337/05 ACKRRSXXXXFXXLNWLTHLKXKXPALNVXXPNNXXFDKLYIWGVHHXXXXXXXISLXAQASGRXTXSTKRSXQXVXXXIXSR
A/NewYork/2782/04 XXKRRXXXXXXSRLNWLTHXKXXXPALNVTMPNNEKXDXXXXXGXXXXXXXXDQISLYXQAXGRITVSTKRSQQTVIPNIGSR
 A/Qater/2039/05 ACKRRSXXXXFXXLNWLTHLKXKXPALXVXMPXNEKFXXLXIWGVHHPGXXXXQXSLXAQASGRXTXSTKRSXQTXXPNIGSR
 A/Japan/1383/05 ACKRRSXXXXFSRLNWLTHLKFKXPALNVTMPNNXXXXXXYIWGVHHPXXXXDQISLYAQASGRITVSTKRSQQTVIPNXXXX
      A/HA3/probe ACKRRSNKSFFSRLNWLTHLKYKYPALNVTMPNNEKFDKLYIWGVHHPGTDSDQISLYAQASGRITVSTKRSQQTVIPNIGSR
```

Figure 2. Alignment of hemagglutinin peptide sequences containing an influenza A/H3N2 prototype and the translated sequences from 12 A/H3N2 isolates generated from respiratory pathogen microarray version 1. A, antibody-binding site; TH, antibody-binding site Fujian-like lineage amino acid substitutions threonine and histidine; B, antibody-binding site; D, antibody-binding site. Asterisks indicate conserved amino acids.

intranasal vaccine. Figure 1D shows that 8 tiled influenza sequences on RPM version 1 were strongly hybridized by randomly amplified FluMist nucleic acids, and the resulting sequence data confirmed that FluMist includes immunogenic surface protein (HA and NA) genes from influenza H1N1, H3N2, and influenza B strains. Sequence analysis showed that these antigen-encoding genes matched those of 3 wild-type influenza strains recommended by WHO for making vaccine for the 2004–2005 season (Table 4). Two types of M genes from FluMist were identified by RPM version 1 as those in the cold-adapted Ann Arbor strains of influenza A and B, both of which are essential components in the cold-adapted master donor virus vaccine strain (*23*).

Discussion

Because of the relative ease of transmission of respiratory pathogens, tremendous pressure exists to develop rapid and sensitive tools to identify them. The surveillance of influenza virus outbreaks requires identification not only on the species level but also on the subtype or strain level. Current molecular methods, such as PCR and multiplex PCR, have dramatically improved detection sensitivities and efficiency compared with culture and serologic methods (*24*). However, they require multiple diagnostic tests to discriminate between organisms at multiple phylogenetic levels and are inherently limited in scope and resolution (i.e., increases in resolution necessitate corresponding decreases in scope). Furthermore, these tests rely on the conservation of primer-targeted sequences and as such can be rendered completely ineffective by as little as a single base mutation.

Currently, most microarrays used for microbial detection are spotted arrays that use redundant oligonucleotides as independent probes. For these methods, 2 types of probe targets are usually considered. The first are conserved gene sequences such as 16S rRNA and gyrase (*25*,*26*), which are chosen for identification at the genus or family levels. The second are relatively unique sequences such as virulence factor genes and antigenic determinant genes (*27*,*28*), which are used for species or serotype identification. In this way, pathogen recognition by microarray becomes as reliant on specific hybridization patterns as PCR is on primer-target conservation. Thus, a microarray is only able to resolve identity to the level of divergence represented by the diversity of probes present on the array. With resequencing arrays such as RPM version 1, multiple contiguous sequences (range 100 bp to 2 kb) containing both conserved and unique target genes from each species or subtype can be selected as prototype regions, and every nucleotide from the hybridized target regions can be potentially read as an independent data point using resequencing algorithms (*5*). The key advantage of the resequencing array is that it does not require a specific match between the analyzed sample and the probe, and mismatches actually add value because they can be identified and used as strain-specific markers.

Since the antigen-encoding HA and NA genes are highly variable between different subtypes, sequences specific

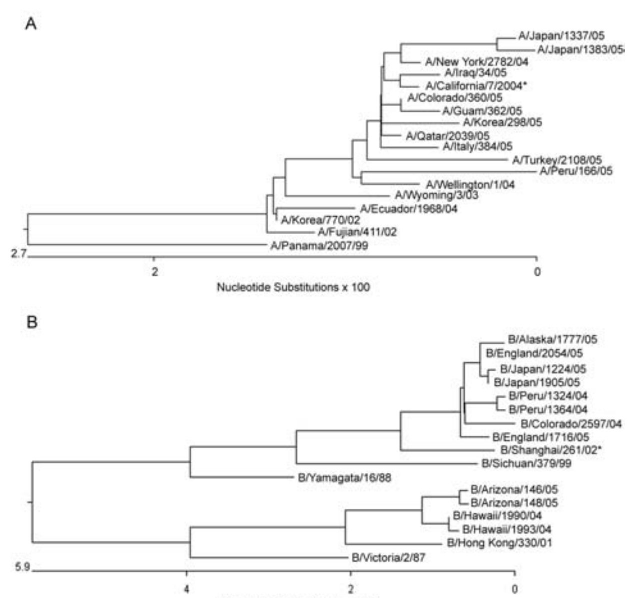

Figure 3. Unrooted phylogenetic analysis of the hemagglutinin 1 (HA1) gene of A) 11 influenza A/H3N2 isolates and B) 12 influenza B isolates compared with vaccine and reference strains. All clinical isolates are available from GenBank under accession nos. DQ265706-DG265730. *denotes the 2005–2006 influenza A/H3N2 and influenza B vaccine strains.

Table 4. Strain component analysis of the FluMist vaccine with respiratory pathogen microarray (RPM) version 1*

Segment	Base call rate† (%)	Strain identification	GenBank accession no.
A/HA1	86.8	A/New Caledonia/20/99	AJ344014
A/NA1	65.6	A/New Caledonia/20/99	AJ518092
A/HA3	86.5	A/Wyoming/3/03	AY531033
A/NA2	78.2	A/Wyoming/3/03	AY531034
A/M	75.9	A/Ann Arbor/6/60	M23978
B/HA	77.1	B/Jilin/20/2003	ISDN40908
B/NA	83.5	B/Yamagata/1246/2003‡	AB120256
B/M	78.4	B/Ann Arbor/1/66	M20175

*HA, hemagglutinin; NA, neuramindase; M, matrix.
†No. of base calls generated from the RPM version 1 divided by the length of the tiled probe sequence.
‡The NA sequence of B/Jilin/20/2003 strain was not available in the influenza database.

for HA1, HA3, HA5, NA1, and NA2 were all tiled on RPM version 1 independently so that influenza A H3N2, H1N1, and H5N1 viruses could be identified and resequenced. Further analysis of the generated sequences showed variations between target and prototype sequences and accurately identified tested isolates at the strain level and as members of recognized circulating variants (Table 2). With its capability to identify strains, the resequencing microarray is a powerful tool for analysis of genetic characteristics of circulating and emerging influenza viruses and can be used to track movement of known variants. Although only 1 type of M gene (H1N1), which is relatively conserved among influenza A viruses, was tiled on RPM version 1, it was still able to cross-hybridize and differentiate M genes from different subtypes (Figure 1 and Table 4). This tiled gene would theoretically allow detection of any other type of influenza virus for which antigenic HA and NA sequences were not tiled on the array.

Another powerful feature of RPM version 1 is its broad-spectrum detection capability, allowing simultaneous resequencing of dozens of gene targets from multiple pathogens in 1 assay. This capability, however, is dependent on an equally broad-spectrum amplification method. With 66 diverse gene probes tiled on RPM version 1 covering 20 common respiratory and 6 biothreat pathogens (*14*), it was logical to use a generic, sequence-independent PCR strategy to amplify all potentially pathogen-derived sequences in an unbiased fashion before hybridization. By adopting a random amplification protocol (*18*) for use with RPM version 1, we could simultaneously detect multiple microorganisms, as shown with trivalent FluMist vaccine.

Correctly identifying 4 different influenza subtypes and their corresponding genes provided a simultaneous demonstration of 3 features of the resequencing microarray: strain identification through pattern recognition, sequence determination, and broad-spectrum capability. Conventional sequencing can determine DNA sequence and has been routinely used for genetic typing in surveillance investigations (*16*,*17*,*21*,*29*). However, it requires designing specific primers and multiple RT-PCRs to determine and amplify individual genes (such as HA, NA and M) before proceeding with sequencing reactions (this is especially true for highly polymorphic RNA viruses such as influenza virus). This requires initial use of other lower resolution techniques to identify strain type. All of these steps are time-consuming and labor-intensive. RPM version 1, combined with a random amplification protocol, can provide sequence information about a wide variety of genes representing many pathogens simultaneously and rapidly without knowledge of the identity of the tested sample. With the current possibility of an avian influenza virus A/H5N1 pandemic (*30*), surveillance for and characterization of emerging variants are essential to the rapid implementation of control measures.

In conclusion, we have combined a random amplification strategy with a resequencing microarray to efficiently and simultaneously detect, type, and genetically characterize geographically diverse influenza viruses. Application of this and similar methods may aid in a better understanding of the incidence, prevalence, and epidemiology of influenza infections and simultaneously allow more rapid identification of epidemic and pandemic outbreaks.

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Dr Wang is a molecular biologist at the Naval Research Laboratory, Washington DC. His research interests include molecular diagnosis of infectious diseases, genomics, and bioinformatics.

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Animals as Sentinels of Bioterrorism Agents

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We conducted a systematic review of the scientific literature from 1966 to 2005 to determine whether animals could provide early warning of a bioterrorism attack, serve as markers for ongoing exposure risk, and amplify or propagate a bioterrorism outbreak. We found evidence that, for certain bioterrorism agents, pets, wildlife, or livestock could provide early warning and that for other agents, humans would likely manifest symptoms before illness could be detected in animals. After an acute attack, active surveillance of wild or domestic animal populations could help identify many ongoing exposure risks. If certain bioterrorism agents found their way into animal populations, they could spread widely through animal-to-animal transmission and prove difficult to control. The public health infrastructure must look beyond passive surveillance of acute animal disease events to build capacity for active surveillance and intervention efforts to detect and control ongoing outbreaks of disease in domestic and wild animal populations.

Most priority bioterrorism agents are zoonotic in origin. As a result, an attack on human populations with a bioterrorism agent would likely pose a health risk to animal populations in the target area; therefore, integrating veterinary and human public health surveillance efforts is essential. The Centers for Disease Control and Prevention (CDC), in planning for the early detection and management of a biological terrorism attack, has recommended the "prompt diagnosis of unusual or suspicious health problems in animals," as well as establishing "criteria for investigating and evaluating suspicious clusters of human and animal disease or injury and triggers for notifying law enforcement of suspected acts of biological or chemical terrorism" (*1*). Similarly, an indicator of a biological terrorism attack would be "increased numbers of sick or dead animals, often of different species. Some BW (biological warfare) agents are capable of infecting/intoxicating a wide range of hosts" (*2*). In part because of such recommendations, calls have been made for enhanced veterinary surveillance for outbreaks of animal disease caused by bioterrorism agents and better communication between animal health and human health professionals. For such efforts to succeed, the relevance to human health of disease events in animals must be established. The potential use of animals as "sentinels" of a human bioterrorism attack can be differentiated from the possibility of a direct attack on animals of agricultural importance (agroterrorism) and is the subject of this review.

First, animals could provide an early warning to humans if clinical signs could be detected before human illness emerged or soon enough to allow preventive measures to be initiated. This early detection could occur because an animal species had increased susceptibility to a particular agent, because the disease caused by the agent had a shorter incubation period, or because animals were exposed sooner (or at more intense and continuous levels) than the human population (*2*). The simultaneous appearance of disease signs and symptoms in animals may contribute to the more rapid identification of a biological warfare agent that was producing nonspecific effects in nearby persons.

Second, if a released biological agent persists in the environment (such as soil, water, or air), active surveillance for sporadic illness in animals could help detect ongoing exposure risks. Additionally, the geographic pattern of sick or dead animals could indicate the persistence of a biological threat (*2*).

Finally, animal populations such as wild birds, commercially shipped livestock, and animals involved in the

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local or international pet trade, could play a role in the maintenance and spread of an epidemic attributable to an intentional release of a biological agent. Detecting the agent in such mobile populations could therefore signal the ongoing spread of the agent and provide an opportunity for interventions to prevent further spread.

Previous reviews have discussed the implications of bioterrorism attacks on human and animal health (*3*). Yet these reviews did not examine the strength of evidence or attempt to determine whether animals could be effective sentinels for particular agents.

We therefore reviewed the biomedical literature for evidence that animals could fulfill the above criteria for sentinel potential. We also hypothesized that large gaps in knowledge exist in this area, including different levels of evidence regarding specific agents and types of animals.

Methods

We systematically searched the biomedical literature from 1966 to 2005 for reports of adverse health events in animals that were attributed to potential bioterrorism agents. The CDC publication Biological and Chemical Terrorism: Strategic Plan for Preparedness and Response (*1*) contains a list of biological and chemical agents that could be used in a terrorist attack. Infectious agents are categorized as A, B, or C, depending on their risk to public health. We searched the Medline database for reports of animal exposure to these biological agents. As a further check, we performed focused searches for individual agents in the CAB Abstracts and Agricultural Online Access (AGRICOLA) databases and also reviewed the bibliographies of recent bioterrorism reviews to locate additional sources.

Our search method used both the name of the agent and the terms "animals, wild" "animals, domestic" and "animals, zoo." For each agent, we searched for peer-reviewed studies of infection in animal populations caused by a specific agent, as well as authoritative subject reviews. The episodes of infection included both actual bioterrorism events as well as naturally occurring epizootics. This search process identified ≈6,000 potential citations, including original journal articles, textbook chapters, and reviews, which were manually culled for relevance to animal sentinel issues; this process resulted in ≈200 citations available for final analysis.

Studies that included data about relative incubation periods and susceptibilities in animals were compared to human data to determine whether evidence supported the use of animals as early warning of bioterrorism agents. We also included in this category reports of animals displaying evidence of infection before nearby human populations did. Data on human incubation periods and infective doses for individual agents were obtained from standard references on biological warfare and terrorism (*4*). Studies that detected symptomatic infection or biomarkers of infection for agents that persist in the environment were reviewed to determine whether they supported the utility of animals for ongoing exposure monitoring. Studies that demonstrated a substantial degree of animal-to-animal or animal-to-human transmission (with or without a vector) were considered to provide evidence that animals could propagate an outbreak of infectious disease caused by a deliberately released pathogen.

Analysis of Evidence

Studies located in the above search were classified according to agent, disease, species, and study method, and these data were then entered into an online database of animals as sentinels of human environmental health hazards (available from http://canarydatabase.org/) for further analysis. For the purposes of this review, we created a taxonomy for evidence regarding animals of sentinels based on existing evidence-based medicine taxonomies that provide guidelines for assigning levels of evidence based on quality and consistency of scientific studies (*5*). We considered level 1 evidence studies to include experimental studies, cohort studies, and systematic reviews of high-quality studies with consistent findings. We classified case-control studies and cross-sectional surveys of animals as level 2 evidence. Evidence from professional consensus statements, textbooks, and descriptive case reports was classified as level 3 evidence. To arrive at an overall strength of recommendation based on a body of evidence, we used these levels of evidence to determine the overall strength of the recommendation that animals could serve in the 3 sentinel capacities of early warning signal, ongoing exposure indicator, and potential propagator and spreader of a bioterrorism agent.

Results

The Table displays the evidence found for animals serving in a sentinel capacity for specific agents or classes of agents.

Evidence That Animals Provide Early Warning of an Acute Bioterrorism Attack

For a number of agents, this review found evidence that animals might be affected before human populations. For *Bacillus anthracis*, whether animals would have a shorter incubation period in the event of an aerosol release was not clear, since the incubation period in the 2001 mail attacks was ≤4 days, while during the 1979 release of *B. anthracis* from a Soviet military laboratory, human symptoms began in 2 days, with death in as few as 6 days (*6*). At the same time, while human cases in Sverdlovsk were concentrated along the path of the prevailing wind ≤4 km from the lab-

Table. Evidence for animals as sentinels of bioterrorism agents*

Agent/disease	Animals provide early warning of acute bioterrorism attack	Animals could be markers for ongoing exposure risk	Animals can propagate/maintain epidemic
Category A			
Anthrax	Yes: sheep, cattle (level 3 evidence [*6*]) No: dogs and pigs (level 1 evidence [*7*])	Yes: sheep, cattle (level 3 evidence [*6*,*8*])	–
Plague	Yes: cats (level 1 evidence [*9*])	Yes: dogs, cats (level 1 evidence [9]), multiple species (level 2 evidence [*10*])	Yes: cats, camels, goats (level 3 evidence [11,12])
Tularemia	No (level 3 evidence [*13*])	Yes: rodents (level 2 evidence *[14*]) No: horses, cows (level 2 evidence [*13*])	Yes: ticks, rodents, prairie dogs (level 2 evidence [*15*])
Botulism	No (level 3 evidence [*16*])	No (level 3 evidence [16])	No (level 3 evidence [*16*)
Filovirus infection	–	–	Yes: wildlife (level 3 evidence [*17*])
Category B			
Q fever	No: sheep (level 1 evidence [*18*])	Yes: wild hogs, goats (level 2 evidence [19,20])	Yes: cats, sheep, goat, cattle (level 3 evidence [*21*])
Brucellosis	No (level 3 evidence [*3*])	Yes: cattle (level 2 [22])	Yes: wildlife, cattle, dogs (level 3 evidence [*23*])
Foodborne illness: *Salmonella* spp; *Shigella* spp.; *Cryptosporidium* spp, etc.	Yes: cattle (level 3 evidence [24])	–	–
Glanders	–	Yes: horses (level 2 evidence [25])	Yes: horses (level 3 evidence [*26*])
Alphaviruses (VEE/EEE)	Yes: horses (level 3 evidence [*26*]))	Yes: birds (level 1 evidence [*27*])	Yes: wild birds (level 2 evidence [*27*])
Rift valley fever	Yes: cattle, sheep (level 3 evidence *[23*])	Yes: sheep (level 1 evidence [*28*])	Yes: mosquitoes, rodents (level 1 evidence [*29*])
Ricin toxin	–	–	–
Epsilon toxin	–	–	–
Category C (emerging diseases)			
Nipah virus	–	Yes: multiple species (level 3 evidence [30])	Yes: pigs (level 1 evidence [*31*])
Hantavirus	No (level 2 evidence [*32*])	Yes: multiple species (level 2 evidence *[32*])	Yes: rodents (level 2 evidence [*33*])
Flavivirus (WN, JE)	Yes: wild birds (level 3 evidence [*34*])	Yes: mosquitoes, birds (level 2 evidence [*35*])	Yes: birds (level 1 evidence [36])

*Level 1 evidence, experimental or cohort study or randomized clinical trial; level 2 evidence, case-control or cross-sectional study; level 3 evidence, case reports or case series, expert opinion; .–, insufficient evidence found; VEE/EEE, Venezuelan equine encephalitis /eastern equine encephalitis; WN, West Nile; JE, Japanese encephalitis.

oratory, livestock, including sheep and cows, began dying 3 days after the release in 6 villages located along the path of the aerosol at a distance ≤50 km downwind from the facility. No human cases were reported in these villages. Calculations of the airborne *B. anthracis* dosage at a town where several sheep and a cow died indicate that the animals were exposed to a dose more than an order of magnitude lower than humans received near the weapons facility. This finding suggests that sheep and cows are more susceptible than humans, although they could have also remained outside in the path of the aerosol for a longer period, which led to greater exposure (*6*).

For *Yersinia pestis*, evidence from experimental inhalation studies in cats indicates that the usual incubation period for symptoms of plague to develop after an inhalation exposure may be shorter (1–2 days) than the presumed incubation time for humans (1–6 days), which provides evidence that symptoms develop in cats at the same time as in humans, and thus may have some sentinel value. In contrast to the findings for anthrax and plague, however, we were unable to find evidence that animals could provide early warning of infection with airborne *Francisella tularensis*. During a prolonged outbreak of pneumonic tularemia in Scandinavia, for example, febrile illness developed in a number of horses, a cow, and a pig, but apparently not before the onset of disease in humans living nearby (*13*).

For foodborne illnesses, including botulism, animals would likely not manifest illness before humans if an attack were directed at humans, since in a typical attack scenario, food would be infected during the distribution pathway before consumption by humans and not necessarily allow for animal consumption before this. We did not locate reports of animals becoming symptomatic with

foodborne illness before the onset of human cases. However, if the attack on the food supply were directed at the animals themselves, they could potentially manifest symptoms before humans who would consume the meat, eggs, or dairy products (*24*). Attacks on water supplies with agents such as *Clostridium botulinum* could put humans at risk as well, although dilution and water treatment would reduce the risk (*37*). An exception may be *Cryptosporidium* spp., which have caused widespread outbreaks through the water supply. Waterfowl die-offs from type C and type E botulism have been well documented, although these types are not well recognized as causes of clinical *C. botulinum* poisoning in humans, but the fact that primates are susceptible to type C makes *C. botulinum* poisoning a possibility. On the whole, however, an attack on human populations with *C. botulinum* would probably not be first detected in animals; the illness would have such a short incubation period in humans that they would become symptomatic at the same time as or before the animals.

For alphaviruses, natural outbreaks have often appeared in animal populations before they affected humans, for example, eastern equine encephalitis virus often appears in equines 2 weeks before humans become symptomatic (*28*). Whether the same pattern would hold true during an attack with an aerosol is not clear. For certain newer agents, such as filoviruses and Nipah virus, current evidence is insufficient to state whether after a generalized release of an aerosolized agent, animal infection would precede that in humans. Studies in Africa have demonstrated that Ebola virus outbreaks can be preceded by deaths in primates as well as in other animal species such as duikers (type of antelope) (*17*), but whether a generalized attack that used Ebola virus in the United States would affect certain animal species first is unknown.

For a number of agents, including *Brucella* spp., *Coxiella burnetii*, and hantavirus, infection in animals is either asymptomatic or develops so slowly that recognizable human cases seem certain to precede animal cases if the agents are released as an aerosol. Finally, the illnesses caused by some agents appear to have shorter incubation times in animals, for example, the 12-hour incubation period for Rift Valley fever in calves and lambs (*23*) compared to the incubation period of several days in humans.

Evidence That Animals Could Be Markers for Ongoing Exposure Risk

After the acute release of a bioterrorist agent, public health officials could be faced with the possibility of an agent persisting in the environment. Anthrax spores can survive for years in soil. Therefore, monitoring for sporadic cases in animal populations such as livestock could indicate so-called exposure hot spots. Although dogs and cats are less susceptible to *B. anthracis* than ruminants, their proximity to humans and their contact with soil could make them sentinels; for example, anthrax developed in a Labrador retriever after the dog hunted in a freshly plowed field (*8*). In the case of ongoing exposures to an agent that has become established in an animal population, case detection could be useful; this situation has been seen for plague in cats (*9*). Similarly, agents like *Brucella* spp. and *C. burnetii*, although they do not cause severe acute illness in animals, could be detected by recognizing increased rates of abortion among a variety of species.

Aside from case detection, active surveillance with surveys of animals may be useful; this surveillance may require testing wildlife as well. Such testing could involve antibody seroprevalence or use of polymerase chain reaction techniques to detect antigen. Evidence about the usefulness of such an approach was inconsistent. During an epidemic of pneumonic tularemia, attributable to contaminated hay, the etiologic agent, *F. tularensis*, can persist in the environment; a serosurvey of asymptomatic livestock (horses and cows) did not show evidence of exposure (*13*). By contrast, in a more recent tularemia outbreak, serosurveys in the wildlife population did show antibodies in a skunk and a rat that lived near persons who had become infected after mowing fields (*14*).

Evidence That Animals Could Propagate an Epidemic Caused by a Bioterrorism Agent

A number of biological terrorism agents have little potential for secondary spread in either animal or human populations, including *B. anthracis* and *C. botulinum*. For other agents, however, we found evidence that their introduction into an animal population could cause an epizootic that would then place additional human populations at risk. For example, studies of mosquitoes native to the United States have demonstrated their potential to spread a disease such as Rift Valley fever through livestock and other animal populations (*29*), even though person-to-person transmission does not occur. The results of animal surveillance for Ebola virus in Africa found that ongoing outbreaks in both primates and duikers suggest that the virus may be able to propagate in a wildlife population (*17*), however, this characteristic has not been demonstrated in US wildlife species.

Agents such as *C. burnetii* and *Brucella* spp. spread easily in animal populations through direct contact and can then pose a wider risk to humans, even though human-to-human transmission does not occur. Agents such as alphaviruses that are prevalent in wild bird populations can spread over a wide area in a short time (*27*). Experimental studies have documented that viruses such as West Nile virus can easily spread from animal to animal in bird populations (*36*).

Discussion

For a number of biological terrorism agents, we found evidence that animals could provide early warning of an acute attack. For the agents for which we found evidence of sentinel potential, a key factor was the relative exposure risk of an animal compared to that of a nearby human population. However, in an actual event involving both humans and animals, the fact that disease was detected sooner in animals could be due to an interplay of a number of factors, including local infrastructure of animal and human health services, public awareness, and laboratory capacity. For other agents, however, humans would demonstrate symptoms at the same time as nearby animals or before. Therefore, the strength of evidence regarding animals serving as early indicators of an attack depends strongly on the agent and species involved. For some agents for which animals would not provide early warning, however, animals could help detect pockets of ongoing exposure risk. For the remainder of agents, evidence regarding the value of animals as sentinels is insufficient at this time.

Overall, according to our classification taxonomy, the strength of the recommendation that animals could provide early warning of an acute bioterrorism attack seems to be, at best, "fair" because of the inconsistency of the evidence. A somewhat more consistent level of evidence appeared to support the recommendation that animals could be markers for ongoing exposure risk and also that animals could play a strong role in propagating outbreaks caused by particular agents. At the same time, our ability to assess the overall strength of evidence for such recommendations was hampered by large gaps in current knowledge.

These findings suggest the need for certain steps related to preparedness for biological agent attacks. First, improved communication is needed between animal health and human health professionals, so that sentinel events could be rapidly detected. Such improvement would mean overcoming existing barriers to communication; a recent survey found that physicians and veterinarians communicate little about zoonotic issues (*38*). Also, an adequate surveillance network should be developed to detect unusual health events in animal populations. Data on usual trends is missing for most animal species that could be potential sentinels. Whether public health resources can be committed to gathering such baseline data remains an open question.

Second, the results of this review indicate that active surveillance of animal populations, including wildlife and companion animals, could fill a critical need in the aftermath of an attack involving certain bioterrorism agents by helping identify persistent sources of infection in the environment. Third, better approaches for intervention are needed to be able to stem the propagation and amplification of an introduced biological warfare agent into a wild or domestic animal population. The US experience with West Nile virus reflects the difficulties of controlling an emerging zoonotic threat as it spreads through animal populations (*39*).

Finally, the results of this review point out the need for additional research to fill knowledge gaps about animals as sentinels of human disease threats, including data on relative susceptibilities and exposure pathways for animal species living near human populations. Concrete steps could include establishment of surveillance veterinary clinics in strategic areas with incentives for practitioners to report unusual events. Another approach would be to make greater use of electronic databases of animal diseases such as those used by the Banfield Clinics, a nationwide chain of veterinary practices. Similar efforts could be useful with wildlife populations.

Such steps would foster ongoing communication between community practitioners and regional public and private veterinary diagnostic laboratories to establish baseline disease incidence trends and algorithms to identify outbreaks. Common links or web-based interfaces should be developed to integrate human and animal disease surveillance information. Reporting systems for wildlife professionals and the public should be created, and their use should be encouraged to document unusual disease events and die-offs. Another constructive step would be to improve the capacity of existing veterinary rapid-response teams, which exist in many states, to carry out active surveillance with animal populations as well as to improve the coordination of veterinary diagnostic laboratories. Again, barriers to funding and cooperation between animal and human health agencies need to be addressed. In the past, these have hampered efforts to have a coordinated approach to collection of animal surveillance data). In addition, state-based efforts would need to be coordinated on a regional and national scale. The growing awareness that animal health and human health are inextricably linked, however, makes cooperation between human and animal health professionals imperative to strengthen the evidence base that will allow for rational use of animal data in public health decision-making.

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Economic Impact of Lyme Disease

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To assess the economic impact of Lyme disease (LD), the most common vectorborne inflammatory disease in the United States, cost data were collected in 5 counties of the Maryland Eastern Shore from 1997 to 2000. Patients were divided into 5 diagnosis groups, clinically defined early-stage LD, clinically defined late-stage LD, suspected LD, tick bite, and other related complaints. From 1997 to 2000, the mean per patient direct medical cost of early-stage LD decreased from $1,609 to $464 ($p<0.05$), and the mean per patient direct medical cost of late-stage LD decreased from $4,240 to $1,380 ($p<0.05$). The expected median of all costs (direct medical cost, indirect medical cost, nonmedical cost, and productivity loss), aggregated across all diagnosis groups of patients, was ≈$281 per patient. These findings will help assess the economics of current and future prevention and control efforts.

Lyme disease (LD) is a multisystem, multistage, inflammatory tickborne disorder caused by the spirochete *Borrelia burgdorferi*. LD usually begins with an initial expanding skin lesion, erythema migrans (EM), which may be followed by musculoskeletal, neurologic, and cardiac manifestations in later stages of the disease (*1–3*). Enzyme-linked immunosorbent assay and Western blotting test are widely used to diagnose LD (*4–6*). LD is most responsive to antimicrobial drugs in the early stage, while further intensive therapy may be necessary in the late stage (*7,8*). A variety of prevention and control procedures can be implemented to prevent and reduce LD incidence, including, but not limited to, public education; personal protection measures such as wearing protective clothing (gloves, long clothes), checking one's body daily for ticks, avoiding tick-infested areas, and applying tick repellent (DEET, permethrin); host management; habitat modification; and chemical control (*9,10*). In 1998, the Food and Drug Administration approved a recombinant outer-surface protein A (rOspA) LD vaccine (LYMErix, SmithKline Beecham Biologicals, Rixensart**,** Belgium) for persons 15–70 years of age (*11*). However, in 2002, SmithKline withdrew the vaccine, citing low demand. Therefore, personal protection measures, early diagnosis, and early treatment are extremely important in preventing and controlling LD.

Since the first case reported in 1975 (*12*), LD has become the most common vectorborne inflammatory disease in the United States. Foci of LD are widely spread in the northeastern, mid-Atlantic, and north-central regions of the United States (*13*). Despite federal, state, and local efforts to prevent and control LD, total reported cases of LD increased almost 3-fold from 1991 to 2002 (Figure 1). In 2002, the Centers for Disease Control and Prevention (CDC) received reports of 23,763 LD cases, 95% of which were from Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, and Wisconsin (*14*). In Maryland, the overall incidence of LD was more than twice as high as the overall incidence of LD in the United States (13.0 vs. 6.3 cases per 100,000 population) (*13*).

Assessing the economic impact of LD will help assess the economics of current and future prevention and control efforts. Although several studies of cost estimates of LD have been published (e.g., *15*), information on the economic impact of LD is limited. Therefore, we conducted a 4-year study to estimate the economic impact of LD on the Maryland Eastern Shore.

Methods

Study Population and Data

This study was conducted in 5 counties (Caroline, Dorchester, Kent, Queen Anne, and Talbot) on the Maryland Eastern Shore, an area where LD is endemic

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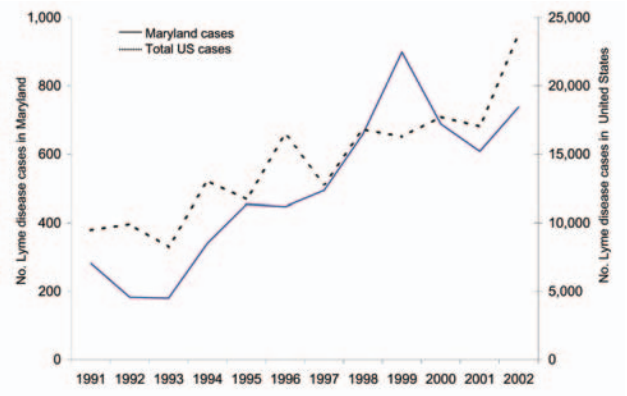

Figure 1. Lyme disease (LD) cases reported to the Centers for Disease Control and Prevention by state health departments in the United States (1991–2002). Reported cases were defined according to the national surveillance definition. For the purpose of surveillance, a case of LD is defined as physician-diagnosed erythema migrans ≥5 cm or ≥1 late rheumatologic, neurologic, or cardiac manifestation with laboratory evidence of *Borrelia burgdorferi* infection. Available from http://www.cdc.gov/ncidod/dvbid/lyme/epi.htm (*14*).

(Table 1). The study population includes patients living in the 4 counties enrolled in Delmarva Health Plan (DHP, a managed healthcare organization) and non-DHP patients receiving health care from office-based physicians in Kent County from 1997 to 2000. Eligible patients were identified through records of encounters for LD, tick bites, insect bites, and serologic testing for LD antibodies. During 1997 and 1998, identified patients were contacted for informed consent. Patients who indicated that they did not wish to participate were excluded from our database. A cost and risk questionnaire (Appendix 1 available online at www.cdc.gov/ncidod/EID/vol12no04/05-0602_app1.htm) was sent to patients who gave informed consent. The response rate of the survey was ≈22%. Interviewers then reviewed patients' charts and consulted relevant sources (e.g., hospital, physician office, laboratory) to obtain the following information: patient demographics; insurance coverage; diagnosis; symptoms; dates of onset and diagnosis; dates of tick bite exposure; dates and costs of primary provider and consultant visits; dates and costs of hospitalizations and emergency department visits; dates, results, and costs of laboratory tests; and dates and costs of antimicrobial drug treatment. All abstracted information was kept confidential. After 1999, an anonymous abstraction of medical records was approved by the institutional review board (IRB) and implemented, allowing inclusion of more patients for all 4 study years, with the exclusion of the records of those who had previously declined participation. All protocols of this study were approved by IRBs from CDC, the state of Maryland, and the University of Maryland. Those patients identified as having received an LD vaccination were not included in this study.

Case Definition

For the purpose of surveillance, a case of LD is defined as physician-diagnosed EM ≥5 cm or at least 1 late rheumatologic, neurologic, or cardiac manifestation with laboratory evidence of *B. burgdorferi* infection (*16*). These criteria were developed as an epidemiologic case definition intended for surveillance purposes only. Although such a standard may aid comparison across clinical studies and facilitate development of research, exposure history and clinical features are critical. For example, treating patients with seasonal (summer) musculoskeletal flulike symptoms in areas where LD is endemic may be clinically appropriate (*12*). Because the data for this study were collected directly from healthcare organizations and physicians, we used a clinical definition of LD. This definition was based on physicians' determination in the medical record, according to patients' clinical findings, tick exposure, and other relevant details (e.g., laboratory results).

In our study, LD patients were identified by using a final diagnosis code in their medical records. LD patients were then divided into 5 diagnosis groups: clinically defined early-stage LD, clinically defined late-stage LD, suspected LD, tick bite, and other related complaints. Most clinically defined early-stage LD patients had EM; some also had musculoskeletal flulike symptoms such as malaise, fatigue, headache, fever, and chills (*12*). In this study, clinically defined late-stage LD patients included those with later manifestations (neurologic involvement, cardiac involvement, and arthritis) and patients with chronic LD. The diagnosis groups of suspected LD, tick

Table 1. Reported cases* of Lyme disease (LD) in Maryland Eastern Shore, 1997–2000†

County	1997	1998	1999	2000	Total
Caroline	18	17	26	21	82
Dorchester	3	4	3	4	14
Kent	24	47	20	34	125
Queen Anne	32	31	40	35	138
Talbot	13	22	33	37	105
Total	90	121	122	131	464

*Reported cases defined according to the national surveillance definition. For the purpose of surveillance, a case of LD is defined as physician-diagnosed erythema migrans ≥5 cm or ≥1 late rheumatologic, neurologic, or cardiac manifestation with laboratory evidence of *Borrelia burgdorferi* infection.
†Source: Maryland Department of Health and Mental Hygiene. Available from http://www.edcp.org/vet_med/lyme_disease.html

bite, and other related complaints involved all patients without a clear final diagnosis of LD. Suspected LD referred to patients who had some symptoms that could be indicative of LD without further evidence and thus no definitive diagnosis of LD. Patients with tick bites without symptoms were placed in the tick bite group. The diagnosis group of other related complaints included all other diagnoses that were different from the above 4 diagnosis groups, such as unknown insect bites and screening among asymptomatic persons.

Study Design

We calculated the following total costs of LD: 1) direct medical costs of LD diagnosis and treatment, 2) indirect medical costs, 3) nonmedical costs, and 4) productivity losses. Intangible costs (e.g., costs incurred because of pain and suffering) were not incorporated. Consumer price index (CPI) for medical care was used to adjust all medical payments into year 2000 dollars (*17*). For nonmedical costs and productivity losses, we adjusted costs by using the general CPI. We took a societal perspective, which incorporates all costs and all benefits no matter who pays costs or who receives benefits.

Charges were used to estimate the direct medical cost. To determine the direct medical costs associated with LD, we used charge data from both DHP and office-based healthcare providers in Kent County. Direct medical costs of LD included costs (charges) of physician visits, consultation, serology, procedure, therapy, hospitalization/emergency room (ER), and other related costs (Appendix 2 available online at www.cdc.gov/ncidod/EID/vol12no04/05-0602_app2.htm).

Indirect medical costs, nonmedical costs, and productivity losses were all acquired from a patient questionnaire used in 1997 and 1998. The questionnaire was sent to LD patients with informed consent forms. Collection of these data was restricted to those 2 years. In this study, indirect medical costs refer to extra prescription and nonprescription drug costs that patients paid out of pocket.

The patient's questionnaire also collected information on nonmedical payments made for home or health aides and miscellaneous services, such as travel (transportation) and babysitting. Each patient's transportation costs to a physician's office were estimated by using the US federal government reimbursement rate, multiplying the reported total travel miles per patient by $0.365/mile. Total travel mileage per patient was calculated by counting the number of physician visits and multiplying total visits by the distance of a round trip to the physician's office.

We used patient-reported time lost from work to estimate productivity losses due to LD on the basis of the human capital method and valued the time lost by using age- and sex-weighted productivity valuation tables (*18*). Because of the potential complexity of accurately answering the question, we did not ask patients to estimate the time they lost from household production. We did, however, ask patients if they paid anybody to do household tasks because their LD-related infirmities prevented them from doing those tasks. For patients <15 years of age, we assumed that their parents (usually the mother) had to take time off from their work to take care of them. Therefore, their mothers' values of lost days of work were included.

Analysis

We used the following formula to estimate the average per capita cost of LD, i.e., the mean cost (direct medical costs, indirect medical costs, nonmedical costs, and productivity losses) aggregated across all diagnosis groups of patients:

$$\text{Expected mean cost of a LD outcome} = \Sigma_{\text{direct medical costs, indirect medical costs, nonmedical costs, and productivity losses}} (\text{Mean cost of outcome}_{\text{clinically defined early-stage LD, clinically defined late-stage LD, suspected LD, tick bite, and other related complaints}} \times \text{Probability of outcome}_{\text{clinically defined early-stage LD, clinically defined late-stage LD, suspected LD, tick bite, and other related complaints}}).$$

Because the distribution of cost data is often not normal, we also calculated the medians of these costs and used both mean and median to estimate the most likely per capita cost of LD on the Maryland Eastern Shore. The median cost of an LD outcome was calculated by using the following formula:

$$\text{Expected median cost of a LD outcome} = \Sigma_{\text{direct medical costs, indirect medical costs, nonmedical costs, and productivity losses}} (\text{Median cost of outcome}_{\text{clinically defined early-stage LD, clinically defined late-stage LD, suspected LD, tick bite, and other related complaints}} \times \text{Probability of outcome}_{\text{clinically defined early-stage LD, clinically defined late-stage LD, suspected LD, tick bite, and other related complaints}})$$

Differences between annual mean direct medical costs were analyzed by using 1-way analysis of variance followed by a Bonferroni test. Differences were considered significant for p values <0.05. Additionally, we used a multivariate linear regression model to estimate the relative impact of a number of factors on the direct medical costs of LD. The ordinary linear regression (OLS) method was applied by using SAS 8.2 (SAS Institute, Cary, NC, USA) and Stata SE (StataCorp LP, College Station, TX, USA). The dependent variable was total direct medical cost per LD patient. We transformed total direct medical costs by using natural logarithms because the data were highly skewed. Independent variables of the equation included cohort year, LD diagnosis groups, diagnostic and treatment procedures, and patient characteristics (e.g., sex, age). All independent variables, except age, were binomial (yes = 1, no = 0). Baseline costs (i.e., the intercept term in the regression equation) referred to those costs accrued by a woman who had tick bite only (without EM symptoms) diagnosed

in 1997 during an office visit. Such a patient had no hospital or ER stay, no serologic tests, no consultation from other physicians, no antimicrobial drug therapy, and no other procedures outside a physician office and hospital/ER. Additional direct medical costs were added or subtracted to the baseline costs for each independent variable of interest if significant (Appendix 3, available online at http://www.cdc.gov/ncidod/EID/vol12no04/05-0602_app3.htm). We tested heteroscedasticity in Stata and corrected mild heteroscedasticity by using "robust" and "hc3" procedures. We also tested both linearity and multicollinearity in SAS and Stata.

Results

From 1997 to 2000, we identified 3,415 LD-relevant patients in the 5 counties studied on Maryland Eastern Shore (Table 2). Among them, 10% had clinically defined early-stage LD while almost 5% of all patients had clinically defined late-stage LD. Of 284 patients who returned a completed patient questionnaire, 59 patients had clinically defined early-stage LD; 25 patients had clinically defined late-stage LD.

Table 3 provides cohort years, medians, means, and standard deviations of direct medical costs comparing the different diagnosis groups. During the study's time frame, the mean (range) direct medical cost of clinically defined early-stage LD decreased from $1,609 ($95–$11,286) in 1997 to $464 ($5–$5,338) in 2000 ($p<0.05$). The mean direct medical cost of clinically defined late-stage LD decreased from $4,240 ($275–$24,985) in 1997 to $1,380 ($45–$6,918) in 2000 ($p<0.05$).

From 1997 to 2000, the mean cost of therapy of all diagnosis groups decreased 75%, from $189 to $47, and the mean cost of hospitalization/ER decreased 61%, from $41 to $16 (Figure 2). During the same period, the mean cost of an office visit, consultation, and serologic tests also decreased 20%, 15%, and 4%, respectively. Additionally,

Table 2. Distribution of Lyme disease (LD) cases* in Maryland Eastern Shore, 1997–2000

Diagnosis group†	No. LD cases (%) from medical record abstraction‡	No. LD cases (%) from follow-up patient survey§
Early stage	334 (10)	59 (21)
Late stage	156 (5)	25 (9)
Suspected LD	718 (21)	54 (19)
Tick bite	539 (16)	62 (22)
Other	1,668 (49)	84 (30)
Total	3,415 (100)	284 (100)

*LD cases in the study are clinically defined LD cases, which may not fit surveillance definition because the data were collected directly from healthcare organizations and physicians.
†Patients were divided into 5 diagnosis groups: clinically defined early-stage LD, clinically defined late-stage LD, suspected LD, tick bite, and other related complaints.
‡Number of patients (1997–2000) who were identified through records of encounters for LD, tick bites, insect bites, and serologic testing.
§Number of patients (1997–1998) who answered a questionnaire recording indirect medical costs, nonmedical costs, and productivity losses.

Table 3. Summary of direct medical cost*† per Lyme disease (LD) patient in Maryland Eastern Shore, 1997–2000

Diagnosis group‡	Cohort	No. cases	Cost per case (US$)					Significance§			
			Median	Mean	Minimum	Maximum	SD	1997	1998	1999	2000
Early-stage LD	1997	77	565	1,609	95	11,286	2,010	NA			
	1998	63	337	869	78	9,720	1,542	S	NA		
	1999	122	282	455	42	3,574	630	S	NS	NA	
	2000	72	288	464	5	5,338	738	S	NS	NS	NA
Late-stage LD	1997	28	3,673	4,240	275	24,985	5,132	NA			
	1998	24	654	1,472	125	6,417	1,839	S	NA		
	1999	59	588	1,286	74	5,402	1,334	S	NS	NA	
	2000	45	589	1,380	45	6,918	1,652	S	NS	NS	NA
Suspected LD	1997	153	169	326	45	9,564	948	NA			
	1998	79	174	255	48	2,285	281	NS	NA		
	1999	242	198	321	51	3,869	445	NS	NS	NA	
	2000	244	238	361	42	7,816	601	NS	NS	NS	NA
Tick bite	1997	143	92	140	33	836	129	NA			
	1998	55	93	227	34	3,432	502	S	NA		
	1999	202	87	120	17	527	98	NS	S	NA	
	2000	139	70	121	16	1,181	141	NS	S	NS	NA
Other	1997	490	196	319	8	6,236	495	NA			
	1998	154	273	479	34	3,721	561	S	NA		
	1999	573	215	321	36	5,091	435	NS	S	NA	
	2000	451	256	381	17	4,157	452	NS	NS	NS	NA

*Direct medical costs were collected from medical record abstraction (1997–2000). Direct medical costs of LD included costs of physician visits, consultation, serologic testing, procedure, therapy, hospitalization/ER, and other relevant costs.
†All costs were converted to 2000 equivalent.
‡Patients were divided into 5 diagnosis groups: clinically defined early-stage LD, clinically defined late-stage LD, suspected LD, tick bite, and other related complaints.
§Differences between annual mean direct medical costs were analyzed by using 1-way analysis of variance followed by Bonferroni test; $p<0.05$; SD, standard deviation; NA, not available; S, significant; NS, not significant.

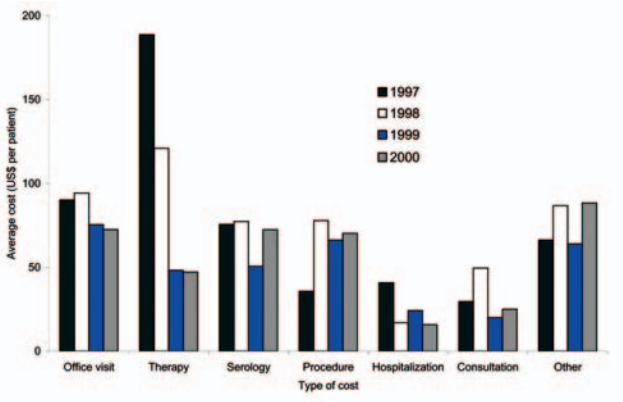

Figure 2. Distribution of elements of direct medical cost (US$) per Lyme disease (LD) patient in Maryland Eastern Shore (1997–2000). Mean is based on direct medical costs of LD patients. Direct medical costs were collected from medical record abstraction (1997–2000). Direct medical costs of LD included costs of physician visits, consultation, serologic tests, procedure, therapy, hospitalization/emergency room, and other relevant costs. All costs were converted to 2000 equivalent.

the proportion of patients within the highest percentile (95th percentile for all 4 years) of therapy cost gradually decreased from 8% in 1997 to 7% in 1998, to 4% in 1999, and 3% in 2000 (data available upon request).

A patient with clinically defined early-stage LD paid an average of $164 in 1997 and $307 in 1998 (in 2000 dollars) for extra prescription and nonprescription drugs (Table 4). Those with clinically defined late-stage LD paid, for similar items, an average of $579 in 1997 and $389 in 1998. The mean nonmedical cost for clinically defined early-stage LD was $109 in 1997 and $23 in 1998. For patients with clinically defined late-stage LD, mean nonmedical costs were $60 in 1997 and $6,703 in 1998. During the survey period, the mean productivity loss of clinically defined early-stage LD was $411 in 1997 and $88 in 1998, and the mean productivity loss of clinically defined late-stage LD was $7,762 in 1997 and $9,108 in 1998. For all 3 types of costs shown in Table 4, a large difference was seen between mean and median values, with the latter often less than half of the mean value, indicating that a small number of LD patients account for a large portion of total costs.

Using multivariate linear regression analysis, we found that patients with clinically defined early- and late-stage LD had direct medical costs that were ≈50% and 100%, respectively, higher ($p<0.001$) relative to patients who only had tick bite, if the impact from other factors was not considered (Table 5). Moreover, patients who were hospitalized or made ER visits, who underwent serologic testing, who needed therapy, who were referred for consultation, and who had other procedures had substantially ($p<0.001$) higher direct medical cost than those who did not (Table 5). No cost difference was seen between men and women. After controlling for other factors, direct medical costs per LD patient in 2000 were lower than those in 1997 (Table 5).

In year 2000 dollars, the expected mean total cost attributable to LD was $1,965 per patient, and the expected median total cost attributable to LD was estimated at $281 per patient (Figure 3). For LD patients at the clinically defined early stage, the median total cost was ≈$397 (mean $1,310), whereas for patients at the clinically defined late stage, the median cost rose to $923 (mean $16,199). Suspected LD cases, tick bite cases, and other LD-related complaints had median costs of $238 (mean $461), $108 (mean $316), and $256 (mean $714), respectively.

Table 4. Indirect medical cost, nonmedical cost, and productivity loss*† per Lyme disease (LD) patient in Maryland Eastern Shore, 1997–1998

Diagnosis group‡	Cohort	No.	Indirect medical cost (US$)§			Nonmedical cost (US$)¶			Productivity loss (US$)#		
			Median	Mean	SD**	Median	Mean	SD	Median	Mean	SD
Early-stage LD	1997	20	20	164	428	27	109	219	28	411	1,095
	1998	39	8	307	1,773	8	23	71	49	88	85
Late-stage LD	1997	6	35	579	1,295	22	60	85	273	7,762	17,458
	1998	19	11	389	1,448	37	6,703	22,405	46	9,108	28,284
Suspected LD	1997	22	5	25	49	8	24	37	26	83	164
	1998	32	0	12	22	4	12	17	44	109	197
Tick bite	1997	31	0	37	105	9	155	731	7	73	151
	1998	31	0	11	40	8	17	50	19	66	79
Other	1997	33	0	31	102	11	143	696	28	233	605
	1998	51	0	11	21	4	23	95	19	300	1,539

*Indirect medical costs, nonmedical costs, and productivity losses were acquired from patient questionnaire (1997–1998).
†All costs were converted to 2000 equivalent.
‡Patients were divided into 5 diagnosis groups: clinically defined early-stage LD, clinically defined late-stage LD, suspected LD, tick bite, and other related complaints.
§Indirect medical costs refer to prescription and nonprescription drug costs patients paid out of pocket.
¶Nonmedical costs are payments made for home/health aides and miscellaneous services, such as transportation and babysitting.
#Productivity losses refer to losses in earning due to illness.
**SD, standard deviation.

Table 5. Impact on direct medical cost* due to cohort year, Lyme disease (LD) diagnosis groups, diagnostic and treatment procedures, and patient characteristics in Maryland Eastern Shore (regression results, n = 3,415)

	Direct medical cost (US$)	5th CI† (US$)	95th CI (US$)	p
Baseline cost‡	60.88	55.94	66.26	<0.0001
Additional direct medical cost§				
Clinically early stage	34.93	22.59	50.65	<0.0001
Clinically late stage	67.05	45.57	94.97	<0.0001
Suspected LD	3.16	-0.68	7.96	0.171
Other LD-relevant complaint	8.33	4.28	13.29	<0.0001
Serologic test¶	38.27	28.20	50.59	<0.0001
Procedure#	26.13	17.68	36.58	<0.0001
Hospitalization/emergency room (ER)**	114.96	89.85	145.83	<0.0001
Consultation††	84.68	68.09	104.56	<0.0001
Therapy‡‡	36.66	29.15	45.56	<0.0001
Miscellaneous§§	46.96	38.21	57.27	<0.0001
Erythema migrans¶¶	–9.56	–13.02	–4.90	<0.0001
Male	–0.68	–2.72	1.84	0.571
Each year of age##	0.11	0.05	0.19	<0.0001
Year 1998	–5.05	–9.28	0.54	0.0003
Year 1999	–12.74	–15.11	–9.50	0.0371
Year 2000	–9.09	–12.09	–5.08	<0.0001

*Direct medical costs of LD included costs of physician visits, consultation, serologic testing, procedure, therapy, hospitalization/ER, and other relevant costs. Patients were divided into 5 diagnosis groups: clinically defined early-stage LD, clinically defined late-stage LD, suspected LD, tick bite, and other related complaints. All costs were converted to 2000 equivalent.
†CI, confidence interval.
‡Baseline costs refer to those costs accrued by a female patient who had tick bite only (with no erythema migrans symptoms), diagnosed in 1997 during an office visit. She had no hospital or ER stay, no serologic tests, no consultation, no therapy, and no other procedures ($R^2 = 0.67$).
§Additional direct medical costs are added or subtracted to the baseline costs for each variable of interest if significant (see online Appendix 3 for details).
¶Serologic test (yes = 1, no = 0) refers to patients who had serologic test (e.g., enzyme-linked immunosorbent assay or Western blotting test).
#Procedure (yes = 1, no = 0) refers to patients who had other procedures that were not performed in hospital/ER, consultation, or physician office.
**Hospitalization/ER (yes = 1, no = 0) refers to patients who had hospital or ER stay.
††Consultation (yes = 1, no = 0) refers to patients who received consultation from other physicians.
‡‡Therapy (yes = 1, no = 0) refers to patients who had therapy charges including antimicrobial agents and additional costs associated (e.g., registered nurse home visits).
§§Miscellaneous (yes = 1, no = 0) refers to patients who had other appropriate charges such as charges for additional laboratory tests.
¶¶ Refers to patients with erythema migrans (yes = 1, no = 0).
##Age is a continuous variable and refers to each additional year of age of the patient.

Discussion

Previous studies of the economic impact of LD were often based on numerous assumptions and experts' suggestions (e.g., Maes et al. [*15*]). Only a few studies provided cost estimates of LD based on data collected from the field (e.g., Fix et al. [*19*], Strickland et al. [*20*]). Even in those studies, however, cost estimates only related to direct medical charges or certain diagnosis or treatment procedures. By combining data from medical records with results from a patient survey, this study more comprehensively documents the economic impact of LD from a societal perspective.

To approximate the annual economic impact of LD nationwide, we extrapolated our results to the total number of LD cases reported nationwide. In this study, the annual total direct medical cost of LD cases on Maryland Eastern Shore was $1,455,081; 490 cases were in the clinically defined early or late stage of LD. Total indirect medical costs, nonmedical costs, and productivity losses were $436,949; 84 cases were clinically defined early- or late-stage LD. Therefore, in general, an LD patient (clinically defined early or late stage) costs $2,970 in direct medical costs plus $5,202 in indirect medical costs, nonmedical costs, and productivity losses. In 2002, 23,763 LD cases were reported to CDC. Hence, the estimated nationwide annual economic impact of LD and relevant complaints was ≈$203 million (in 2002 dollars). However, since LD cases reported on the basis of the surveillance case definition are believed to be underreported (*13*,*21*), this nationwide estimate is likely to be low.

We found that the average cost per LD case decreased over the study period. In LD-endemic areas, personal protection measures are frequently emphasized and insecticides are widely used (*22*). Persons in LD-endemic areas likely visit physicians more frequently whenever they have an exposure or an insect bite, and physicians attending patients from an LD-endemic area likely order serologic testing for possible LD patients and provide prompt treatment. However, our current evidence was limited in that we were only able to find a decrease in per capita cost within diagnosis groups (e.g., clinically defined early- and late-stage LD), but we could not find a shift in the number of cases from late to early stage. Therefore, we don't know what caused the decrease in average cost per LD case.

This study has certain limitations. First, we used clinical case definition (physician determination) instead of

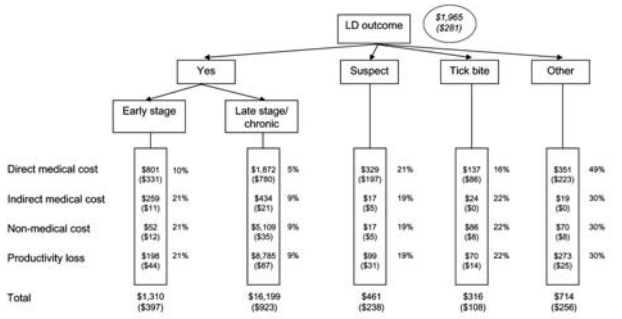

Figure 3. Expected mean (median) cost per Lyme disease (LD) patient in Maryland Eastern Shore by using LD outcome tree. Direct medical costs collected from medical record abstraction (1997-2000). Indirect medical costs, nonmedical costs, and productivity losses were acquired from patient questionnaire (1998–1999). The mean (median) of all costs was aggregated across all diagnostic groups of patients. Percentages refer to probabilities of outcome of a possible LD case (clinically defined early-stage LD, clinically defined late-stage LD, suspected LD, tick bite, and other related complaints). Total percentages do not add to 100% because of rounding. All costs were converted to 2000 equivalent.

surveillance case definition of LD because of limited data. Thus, we may have overestimated the number of LD cases. As a result of case definition, our estimation of cost not only included the cost of LD (clinically defined early- and late-stage LD) but also the costs of LD-relevant complaints (suspected LD, tick bite, and other related complaints). Second, medical charges used in our study may not reflect the true cost. Third, our results are likely to underestimate the costs per case because some of the costs were not included. Costs that were omitted included any costs incurred by a patient beyond the study period. Likewise, Steer et al. reported that ≈7% of LD cases remained asymptomatic within the 20-month study (*23*). These asymptomatic patients may have costs beyond the study. Public health surveillance and administration costs and intangible costs (e.g., costs incurred because of pain and suffering) were also not incorporated in the study. Fourth, because of the large variance between mean and median costs, using mean cost to estimate national impact could be an overestimation. Finally, this study is also limited in that we only had information for indirect medical costs, nonmedical costs, and productivity losses from ≈8% of total patients in the study. Therefore, the results from survey data were extrapolated to represent the whole study population. This method may have biased our results.

LD is the most common vectorborne zoonotic inflammatory disease in the United States. The longterm sequelae of LD are debilitating to patients and costly to society. The emergence of LD and previous experience predict the feasibility of public health interventions for LD control and prevention (*24*). More research on the social behavior of LD patients and economic evaluation of LD prevention interventions is needed.

Acknowledgments

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1951 Influenza Epidemic, England and Wales, Canada, and the United States

Cécile Viboud,* Theresa Tam,† Douglas Fleming,‡ Mark A Miller,* and Lone Simonsen*

Influenza poses a continuing public health threat in epidemic and pandemic seasons. The 1951 influenza epidemic (A/H1N1) caused an unusually high death toll in England; in particular, weekly deaths in Liverpool even surpassed those of the 1918 pandemic. We further quantified the death rate of the 1951 epidemic in 3 countries. In England and Canada, we found that excess death rates from pneumonia and influenza and all causes were substantially higher for the 1951 epidemic than for the 1957 and 1968 pandemics (by ≥50%). The age-specific pattern of deaths in 1951 was consistent with that of other interpandemic seasons; no age shift to younger age groups, reminiscent of pandemics, occurred in the death rate. In contrast to England and Canada, the 1951 epidemic was not particularly severe in the United States. Why this epidemic was so severe in some areas but not others remains unknown and highlights major gaps in our understanding of interpandemic influenza.

Influenza is responsible for large increases in deaths in pandemic seasons when emerging viral subtypes with novel surface antigens become predominant, and also in some interpandemic seasons, when established subtypes exhibit antigenic drift (*1*). The circulating viral subtype is associated with varying severity of influenza epidemics (*2*): in the last 2 decades in the United States, estimated excess death rates were on average 2.8-fold higher in A/H3N2-dominated seasons than in A/H1N1 and B seasons (*3*). Within a given subtype, however, the strain-specific determinants of epidemic severity are still poorly understood. For instance in the United States in the same period, excess death rates varied nearly 4-fold among A/H3N2 seasons, even after adjustments for population aging (*3*). Better characterizations of past severe influenza epidemics can help understand and perhaps help predict the occurrence of severe epidemics.

Anecdotal accounts exist in the literature of historical influenza epidemics associated with unusual numbers of deaths, such as occurred in the 1951 epidemic in England in the midst of the first era of A/H1N1 viruses (1918–1957) (*4*). In Liverpool, where the epidemic was said to originate, it was "the cause of the highest weekly death toll, apart from aerial bombardment, in the city's vital statistics records, since the great cholera epidemic of 1849" (*5*). This weekly death toll even surpassed that of the 1918 influenza pandemic (Figure 1).

The international pattern of influenza-related deaths in 1951 has not been adequately quantified in the past because of lack of methodologic tools and historical death records. However, this historical epidemic is a good example to illustrate major gaps in our current understanding of influenza virus epidemiology. We revisited the 1951 epidemic by quantifying its death rate in 3 countries (England and Wales, Canada, the United States) and comparing its age-specific mortality pattern with that of surrounding epidemic and pandemic seasons (*1*).

Methods

Data

We obtained monthly pneumonia and influenza (P&I) and all-cause numbers of deaths for 1950 to 1999 from Health Canada (*6*), by 5-year age groups (details on the International Classification of Diseases codes used are given in Table 1). Canada was the only country with detailed age-specific mortality data for the 1950s readily available in electronic format.

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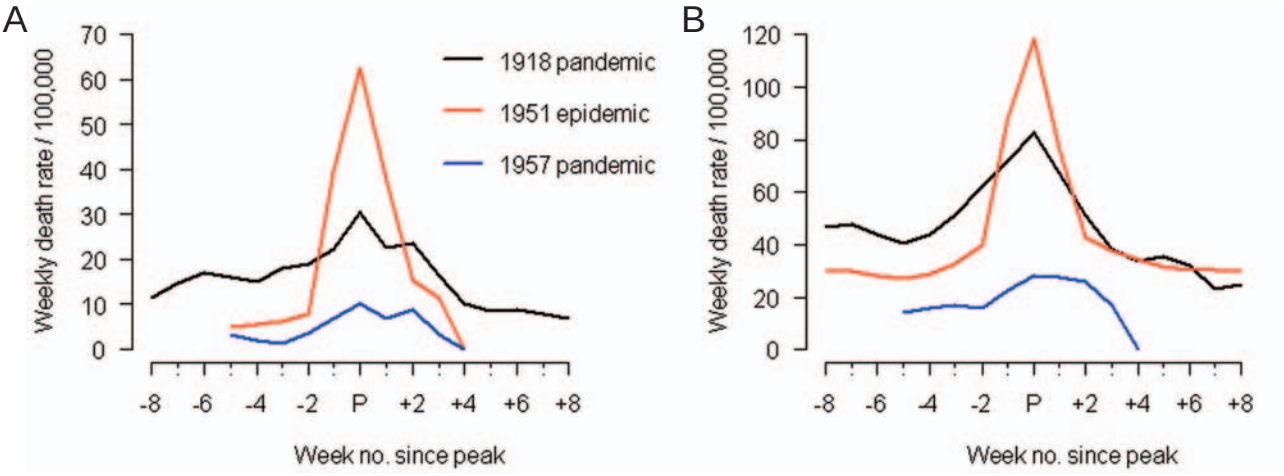

Figure 1. Comparison of 1951 epidemic (A/H1N1) with the 1918 and 1957 pandemics (A/H1N1 and A/H2N2, respectively) in Liverpool, England. Time series of weekly death rates from A) respiratory causes (pneumonia, influenza and bronchitis) and B) all causes. Epidemics were aligned at the week of peak mortality (peak week = week ended Feb 22, 1919; Jan 13, 1951; Oct 12, 1957). The 1918 pandemic occurred in 3 waves in Liverpool (summer 1918, autumn 1918, winter 1919); the "third wave" was associated with the highest death rate and is represented here.

For England and Wales (referred to as "England" for simplicity), we compiled P&I and all-cause deaths by month for 1950 to 1999 from the Registrar General (1950–1958, [*7*]), and National Statistics (1959–1999, [*8*]). In both countries, monthly deaths were normalized by population size to obtain comparable death rates over time and these were standardized to 30.5-day months (Figure 2). Population data were obtained from the same agencies (*6*,*8*).

As US monthly vital statistics were not available electronically since 1950, we compiled excess death estimates from various historical publications (*9*–*12*). These estimates were based on National Vital Statistics and death records from P&I and all causes in major American cities compiled by the Centers for Disease Control and Prevention and derived from excess mortality models similar to ours (see below).

We also conducted a literature search to compile reports describing the local patterns and geographic spread of the 1951 influenza epidemic in the 3 countries (*5*,*9*,*13*,*14*). Moreover, we obtained mortality data specifically for Liverpool, where the 1951 epidemic had the highest impact and death records have been previously described (*5*,*13*,*15*,*16*) (Figure 1).

Seasonal Excess Death Rate Estimates, Canada and England

Our primary goal in this study was to compare the death rate of the 1951 epidemic with that of the 1957 and 1968 pandemics. For this purpose, we fit a seasonal model to P&I and all-cause deaths for 1950 to 1971, capturing all 3 influenza seasons of interest, as described below. We present monthly time series and seasonal estimates for this period (20 seasons, see Figure 2 for P&I). A secondary goal was to compare the age mortality pattern of the 1951 epidemic with that of other influenza seasons. To have more statistical power and analyze several influenza seasons with substantial death rates, we also used an extended study period, 1950–1999.

For Canada and England, we applied a modified version of Serfling's classical seasonal regression model to monthly data on death rates for each country (*17*), as described elsewhere (*3*,*18*). We obtained a baseline for deaths in the absence of influenza, separately for each outcome (P&I and all-cause) and available age group (see Figure 2 for P&I). Seasonal excess deaths were then estimated as the number of deaths in excess of the baseline during months of increased influenza activity.

Table 1. Codes from the International Classification of Diseases (ICD) used for selecting deaths from pneumonia and influenza (P&I) in Canada and England & Wales, 1950–1999

ICD revision	Canada	England & Wales	Codes used for P&I deaths
ICD-6	1950–1957	1950–1957	480–483; 490–493
ICD-7	1958–1968	1958–1967	480–483; 490–493
ICD-8	1969–1978	1968–1978	470–474; 480–486,
ICD-9	1979–1994	1979–1999	480–487
ICD-10	1995–1999		J10.0–J11.8; J12.0–J18.9

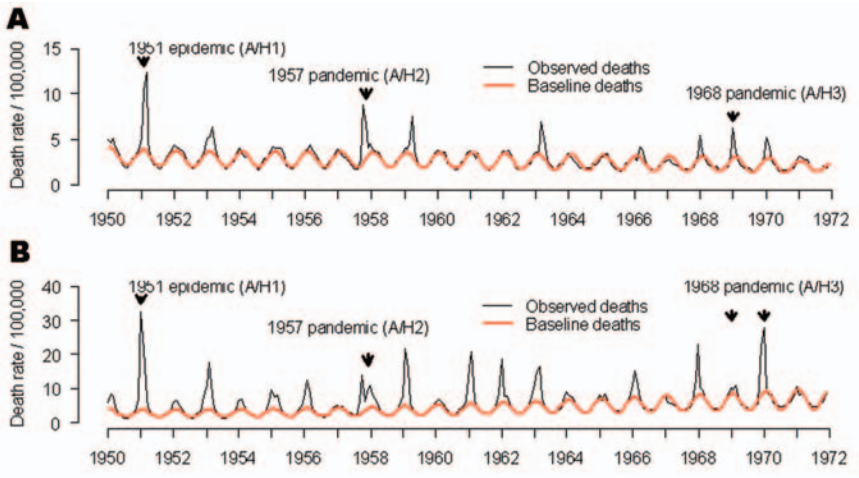

Figure 2. Time series of monthly mortality from pneumonia and influenza (P&I, represented as death rate/100,000) from 1950 to 1972 in A) Canada and B) England and Wales. Black line: observed deaths, Red line: baseline deaths predicted by a seasonal regression model. Note the 2 arrows for the 1968 pandemic in England, representing the 2 waves of the smoldering A/H3N2 pandemic (1968–69 and 1969–70, respectively) (*18*).

Standardization of Seasonal Excess Death Rates

Since our goal was to compare influenza deaths across multiple seasons and countries, we had to control for baseline differences in demography, healthcare, and socioeconomic status that may affect influenza-related deaths. To this end, we calculated age-adjusted seasonal excess death estimates in a manner previously described (*3*,*18*). Further, to control for residual differences in baseline death rates related to health and socioeconomic status, we adjusted the seasonal estimates for temporal changes in mortality in the summer months, when influenza is absent (*18*). We used year 1960, midpoint of the main study period 1950–1971, as an index.

Age-Specific Patterns of Seasonal Excess Death Rates, Canada

We examined whether the 1951 epidemic had an epidemic or pandemic mortality age pattern, as indicated by a shift in the age distribution of deaths towards younger age groups (*1*). In Canada, the 1950–51 season was the first season in our mortality records with complete age details. Hence, we could not evaluate a potential age shift between earlier seasons and the 1950–51 season, as described elsewhere (*1*).

We therefore developed an alternative method to identify a pandemic signature, in which we compared the gradual increase of influenza-related deaths with age between epidemic and pandemic seasons. We first used all moderate-to-severe influenza seasons in the interpandemic periods to obtain a null distribution of mortality age patterns during epidemics (we chose the 17 seasons above the median). Second, we checked that we could actually detect a pandemic age pattern by comparing the null epidemic pattern with those of the 1957 and 1968 pandemics. Then, we compared the null pattern with that of the 1951 epidemic. To model the gradual increase of influenza-related deaths with age in adults, we fitted an exponential to unadjusted P&I excess death rates by 5-year age groups for persons ≥55 years of age. The test then relied on comparing between seasons the values of the age and intercept coefficients of the exponential models. Bootstrap resampling of influenza seasons in the interpandemic periods yielded a p value for the test.

Results

Geographic and Temporal Spread

Influenza activity started to increase in Liverpool, England, in late December 1950 (*5*,*13*). The weekly death rate reached a peak in mid-January 1951 that was ≈40% higher than the peak of the 1918–19 pandemic, reflecting a rapid and unprecedented increase in deaths, which lasted for ≈5 weeks ([*5*] and Figure 1). Since the early 20th century, the geographic spread of influenza could be followed across England from the weekly influenza mortality statistics in the country's largest cities, which represented half of the British population (*13*). During January 1951, the epidemic spread within 2 to 3 weeks from Liverpool throughout the rest of the country.

For Canada, the first report of influenza illness came the third week of January from Grand Falls, Newfoundland (*19*). Within a week, the epidemic had reached the eastern provinces, and influenza subsequently spread rapidly westward (*19*).

For the United States, substantial increases in influenza illness and excess deaths were reported in New England from February to April 1951, at a level unprecedented since the severe 1943–44 influenza season. Much milder epidemics occurred later in the spring elsewhere in the country (*9*).

Local disparities were found in all 3 countries, with a consistent pattern of higher numbers of deaths in locations affected earlier (*9*,*13*,*14*). In England, influenza-related death rates were ≈3-fold higher in Liverpool than in the

rest of the country (*13*). In Canada, death rates were ≈2.4-fold higher in the eastern seaboard provinces than in the rest of the country (*14*). Similarly, in the United States, rates were ≈2.3-fold higher in New England than in the rest of the country (*9*).

Patterns of Seasonal Excess Death Rates, All Ages, 3 Countries

Crude and adjusted seasonal excess death estimates in the 3 countries are presented for the period 1950–1971 in Figure 3 (P&I). A specific comparison of the 1951 epidemic and 1957 pandemic is provided in Table 2 (P&I and all-cause).

For Canada, the 1951 epidemic was the most severe influenza season in the period 1950–1999, as indicated by crude seasonal excess death rates from P&I and all causes (data not shown). On the basis of both outcomes, the 1951 epidemic caused a 1.5-fold higher death rate than the 1957 pandemic; the rate was 3- to 4-fold higher than that seen in the 1968 pandemic. Adjusting for factors unrelated to influenza, such as demographics, health, and socioeconomic status only marginally modified our estimates (by ≤11%).

In England, the 1951 epidemic had similar death patterns. It was responsible for the largest increase in winter deaths from P&I and all causes in the period 1950–1999 (data not shown), with 1.3- to 1.4-fold higher crude excess death rates than those seen in the 1957 and 1968 pandemics. In contrast to Canada, adjusting for trends in demographics and health care substantially changed our excess death estimates, exacerbating the impact of the 1951 epidemic. Baseline P&I summer death rates doubled from 1950 to 1970 (Figure 2), probably because of rapid aging of the British population. During these 2 decades, the proportion of persons ≥65 years of age increased by 2.3% in England, which explains the trend in British death rates; in comparison, it increased by only 0.3% in Canada (*6*,*8*).

In the United States, by contrast, the 1951 epidemic was not particularly severe, except possibly in the New England region, adjacent to Canada. In the United States, this epidemic ranked with low-to-moderate influenza seasons, with only half the impact of the 1957 pandemic for P&I deaths and even less for all-cause deaths.

Estimation of crude and adjusted excess deaths suggests that the 1951 epidemic was unusually severe in England and Canada but not in the United States. The absolute rates of excess deaths were very different between countries, with systematically higher rates in England (by 3- to 5-fold, Table 2). The difference remained even after adjusting for international differences in demographics and healthcare and was also found for deaths from all causes, which controls for potential differences in the coding of death certificates (Table 2). Such international discrepancies in influenza-related death rates have been highlighted on several occasions in the past, although not elucidated (*18*,*20*–*22*). Because of these unresolved differences, this

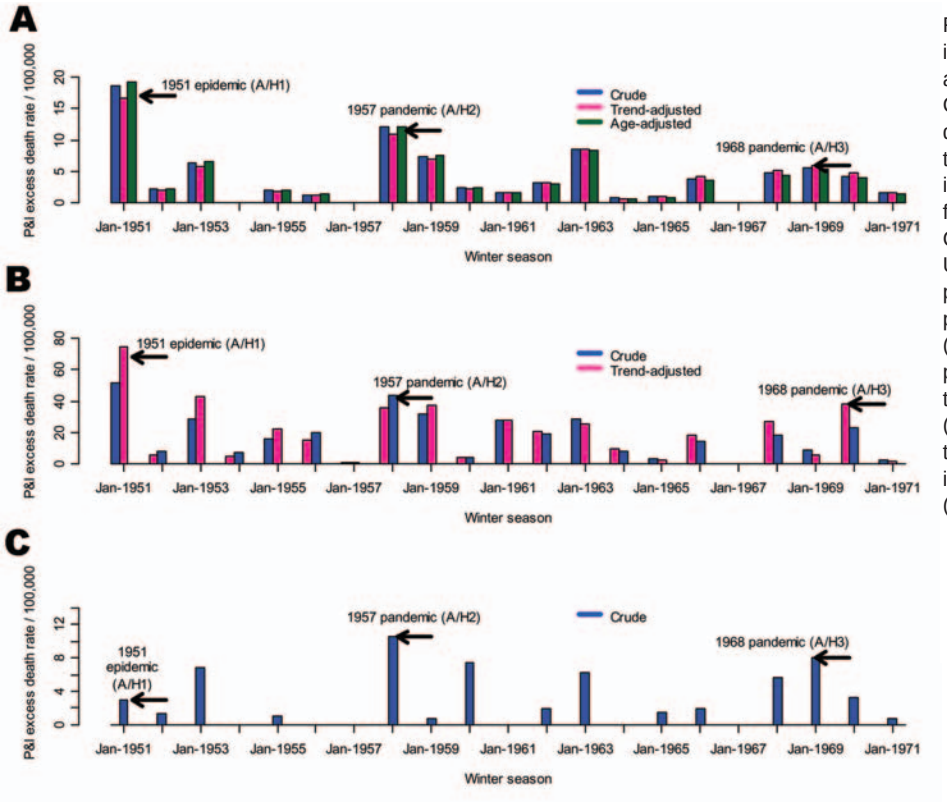

Figure 3. Seasonal pneumonia and influenza (P&I) excess death rates, all ages, 1950–51 to 1970–71. Crude death rates (blue bars), death rates adjusted for summer trends in mortality unrelated to influenza (pink bars) and adjusted for demographics (green bars). A) Canada. B) England and Wales. C) USA. Note that for England, comparisons used the second A/H3N2 pandemic wave of major impact (*18*). Note the 2 arrows for the 1968 pandemic in England, representing the 2 A/H3N2 pandemic waves (1968–69 and 1969–70, respectively); the second wave of major impact was used in comparisons (*18*).

Table 2. Comparison of influenza-related death rates in the 1951 influenza epidemic (A/H1N1) and 1957 influenza pandemic (A/H2N2), Canada, England and Wales, and United States*

Setting	Age group	P&I† excess death rate/ 100,000 (RR‡)	All cause excess death rate/ 100,000 (RR‡)
Canada (13.7 million)§			
1951 epidemic (Jan –Apr)	All ages	18.6 (1.54)	34.1 (1.47)
	<65 y	6.6 (0.90)	14.8 (1.09)
	≥65 y	164 (2.41)	329 (2.22)
1957 pandemic (Sep–Dec)	All ages	12.1 (1.00)	23.2 (1.00)
	<65 y	7.3 (1.00)	13.6 (1.00)
	≥65 y	68.0 (1.00)	148 (1.00)
England & Wales (43.8 million)§			
1951 epidemic (Jan –Mar)	All ages	50.1 (1.40)	178 (1.45)
1957 pandemic (Oct 1957–Mar 1958)	All ages	35.8 (1.00)	123 (1.00)
United States (154.9 million)§			
1951 epidemic (Feb–Apr)	All ages	5.5 (0.48)	9.0 (0.25)
1957 pandemic (Oct 1957–Mar 1958)	All ages	11.5 (1.00)	36.3 (1.00)

*Unadjusted death rates (see Figure 3 for rates adjusted for demographics and trends in health care and socioeconomic status).
†P&I, pneumonia and influenza.
‡RR, ratio of excess death rate in 1951 to excess death rate in 1957.
§Population in 1951.

analysis focused on the relative impact of the 1951 epidemic as compared with surrounding influenza seasons.

Age-specific Patterns of Seasonal Excess Death Rates, Canada

Given the unusual death rate of the 1951 influenza epidemic in England and Canada, we hypothesized that an emerging virus subtype may have circulated there, perhaps with pandemic potential. We investigated the age-specific mortality pattern of the 1951 epidemic in Canada in relation to other seasons to address this aspect.

Inspection of P&I excess death rates by age shows that the 1951 epidemic had the typical pattern of death rates found in other epidemics, steeply increasing with age after infancy (Figure 4). Statistical analysis showed that the age pattern in 1951 was well within the range of the null distribution of reference epidemic seasons in influenza interpandemic periods (Table 3). Conversely, we found lower age coefficients for the 1957 and 1968 pandemics as compared with reference epidemic seasons, illustrating that deaths increased less with age in pandemics than in epidemics and that our statistical approach could detect a pandemic signature ($p<0.001$) (*1*).

Discussion

We have shown that the 1951 influenza epidemic had greater death rate than all subsequent influenza epidemics or pandemics in England and Canada. In Canada, where age-detailed data were available, deaths in persons <65 years of age attributable to the 1951 epidemic were nearly equivalent to those of the 1957 pandemic. But what sets the 1951 epidemic apart from pandemics is that the older population was also severely affected, with twice the deaths as occurred in the 1957 pandemic. By contrast, the 1951 epidemic had minor impact in the United States, except possibly in New England.

To study influenza death patterns, we used P&I deaths, a reliable proxy for the timing and relative impact of influenza epidemics, as well as all-cause deaths, an indicator of their overall impact (*23*). Cardiovascular deaths are also widely used to quantify the impact of influenza (*24,25*); however, they were not available to us for this study. But since winter increases in P&I, cardiovascular, and all-cause deaths are synchronized and correlated in amplitude during influenza epidemics (*25*), we capture here the timing and death rate of these epidemics.

Influenza-related death rates reflect the combination of 2 underlying epidemiologic parameters: the attack rate, a measure of a pathogen's transmissibility, and the case-fatality rate, a measure of a pathogen's virulence. The unusual severity of the 1951 epidemic in England and Canada may stem from higher attack rates, higher case-fatality rates, or both. To isolate these factors, we examined Liverpool, England, where comparable data on illness and death exist for the 1951 and 1957 pandemics (*5*). In Liverpool, influenza attack rates in schoolchildren were 3-fold lower in 1951 than in 1957, which suggests lower transmissibility in this age group in 1951. By contrast, an equal number of influenza-related deaths occurred in the 2 seasons in children in Liverpool, which suggests a higher case-fatality rate in 1951 than in 1957. Similar findings were observed in the working adult population (*5*), an indication of unusual virulence in the influenza virus circulating in 1951 in Liverpool. This argument must be taken with caution, however, since most influenza-related deaths occur in the elderly (*3*), and attack rates are not available for this age group. Estimation of the transmissibility of the 1951 virus using a mathematical model for influenza

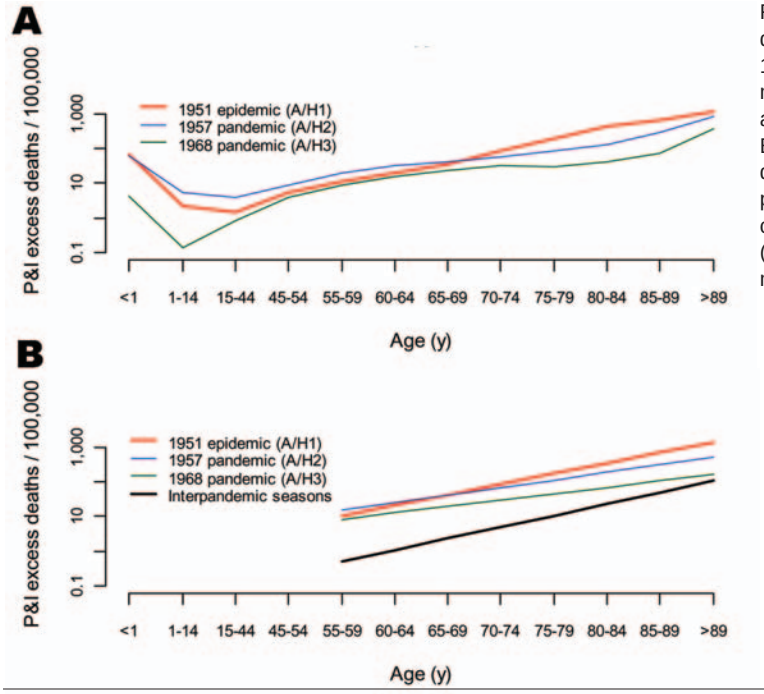

Figure 4. Age-specific pneumonia and influenza (P&I) death rates in the 1951 influenza epidemic, 1957, and 1968 pandemics, Canada. A) Observed. B) Exponential models using 5-year age groups starting at age 55 years and ending at ≥90 years (R^2 ≥0.85 for all seasons). Black curve: "null distribution" of expected pattern in epidemic seasons, based on major epidemics in the interpandemic periods, 1950–1999 (N = 17). The age coefficient was set at the mean of the "null" distribution (see Table 3 for values). The intercept was set at the minimum of the distribution for legibility.

transmission fitted to mortality data (*26*) might answer this question, not only in Liverpool but also in the rest of England and Canada.

Laboratory surveillance data from the World Health Organization (WHO) indicate that influenza A viruses circulating at the time were characterized as H1N1 (*27*), a subtype circulating since the 1918 pandemic (*28*). Although an unusually large drift event in the hemagglutinin of A/H1N1 viruses was reported in 1947 (*29*), subsequent changes in this protein remained minor until after 1951 (*27*). Hence, no virologic evidence of a shift or unusual drift in the hemagglutinin antigen exists for 1951 viruses. In support of the virologic evidence, we have shown that no epidemiologic pandemic signature occurred in 1951, as indicated by an age shift of deaths towards younger age groups (*1*).

The 1951 epidemic exhibited geographic disparities in influenza-related deaths, as illustrated by the contrast between England and Canada (countries with high death rate) and the United States (low death rate). These disparities are in part explained by laboratory surveillance reports by WHO (*27*,*30*), indicating that 2 antigenically distinct influenza A/H1N1 strains cocirculated in the Northern Hemisphere during the 1951 epidemic (*27*,*30*). The so-called "Scandinavian strain" was isolated in northern Europe and associated with mild illnesses. By contrast, the "Liverpool strain" was associated with severe illnesses and high deaths in Great Britain, Canada, southern Europe, and Mediterranean countries (*27*). As both strains cocirculated in some countries (*27*), intrasubtypic cross-immunity might have existed, with these 2 strains competing for susceptible hosts.

Table 3. Test for a pandemic signature in age-specific, influenza-related death rates of 1951 influenza epidemic, Canada*

Influenza seasons	Age coefficient	Intercept	p value†
Major epidemic seasons, 1950–1999‡			
Average (SD)§	0.15 (0.02)	–7.9 (1.9)	Reference
Minimum-maximum	(0.11–0.18)	(–9.9; –4.1)	Reference
1951 epidemic (A/H1)	0.14	–5.2	0.30; 0.13
1957 pandemic (A/H2)	0.10	–2.7	<0.001¶
1968 pandemic (A/H3)	0.08	–2.5	<0.001¶

*Coefficients of exponential models explaining the increase in pneumonia and influenza (P&I) excess death rate with age for the 1951 epidemic, 1957 and 1968 pandemics, and other major epidemics in the interpandemic periods, 1950–1999. Exponential models use 5-year age groups starting at age 55 years and ending at age ≥90 years (see Figure 4). All R^2 between 0.85 and 0.99 (R^2 of linear model applied to log-transformed P&I excess death rate, equivalent to an exponential model for untransformed P&I).
†p value for comparison of respectively age and intercept coefficients with the "null" distribution of major epidemic seasons. Based on 1,000 bootstrap samples.
‡"Null" distribution of major influenza seasons in the interpandemic periods, 1950–1999. Based on 17 seasons that had seasonal P&I excess mortality rates above the median of all seasons (including seasons in the A/H1N1, A/H2N2, and A/H3N2 era but excluding the 1951 epidemics and the 2 pandemics.)
§SD, standard deviation based on bootstrap resampling of the null distribution of observed epidemic patterns.
¶Evidence of pandemic signature (not consistent with an epidemic mortality age pattern).

The precise reasons for the unusually high death rate associated with the Liverpool strain remain elusive. The genetic markers of influenza virulence are still unclear today, but a multibasic cleavage site in the hemagglutinin, as well as minor changes in internal genes, are believed to enhance viral pathogenicity (*31*,*32*). Only hemagglutinin inhibition tests could be performed in 1951, and to our knowledge, no influenza virus isolate or genetic sequence from 1951 is available in the public domain. Further molecular analysis of 1951 influenza specimens could help explain the extreme local pathogenicity in that season.

We have described an influenza season that was unexpectedly severe in some countries and mild in others. This geographic disparity in influenza-related deaths is not common; influenza mortality is generally correlated between the United States and Europe and within the United States (*33*,*34*). Occasional disparities have been reported, however. For instance, the impact of the 2 waves of the 1968 pandemic differed markedly between North American and Eurasian countries, perhaps because of differences in preexisting immunity and evolving viruses (*3*,*18*). In this context, the 1951 epidemic appears as another striking example of geographic disparities in influenza impact, perhaps explained in this case by cocirculation of 2 influenza A/H1N1 strains. Other competing hypotheses include differences in preexisting population immunity or socioeconomic factors, but these are less parsimonious explanations.

Many countries are actively preparing for the next influenza pandemic (*35*–*37*). Previous pandemics in the 20th century have been responsible for large numbers of deaths in all age groups (*1*); however, the age pattern of deaths in the 1918 and 1968 pandemics suggest that the elderly may actually be relatively protected against an emerging pandemic virus (*35*,*38*,*39*). By contrast, we have shown that the 1951 epidemic was not associated with the emergence of a new influenza subtype, yet had a higher death rate than 2 of the 3 pandemics of the past century in England and Canada, especially among the elderly, and higher death rate than all 3 pandemics in Liverpool. We conclude that pandemics are not always more severe in terms of deaths than epidemics, for reasons still unclear. A thorough investigation of the full genome of the influenza viruses involved in the unusually severe 1951 epidemic could shed light on the virulence and transmissibility factors at play and fill key gaps in our current understanding of interpandemic influenza (*40*).

Acknowledgments

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Dr Viboud is a mathematical epidemiologist with the US National Institutes of Health, with an interest in the patterns of illness and death for infectious diseases. Her research focuses on the spatial and temporal patterns of influenza and rotavirus epidemics and how the genetic make-up of the pathogen relates to its epidemiologic impact.

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HIV Transmission in Correctional Facility

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Acute retroviral syndrome developed in an inmate in a detention center after he had intercourse with 2 HIV-infected inmates. Correctional facilities house a disproportionate number of HIV-infected persons, and most do not provide inmates with condoms. Correctional healthcare providers should be familiar with primary HIV infection and acute retroviral syndrome.

Correctional facilities house a disproportionate number of HIV-infected inmates (*1*) and are a setting for unprotected sexual intercourse (*2*). Although symptoms of acute retroviral syndrome develop in up to 89% of persons newly infected with HIV (3), the timely recognition and diagnosis of primary HIV infection and initiation of antiretroviral treatment before HIV seroconversion have rarely been reported from a correctional facility.

The Case

In October 2003, a man with a history of noninjection multiple-drug abuse (including methamphetamine) was admitted to a detention center (regional jail). His pre-incarceration history included unprotected sex with men in the community, most recently in April 2003; however, results of multiple serologic assays for HIV performed in the community had been negative, most recently in June 2003 and December 2002. He had an unremarkable past medical history.

On December 31, 2003, this patient came to the correctional facility's medical clinic with perianal and rectal discomfort; perianal condylomata were present. He reported that during December he had consensual, unprotected, receptive anal intercourse with 2 male inmates at the correctional facility; both of these inmates had chronic HIV infection. One, who was not receiving antiretroviral treatment, had a plasma HIV RNA level of 53,000 copies/mL; the second, who was receiving antiretroviral treatment, had a plasma HIV RNA level of 92 copies/mL. On January 6, enzyme immunoassay (EIA) testing of the index patient for HIV was negative.

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On January 9, the patient came to the medical clinic with fever, sore throat, myalgia, headache, vertigo, nausea, and vomiting. His oral temperature was 40.7°C, and he was profusely diaphoretic. Posterior pharyngeal erythema was present, as well as tender, minimally enlarged, anterior cervical lymphadenopathy. Laboratory testing showed plasma HIV RNA of 436,000 copies/mL; CD4+ T-lymphocyte count of 616 cells/μL (28%); serum alkaline phosphatase 183 IU/L; and negative serologic test results for hepatitis A, B, and C viruses. He denied participating in tattooing or injection drug use.

On January 13, he reported vertigo and urinary retention; his temperature was 39.4°C, and he was ataxic. Acute urinary retention required urethral catheterization. On January 14, physical examination showed bilateral horizontal nystagmus and perianal ulcerations; plasma HIV RNA was >750,000 copies/mL. On January 15, a second EIA for HIV was negative; however, a Western blot of that serum sample showed an equivocal p24 band.

On January 16, he reported difficulty defecating and urinating. Physical examination showed a scattered macular exanthem of discrete erythematous macules on the trunk and extremities with involvement of the palms; oral mucositis; a tender prostate; and a friable, mildly inflamed anal mucosa with some ulcerations and excoriations. His persistent urinary retention required short-term urethral catheterization. Swab cultures of the rectum for herpesvirus, *Chlamydia*, and *Neisseria gonorrhoeae* were negative. Serologic testing for syphilis was negative.

On January 20, a CD4+ T-lymphocyte count drawn on January 15 showed 338 cells/μL (26%); plasma HIV RNA level was 234,000 copies/mL. His exanthem had become maculopapular, diffuse, and pruritic. Antiretroviral therapy with efavirenz, zidovudine, and lamivudine was initiated at the correctional facility to treat his primary HIV infection. A genotype of his pretreatment HIV isolate collected on January 14 showed sensitivity to all antiretroviral agents.

On January 21, he complained of paresthesias involving the tips of his fingers. On January 22, he was able to urinate spontaneously. On January 30, he reported a poor appetite; his weight had decreased 1.4 kg since January 16; physical examination showed a resolving, salmon-colored macular exanthem across the trunk and upper and lower extremities and resolution of his oral and anal lesions.

On February 4, his plasma HIV RNA was 2,463 copies/mL, and his CD4+ T-lymphocyte count was 1,575 cells/μL. An EIA for HIV was positive, and a Western blot was positive with bands present for p24, gp40, p55, and gpl60. On February 9, he reported that his appetite had recovered and his paresthesias had resolved; his weight had increased 0.5 kg since January 30. On March 19, his plasma HIV RNA was <50 copies/mL, and his CD4+

T-lymphocyte count was 1,056 cells/μL. On April 1, the court ordered that he be released, and he moved to another state.

Conclusions

In April 2005, Lambert et al. (*4*) reported concerns that a resurgence of HIV/AIDS may be imminent, fueled in part by increasing indicators of high-risk behavior in the gay and bisexual population. The March 2005 report by Markowitz et al. (*5*) regarding men who have sex with men, use of methamphetamine, and transmission of HIV underscores these concerns. The high prevalence of HIV infection in overcrowded and understaffed correctional facilities further accentuates these concerns and poses a public health challenge.

On December 31, 2002, 2.0% of state prison inmates were positive for HIV (*1*); among interviewed jail inmates, 1.3% disclosed they were HIV positive. Estimates of the proportion of inmates who indulge in homosexual intercourse while in prison range from 2% to 65%, and most of this sexual contact is likely unsafe because few correctional facilities address the issue of intraprison sex or distribute condoms (*2*). Nevertheless, inmate-to-inmate transmission of HIV has rarely been documented. Taylor et al. (*6*) proposed that the paucity of evidence for transmission of HIV infection within correctional facilities is probably accounted for by the difficulties in determining the time of HIV seroconversion in relation to the period of incarceration, rather than by the rarity of the event.

Krebs and Simmons (*2*) used surveillance data from a 22-year period (January 1, 1978–January 1, 2000) to identify inmates who contracted HIV while incarcerated in the Florida state prison system. They reported that a minimum of 33 inmates contracted HIV while in prison, compared to 238 who contracted HIV after leaving prison; inmates were more likely to have contracted HIV in prison by having sex with other men than through injection drug use.

Additional reports of HIV transmission in correctional facilities have been published from Illinois (8 HIV seroconversions) (*7*), Nevada (2 seroconversions) (*8*), Maryland (2 seroconversions) (*9*), Australia (1 seroconversion) (*10*), and Scotland (*11*). Yirrell et al. (*11*) determined that 13 inmates had acquired HIV infection by sharing needles during their incarceration.

Acute retroviral syndrome and primary HIV infection may be frequently unsuspected by the evaluating clinician because the signs and symptoms are relatively nonspecific. However, within correctional facilities, the diagnosis of primary HIV infection should be considered in the differential diagnosis of any inmate with an acute febrile illness associated with pharyngitis and mucocutaneous lesions. Our report is limited in that virus was not sequenced to document transmission between inmates.

Early diagnosis of primary HIV infection can lead to successful antiretroviral intervention (*12*) and prevention of secondary transmission. Whether antiretroviral treatment of acute HIV infection results in long-term virologic, immunologic, or clinical benefit is unknown. In October 2005, the US Department of Health and Human Services Clinical Practices Panel noted that antiretroviral treatment of acute HIV infection is optional. If the clinician and patient elect to treat acute HIV infection with antiretroviral therapy, treatment should be implemented with the goal of suppressing plasma HIV RNA to below detectable levels; resistance testing at baseline will likely optimize virologic response (*13*).

We urge correctional facilities to address the issue of unprotected sex among inmates and the associated transmission of sexually transmitted diseases within institutions (*14*). In 2001, Wolfe et al. (*14*) reported that from 1991 to 1999, ≥5 outbreaks of syphilis occurred in Alabama prisons; multiple concurrent sex networks involving 4, 7, and 10 inmates were identified in the 1999 outbreak. Wolfe et al. recommended that condom distribution should be used to control sexually transmitted disease in correctional facilities. Nevertheless, in 2006, <1% of US correctional facilities provide inmates with condoms. Reasons for not providing condoms include the conflict with policies forbidding sexual intercourse (or sodomy) and the potential for condoms to be used as weapons or to smuggle contraband (*15*). In contrast, condoms are available to inmates in all Canadian federal prisons and some provincial prisons; few problems related to condom distribution have been reported from those systems (*15*). Wolfe et al. proposed that providing condoms to prisoners may yield additional public health advantages beyond the prison walls if exposure to and experience with condoms in this setting translate into increased use after release.

Correctional staff and inmates should be educated about the consequences of unprotected sex and the signs and symptoms of acute retroviral syndrome. Because many correctional systems contract for medical care, and because staff turnover rates are high, annual education should be implemented. Education for staff who screen sick inmates is critical (*14*), and all inmates should have access to HIV counseling and testing.

Dr Macher is a 30-year veteran of the US Public Health Service. He retired in the summer of 2005 and currently advocates for indigent inmates' access to the standard of care. His research interests include the effects of privatization on correctional health care and postrelease access to continuity of care.

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Fatal Human Infection with *Rickettsia rickettsii*, Yucatán, Mexico

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The first fatal *Rickettsia rickettsii* infection was diagnosed in the southwest of Mexico. The patient had fever, erythematous rash, abdominal pain, and severe central nervous system involvement with convulsive crisis. The diagnosis of *R. rickettsii* infection was established by immunohistochemistry and specific polymerase chain reaction.

Five spotted fever group (SFG) rickettsioses have been documented in the Western Hemisphere: Rocky Mountain spotted fever (RMSF) (*Rickettsia rickettsii*), fleaborne spotted fever (*R. felis*), rickettsialpox (*R. akari*), African tick-bite fever (*R. africae*), and infection with *R. parkeri*. *R. rickettsii* infections have been identified in southern Canada, the United States, northern Mexico, Costa Rica, Panama, Brazil, and Argentina (*1–5*). *R. felis* has been detected in humans, *Ctenocephalides* fleas, and opossums in the United States, Mexico, Brazil, Uruguay, and Peru (*6–9*); *R. parkeri* in the United States, Uruguay, and Brazil (*10*); *R. africae* on islands of the Caribbean Sea; and *R. akari* in the United States. Among these agents, only *R. rickettsii* is known to cause fatal infections. The only SFG rickettsial agent previously documented to cause human infections in the Yucatán Peninsula of Mexico, where >5% of the population have antibodies to SFG rickettsiae, is *R. felis*, which is present in 20% of cat fleas (*Ctenocephalides felis*) (*7*,*11*). Previously, Rocky Mountain spotted fever had been recognized mainly in northern Mexico, beginning in the 1940s (Figure 1). We report a case of fatal RMSF in a previously healthy child in southwestern Mexico, where this infection had not previously been recognized. The case may herald the reemergence of RMSF throughout the Americas or be evidence of a misdiagnosed disease in Latin America.

The Case

In August 2004, a previously healthy girl, age 4 years and 9 months, was found with 2 ticks attached to her left ear lobe 3 days before the onset of fever and headache. She was treated with amoxicillin and had a progressively severe illness with fever, abdominal pain, headache, fatigue, diarrhea, nausea, vomiting, cutaneous paresthesias, myalgia, rigidity of the left arm and both legs, and an erythematous rash involving the extremities and thorax. At the site of tick attachment on the left ear, an eschar was observed in association with tender regional lymphadenopathy at the time of admission. Clinical laboratory evaluation showed elevated serum urea and hepatic transaminase concentrations and neutrophil leukocytosis. Thrombocytopenia was not reported. On day 7 of illness, seizures developed, and the patient died.

All research was approved by the ethics committee of the Faculty of Medicine, Universidad Autonoma de Yucatán. Necropsy showed cerebral edema and hemorrhages in the pleura, lungs, pericardium, endocardium, and gastric mucosa. Histopathologic examination demonstrated many lesions of lymphohistiocytic vasculitis, characteristic of rickettsial infection. Immunohistochemical staining performed with specific monoclonal antibodies against SFG lipopolysaccharide as described previously (*4*) identified SFG rickettsiae in vascular endothelial cells in multiple foci in the brain, lung, spleen, and liver (Figure 2).

The patient lived in an urban area where many dogs and sheep also lived. Two months before the onset of the patient's illness, seizures developed in a dog and a sheep belonging to the family; both died.

DNA was extracted from paraffin-embedded spleen and formaldehyde-fixed liver, lung, and brain tissue by DNeasy Tissue kit (Qiagen, Valencia, CA, USA) as previously described. Polymerase chain reaction (PCR) ampli-

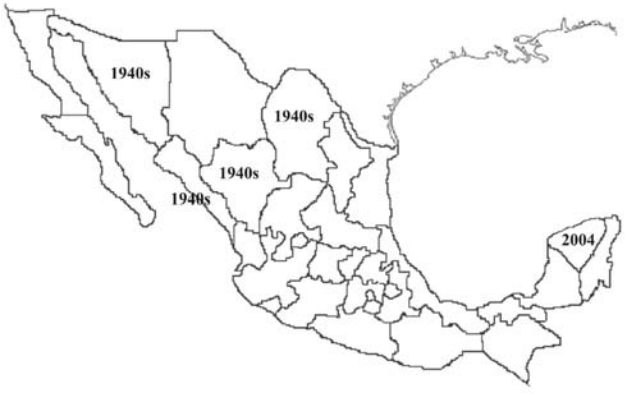

Figure 1. Map of Mexico showing the period and regions where human cases caused by *Rickettsia rickettsii* were detected.

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fication of the extracted DNA used genus-specific primers for the rickettsial 17-kDA protein gene, 5′-TGTCTATCAATTCACAACTTGCC-3′ and 5′-GCTTACAAAATTCTAAAAACCATATA-3′. The fragment was cloned into the TPO TA pCR 2·1-TOPO vector (Invitrogen, Frederick, MD, USA), and selected clones from the same cloning reaction were sequenced 3 times with a ABI Prism 377 automated sequencer (Perkin Elmer, Foster City, CA, USA), and the sequences were compared to those in the GenBank database by using the Basic Local Alignment Search Tool at the National Center for Biotechnology Information (*12*). Two clones (GenBank accession no. DQ176856) identified the DNA sequence of the 434-bp product as *R. rickettsii* (GenBank accession no. AY281069), which differed by only 1 nucleotide.

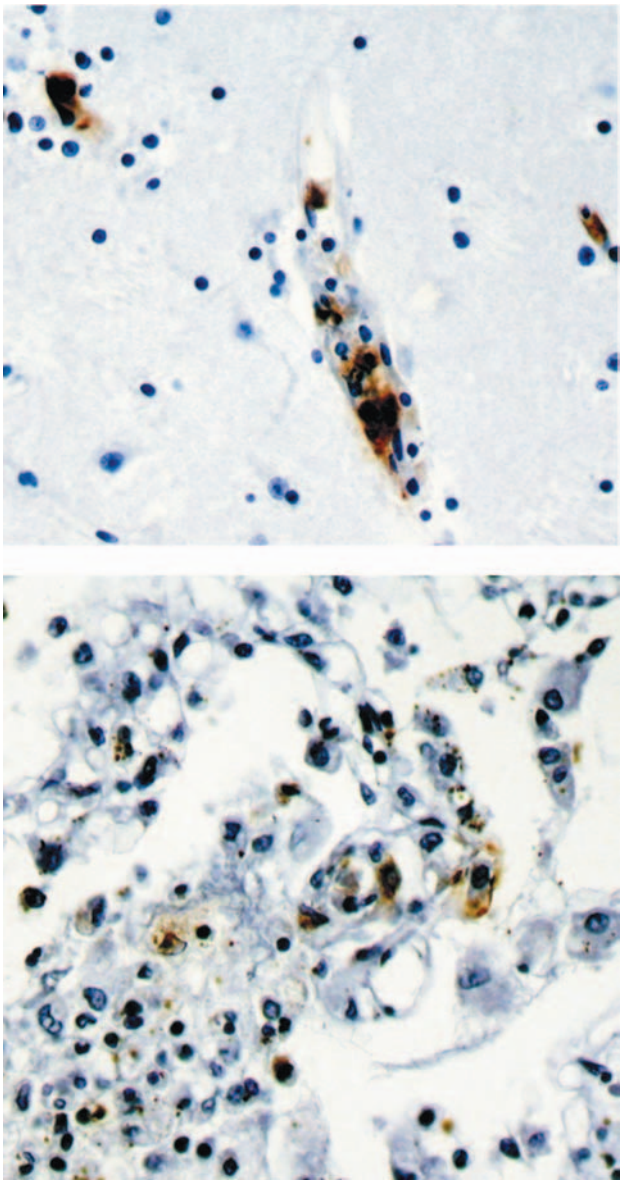

Figure 2. Immunohistochemical stain shows spotted fever group rickettsia in endothelial cells of a blood vessel in brain (top panel) and lung (bottom panel).

Conclusions

The first documentation of RMSF in southwestern Mexico reflects, in part, the development of a regional research laboratory with knowledge and interest in rickettsiology, a situation that is lacking in most parts of Latin America. However, this finding may also represent an early warning of widespread reemergence of RMSF. In the United States, 2 large waves of emergence of RMSF have been documented during the last century; peaks were seen in the mid-1940s and early 1980s. In 2004, a total of 1,514 cases of RMSF were reported, the highest number ever in a single year, including an outbreak in Arizona, where very few cases had been diagnosed previously (*13*).

The recent diagnosis of the first cases of RMSF in Argentina (*5*), reemergence of RMSF in large clusters with a case-fatality ratio of 50% in Brazil (*4*), and reemergence of isolation-documented fatal RMSF in Colombia suggest that the factors responsible for the increased incidence are widespread. This phenomenon was noted for the parallel reemergence of RMSF and Mediterranean spotted fever during the 1970s and 1980s (*14*). The ecologic and epidemiologic factors responsible for the periodically increased transmission of *R. rickettsii* from ticks to humans have not been determined.

Most aspects of this fatal case are typical of RMSF: tick bite, illness in a dog at the residence, disseminated lymphohistiocytic vasculitis, acute renal failure, and fatal seizures associated with cerebral rickettsial endothelial infection, increased vascular permeability, and cerebral edema. However, other features are unusual for RMSF. Although eschars are common in most SFG rickettsioses, they have seldom been documented in RMSF (*15*). Despite the hypothetical spread of SFG rickettsiae from the site of tick feeding through lymphatic vessels to regional lymph nodes, regional lymphadenopathy is not a typical feature of RMSF. Moreover, hemorrhages are not a prominent feature in most cases of RMSF in North America, compared with reports of severe hemorrhages in cases from Brazil. Whether such clinical and pathologic differences are real or not remains to be determined as well as their potential association with genetic differences in rickettsial virulence factors or host factors, including deleterious effects of medications taken early in the course of illness.

This case illustrates the major deficiency in controlling RMSF, the lack of a diagnostic test that is effective early in the course and widely available. Patients seldom have antibodies to *R. rickettsii* when they are first seen by a clinician. PCR detection of rickettsial DNA in blood is insensitive, particularly early in the course. Diagnostic immunohistochemistry and PCR are available in only a

few reference laboratories. Timely consideration of the diagnosis and empiric treatment with doxycycline are the best that can be achieved in most settings in Mexico, the United States, or elsewhere.

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Dr Zavala-Castro investigates rickettsial diseases at the Faculty of Medicine of the University of Yucatán, Mexico. His research interests focus on host-bacteria-vector relationships and mechanisms, rickettsial evolution, and new diagnostic methods and vaccines for rickettsial diseases.

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Shrews as Reservoir Hosts of Borna Disease Virus

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Borna disease virus (BDV) is the causative agent of severe T-cell–mediated meningoencephalitis in horses, sheep, and other animal species in central Europe. Here we report the first unequivocal detection of a BDV reservoir species, the bicolored white-toothed shrew, *Crocidura leucodon*, in an area in Switzerland with endemic Borna disease.

Borna disease (BD) is a severe immunopathologic disorder of the central nervous system induced by infection with Borna disease virus (BDV), the prototype of a new virus family, *Bornaviridae*, within the order Mononegavirales. With 1 notable exception (strain No/98 [*1*,*2*]), all BDV isolates exhibit a highly conserved genome (*3*). BD primarily affects horses and sheep, although many animals can be infected experimentally (*4*–*6*). BD is restricted to well-defined disease-endemic regions in central Europe (*7*). The precise pathogenesis and epidemiology of natural BDV infections are unknown; however, several unique epidemiologic features point towards the existence of BDV reservoir populations other than the final hosts (*4*,*8*,*9*).

Laboratory diagnosis of BD relies on postmortem examination of the brain by histologic techniques, immunohistologic (IHC) testing, reverse transcription–polymerase chain reaction (RT-PCR), and recently TaqMan real-time RT-PCR (*5*,*7*). Histologically, BD manifests as mononuclear inflammation (meningoencephalitis), especially in the hippocampal area, where frequently intranuclear, eosinophilic (so-called Joest-Degen) inclusion bodies can be observed (*7*,*10*). The nucleoprotein (p38/40, open reading frame [ORF] I) and the phosphoprotein (p24, ORF II) are the 2 most important BDV target proteins and genes for IHC, RT-PCR, and TaqMan real-time RT-PCR, respectively (*4*,*5*,*7*,*8*,*10*–*12*).

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The Study

The objective of this study was to search for the putative natural reservoir hosts or vectors of BDV in an environment in which BD is endemic in horses and sheep. Eight moles, 3 shrews, and 87 mice of different species were trapped between 1999 and 2003 in a small village near Chur, Switzerland (Malix, located 1130 m above sea level), an area in which BD is endemic in horses and sheep. The animals were euthanized and stored at –20°C for later examination. Their brains were divided into 2 equal parts, one half was fixed in 4% formaldehyde, cut transversally into several equal parts and embedded in paraffin for microscopic evaluation; the other half was stored in tubes at –20°C. IHC was performed as described previously (*5*,*7*,*10*). A recently established TaqMan real-time RT-PCR system (Applied Biosystems, Rotkreuz, Switzerland) (*5*) was used to detect and quantify BDV nucleic acid in all brain samples and selected heart samples from the mice, shrews, and moles.

All samples that were positive by TaqMan real-time RT-PCR in the Zurich laboratory as well as selected negative samples were reevaluated blind in the Vienna laboratory by conventional RT-PCR, beginning with frozen parallel samples that had been stored. RT-PCR and sequencing were carried out as described by Kolodziejek et al. (*3*). The resulting amplicons of 1 shrew (no. 144) were sequenced by employing the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA) and an ABI Prism 310 genetic analyzer (PE Applied Biosystems).

Histologic examination showed no inflammation or degenerative processes in any of the 98 brains. Three of the 98 brains, however, were positive for BDV antigen by IHC with both monoclonal antibodies. Labeling of inclusion bodies and some intracytoplasmic staining was notable from the forebrain (prosencephalon) to the mesencephalon (Figure, panel A). The 3 BDV-antigen–positive brains originated from the 3 shrews investigated, while all samples from moles and different species of mice proved negative. Identical results were obtained by TaqMan real-time RT-PCR; all 95 mouse and mole brains were negative, while the brain samples of all 3 shrews were positive (Table).

In addition to brain tissues, the hearts of the 3 BDV-positive shrews and of 2 BDV-negative mice were examined. In the heart of shrew 144, BDV-positive labeling was found in a multifocal pattern (Figure, panel B). The myocardiocytes showed intracytoplasmic and intranuclear labeling, but no inclusion bodies could be recognized. The hearts of the other 2 shrews and the 2 mice were negative by IHC. By TaqMan real-time RT-PCR, however, the hearts of all 3 shrews proved positive (Table), while the hearts of the mice were negative. The observed differences

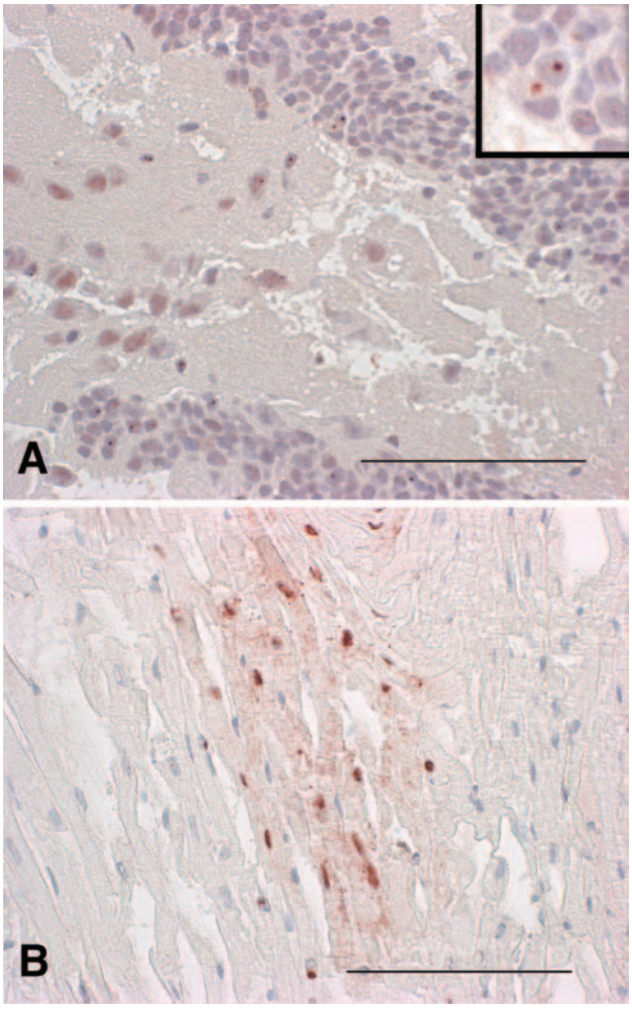

Figure. A) Distribution of Borna disease virus (BDV) p24 antigen in the hippocampus of shrew 144. Note the so-called Joest-Degen intranuclear inclusion bodies in multiple neurons in the gyrus dentatus; in some neurons, a homogenous intracytoplasmic staining can be seen (immunohistochemistry, ChemMate method [DAKO, Cygomation, Zug, Switzerland], ×40 [bar, 100 μm]). Inset: Joest-Degen intranuclear inclusion bodies are visible in multiple neurons in the gyrus dentatus (ChemMate method, ×100). B) Focal distribution of BDV p38/40 antigen in the heart of shrew 144: granular intracytoplasmic and homogenous intranuclear labeling (ChemMate method, ×40 [bar, 100 μm]).

in the results obtained by the 2 methods can be explained by the higher sensitivity of the TaqMan method compared to IHC.

When the brains of the shrews were analyzed by conventional RT-PCR, all 6 assays yielded amplicons of the expected sizes in all 3 shrews, whereas mouse samples negative by TaqMan real-time RT-PCR were also negative by conventional RT-PCR. The compiled sequences of shrew 144 resulted in a stretch of 2,041 nucleotides (nt), composing the complete N, X, and P protein-encoding regions, as well as the 5′-untranslated region of the X/P transcript of the BDV genome. The shrew-derived sequence (GenBank accession no. DQ251041) was verified as the expected BDV sequence by BLAST (available from http://www.ncbi.nlm.nih.gov/blast/) search. The sequence showed 99.9% identity to a BDV sequence derived from a horse (GenBank accession no. DQ251042), which died of BD near the location where the shrews were trapped, and to a BDV sequence from another horse from this region (GenBank accession no. AY374547), which differed by only 1 nt and 2 nt, respectively), confirming the identity of BDVs in this area.

Conclusions

Bicolored white-toothed shrews (*Crocidura leucodon*) are insectivores that are distributed from central Europe eastward to the Caspian Sea. In Switzerland, these shrews are found in the same areas where BD is endemic in horses, sheep, and other animal species. Bicolored white-toothed shrews are 93–125 mm long and weigh 7–13 g. In northern regions, they often live in gardens, outhouses, and farm buildings, and in the Alps, they can be found ≤1,600 m above sea level. Shrews are carnivores, and their diet consists of insects and snails, but they may also eat forage (*13*). They live on the ground and do not climb, which could explain why mainly horses (during the grazing season) or animals located in old-fashioned farmhouses without feeding troughs are affected by BD. BD shows an increased incidence in spring and (early) summer, when most shrews are found in pastures and in close contact with grazing animals. In winter, some species of the genus *Crocidura* show social acceptance and group together, which may be a possible source of infection between shrews (*13*). In an experimental study by Sauder and Staeheli (*14*), rats persistently infected with BDV and naive rats were put together, which led to infection of the naive rats after cohabitation. Most animals showed signs of disease 5–6 weeks after first contact with carrier rats, and high virus titers were found in their urine.

In the case of the shrews, no other organs, urine, or body fluids were available. Nevertheless, the experimental results in rats led to the conclusion that persistently infected rodents, in this case, shrews, most likely transmit the virus by excretions such as saliva or urine deposited on forage for horses and sheep. This suggestion is supported by the observation that the bulbus olfactorius near the ethmoid is heavily inflamed in cases of naturally occurring BD. Levels of viral antigen or viral RNA are also high in this region of the brain.

In conclusion, we postulate that shrews are reservoir hosts of BDV. Shrews also meet the criteria established for potential BDV reservoir species in a recent review article (*9*). Our finding, however, does not exclude the possibility

Table. Summary of immunohistologic and real-time RT-PCR findings*

Case no.	Organ	Immunohistology	Real-time RT-PCR Ct values† p24 and p40 (mean of 2 analyses)	Real-time RT-PCR Ct values 18S rRNA (mean value)	Calibrated values‡ (virus copies; mean of 2 analyses)
134	Brain	p24 positive	p24: 14.93 / 17.53	13.34	p24: 271.9
		p40 positive	p40: 15.87 / 19.35		p40: 40.87
	Heart	p24 negative	p24: 28.07 / 29.54	19.64	p24: 2.866
		p40 negative	p40: 27.94 / 30.05		p40: 1.749
137	Brain	p24 positive	p24: 24.00 / 25.04	24.41	p24: 13,246
		p40 positive	p40: 25.50 / 26.95		p40: 2,218.8
	Heart	p24 negative	p24: 33.88 / 38.07	19.66	p24: 0.0204
		p40 negative	p40: 33.84 / 35.24		p40: 0.0366
144	Brain	p24 positive	p24: 16.27 / 17.44	13.98	p24: 216.04
		p40 positive	p40: 17.09 / 18.88		p40: 46.289
	Heart	p24 positive	p24: 32.32 / 33.92	23.83	p24: 2.8235
		p40 positive	p40: 31.68 / 35.17		p40: 1.3375

*Histologic findings: no lesions on brain or heart for all 3 cases.
†RT-PCR, reverse transcription–polymerase chain reaction; Ct, threshold cycle no.
‡The calibrated values were calculated by using the sum of both normalized Ct-values of Borna disease virus p24 and p40, respectively, and dividing it by the normalized value of the 18s rRNA (5). Each organ was analyzed twice, and the evaluation was performed in duplicate. Please note that the viral load in each animal can be quite variable.

that other animal species living in this environment could also harbor BDV. In further studies, we will examine more shrews and additional organs, excretions, and secretions as well as other possible reservoir and vector species.

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Tularemia Outbreak, Bulgaria, 1997–2005

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The 1997–2005 tularemia outbreak in Bulgaria affected 285 people. Ten strains were isolated from humans, a tick, a hare, and water. Amplified fragment length polymorphism typing of the present isolates and of the strain isolated in 1962 suggests that a new genetic variant caused the outbreak.

Tularemia is a zoonotic disease caused by the gram-negative bacterium *Francisella tularensis* (*1*). During the last 10 years, several outbreaks occurred in different countries, causing tularemia to become a major problem on the Balkan Peninsula (*2–5*).

The first Bulgarian *F. tularensis* strain, isolated in 1962 from a muskrat (*Ondatra zibethica*) found in the lake of Srebarna reserve near the Danube River, was designated Srebarna19 (*6*). The first 4 tularemia cases in Bulgaria were reported in 1963 (*6,7*) after a small epidemic involving mostly employees in the Srebarna reserve. After 35 years of tularemia surveillance with no cases reported, a focal epidemic was detected near the end of 1997 (*2,8*). New cases appeared, and strains were isolated and characterized. A total of 285 cases of tularemia were reported and registered at the Bulgarian Ministry of Health in the period 1997–2004 and the first quarter of 2005. The outbreak areas in 1962 and 1997–2005 in Bulgaria are shown on Figure 1. The first case of tularemia was reported in November 1997 in a patient from a small town in the Slivnitsa region. From 1998 to 2000, 171 cases were reported (*8*). The outbreak seemed to abate during 2001 and 2002, when only 16 cases were documented. The incidence increased again in 2003 when 76 new cases were reported. An area ≈4,000 km^2 near the western border with Serbia and Montenegro was the epidemic focus of the outbreak.

All the patients exhibited the typical clinical picture of oropharyngeal, oculoglandular, or ulceroglandular tularemia. Four (1.4%) of the 285 patients had the oculoglandular form, 6 (2.1%) had the ulceroglandular form, and 275 (96.5%) had the oropharyngeal form. No deaths, complications, or relapses were observed.

Except for 1 seronegative patient, tularemia cases were diagnosed according to the confirmed case definition of the Centers for Disease Control and Prevention (*9*). Clinically relevant information was gathered by interviews, referral to hospitals, and questionnaires sent to general practitioners in the region and submitted to the reference centers for epidemiologic analysis. Three serum samples (acute phase, convalescent phrase, and l collected 3 months ± 15 days after the end of therapy) were collected from all patients (online Appendix; available from http://www.cdc.gov/ncidod/EID/vol12no04/05-0709_app.htm). All samples were tested with hemagglutination and tube-agglutination assays (BulBio-NCIPD, Sofia, Bulgaria) for anti-*Francisella* antibodies.

Fine needle biopsy specimens from enlarged lymph nodes were processed from 20 patients. Half of the volume from each specimen was cultured on modified Thayer-Martin agar (*10*), and the other half was processed for polymerase chain reaction (PCR). Water samples, collected from 41 wells, were also cultured through passage in guinea pigs (Appendix). Ten strains were isolated, 4 from patients, 4 from water, 1 from a hare, and 1 from a tick (Appendix). One of the human isolates (isolate Las) was from a seronegative patient. Identification of the strains was performed according to their microbiologic and antigenic properties by using standard methods (*10*). Direct immunofluorescence assay (IFA) with fluorescein isothiocyanate–conjugated anti-*Francisella* serum (BulBio-NCIPD) was used to detect *F. tularensis* antigens. DNA from biopsy specimens and strains was subjected to PCR with tul4 and RD1 primers (*11*). All investigated biopsy specimens and strains were PCR and IFA positive.

16S-PCR restriction fragment length polymorphisms (RFLP) and amplified fragment length polymorphism (AFLP) methods were used for molecular typing. For 16S-PCR RFLP, the genomic DNA was amplified by 16S rRNA universal primers (*12*). The 948-bp PCR product was digested with *Mbo*I, *Rsa*I, and *Hae*III enzymes. All strains exhibited a characteristic *F. tularensis* fingerprinting pattern, and no variations were found. For AFLP, DNA was digested with *Hind*III and *Mbo*I enzymes, adaptors were ligated, and selective PCR was carried out with Hind+0 and Mbo+C primers. Pearson correlation and unweighted pair group method with arithmetic averages (UPGMA) algorithms were applied for generating dendrogram (Figure 2). Three of the water isolates showed 100% similarity, and only 1 (Aqua D) was included in the dendrogram (Figure 2). A set of DNA samples from 27 *F. tularensis* strains originating from Asia, Europe, America, and Bulgaria were also typed. The AFLP method clearly discriminated the representatives of different

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Figure 1. The outbreak areas, Bulgaria, 1962 and 1997–2005.

phylogenetic *F. tularensis* groups. Although the 27 AFLP patterns show little variability (≤25%, Figure 2), distinctive clusters are seen. All of the subspecies *holarctica* cluster away from the subspecies *tularensis*. The fingerprinting pattern of a strain Srebarna19, isolated in 1962 during an outbreak near the Lake of Srebarna, shows high similarity with fingerprints of strains isolated in Europe (e.g., the 335–64, Italy 1964, Figure 2). The dendrogram clearly shows divergence between the 1962 Srebarna19 strain and

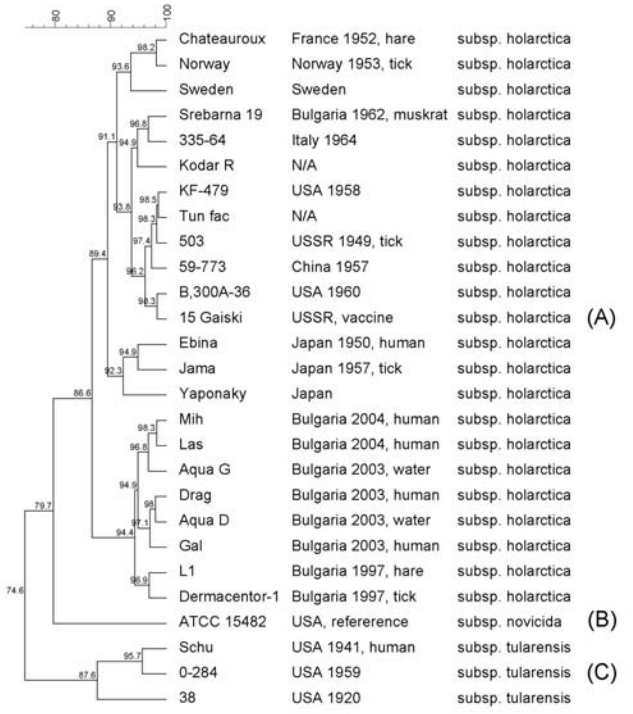

Figure 2. Amplified fragment length polymorphism dendrogram.

the organisms associated with the current outbreak. The human, water, and animal isolates from the current outbreak have ≈95% similarity. The human isolates are closely related to isolates recovered from well water but are more distantly related to isolates from the hare and tick. The AFLP data of isolates from the current outbreak support the hypothesis of a new genotype emerging in Bulgaria. AFLP also shows the emerging isolates to be genetically distinct from the European, Asian, and American isolates evaluated in this study.

Several publications describe outbreaks in the Balkan Peninsula with healthcare importance (*3–5*). Ten *F. tularensis* strains were isolated in Turkey (*3*). However, they were not genetically characterized. No isolation was attempted during the Kosovo outbreak in 1999 (*5*).

The origin of the 10 strains from the current outbreak is controversial, but they are clearly distinct from the other worldwide isolates included in our study. The new outbreak may be a result of the agricultural reorganizations in Bulgaria in 1990s. These changes affected the way in which the arable soil was ploughed, leaving rodent holes intact. As a result, the populations of rodents, considered the main reservoir of the infection, increased substantially (*13*).

Francisella organisms can survive in water for prolonged periods, probably by interaction with protozoa (*14*). The isolation of bacteria from 4 private wells in the affected area points to ingestion of contaminated food or drinking water as the probable route of infection. This finding is further supported by the observation that most of the cases represent the oropharyngeal form. Rodents (or their excrement) could be the source for water contamination, but this hypothesis is not confirmable because of the lack of later rodent isolates for comparison.

The organism might have been introduced by means of rodents and hares through the border with Serbia and Montenegro. Agricultural practices are alike in the neighboring countries, and a similar boom in the rodent population might also have occurred there. Such a migration is bidirectional, but a future collaborative study with colleagues from Serbia and Macedonia, where tularemia is also problematic, is necessary to answer this question. Typing isolates originating from different Balkan countries will show the genetic relatedness and biodiversity among resident *F. tularensis* populations.

The cases reported in 2004 and 2005 suggest that the outbreak is still in progress. These are the first data for genetic identification and typing of isolates from the Balkan region, and they show a new genotype of *F. tularensis* emerging as a cause of human disease in Bulgaria.

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Avian Influenza H5N1 in Naturally Infected Domestic Cat

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We report H5N1 virus infection in a domestic cat infected by eating a pigeon carcass. The virus isolated from the pigeon and the cat showed the same cluster as the viruses obtained during the outbreak in Thailand. Since cats are common house pets, concern regarding disease transmission to humans exists.

Highly pathogenic avian influenza (HPAI) H5N1 causes death in many avian species and mammals, including humans (*1*–*5*). In Thailand, infection by HPAI H5N1 has been reported in mammalian species such as tigers (*1*,*3*) and cats (*6*). Most infected mammals had high fever, panted, and showed symptoms of depression, myalgia, and nervousness (*4*). This article reports H5N1 infection in a cat during the early H5N1 outbreaks in Thailand and characterizes the genome of H5N1 virus isolated from the infected domestic cat.

The Study

In early February 2004, during the outbreak of HPAI (H5N1) in Thailand, a carcass of a 2-year-old male cat (*Felis catus*) was taken in an icebox 6 hours postmortem to the Faculty of Veterinary Medicine at Kasetsart University, Nakornpathom, Thailand. The cat's owner volunteered the information that the cat had eaten a pigeon (*Columba levia*) carcass 5 days before illness onset. The owner reported that the cat had a temperature of 41°C, was panting, and appeared to be depressed. Furthermore, the cat had convulsions and ataxia and died 2 days after onset of illness. The cat was given a single dose of 75 mg aspirin 1 day before it died; however, its body temperature remained elevated. Many dead pigeons were found in the area where the cat lived. Necropsy of the cat showed cerebral congestion, conjunctivitis, pulmonary edema, severe pneumonia, renal congestion, and hemorrhage in the intestinal serosa. Tissues from brain, trachea, lungs, mesenteric lymph nodes, intestines (duodenum, jejunum, and ileum), kidneys, liver, pancreas, spleen, and heart were collected, fixed with 10% buffered formalin, and processed for histopathologic examination. Histopathologic examination results showed nonsuppurative encephalitis, gliosis, mononuclear infiltration into the Virchow-Robin space, vasculitis, and congestion in both cerebrum and cerebellum. A microscopic lesion in the lung was caused by severe pulmonary edema, interstitial pneumonia, and congestion (Figure 1A). Multifocal necrosis in the liver (Figure 1B), tubulonephritis, and lymphoid depletion in the spleen were found. No abnormalities were detected in any other organs.

The paraffin-embedded tissues, including brain, lung, kidney, heart, spleen, pancreas, liver, and intestine tissue, were examined immunohistochemically.A polyclonal goat anti-HPAI H5N1 (Kasetsart University, Nakornpathom, Thailand) diluted 1:400 in phosphate-buffered saline was used as the primary antibody. The secondary antibody was polyclonal mouse anti-goat immunoglobulin G (Zymed Laboratories, Inc., San Francisco, CA, USA) diluted 1:200 in phosphate-buffered saline. Diamino benzidine was the substrate developed as a chromogen. Tissue from a cat that had been hit and killed by a car was used as the negative control. Sites displaying a positive H5N1 antigen reaction were in cerebral neurons (Figure 1C), heart (myocardial cells) (Figure 1D), pneumocytes, renal tubular epithelial cells, hepatic cells, and white pulp of the spleen

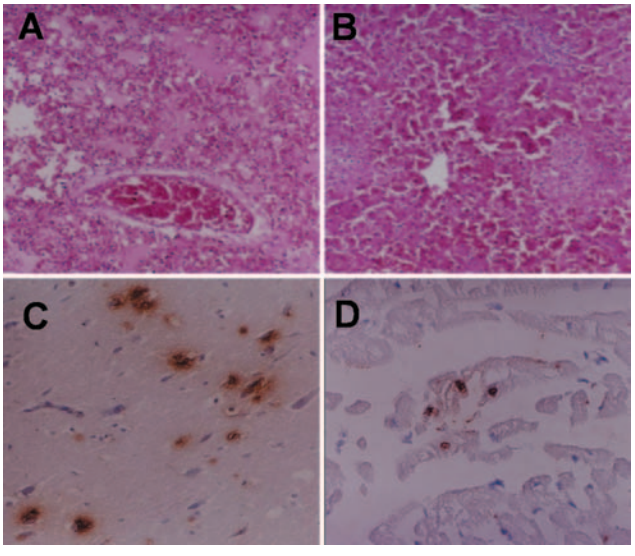

Figure 1. Microscopic lesions of the infected cat, lung edema with homogeneous pink material and congestion (A) and multifocal necrosis in the liver (B). Positive sites are shown by immunohistochemical examination of the infected cat in neurons (C) and cardiac muscle cells (D) (magnification ×100).

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(macrophages). The pancreas and intestine were negative for H5N1 antigen.

Parts of frozen brain, lung, liver, kidney, spleen, and duodenum content were ground separately, and virus isolation testing was conducted by using embryonated egg injection. Virus isolation testing was also conducted on pleural fluid and urine. Virus isolation testing was conducted by injecting pleural fluid, urine, and filtrates obtained from the ground tissues into the allantoic sac of 10-day-old embryonated chicken eggs. Embryonic death occurred 18 hours after injection. The allantoic fluids of the dead embryos were subjected to hemagglutination (HA) and hemagglutination inhibition tests. All fluids from the dead embryos were positive for avian influenza A (H5). The virus could be isolated from all injected specimens. To identify the subtype, reverse transcription–polymerase chain reaction was conducted, and the virus was confirmed to be influenza A H5N1 (*7,8*). The HPAI H5N1 isolate recovered from the infected cat's lung was labeled A/Cat/Thailand/KU-02/04. In addition, an isolate of HPAI H5N1 from an infected pigeon in the area where the cat lived was included in the study and labeled A/Pigeon/Thailand/KU-03/04.

H5N1 viruses isolated from the cat's (KU-02) and the pigeon's (KU-03) lung tissue were characterized in this study. The entire genome sequence was determined in the H5N1 isolate from the cat, while the H5N1 isolate from the pigeon was sequenced to specifically obtain the HA, neuraminidase, and PB2 genes. The sequences obtained from the cat (H5N1) (A/Cat/Thailand/KU-02/04) were submitted to the GenBank database under accession numbers PB2 (DQ236079), PB1 (DQ236080), PA (DQ23 6081), HA (DQ236077), NP (DQ236082), NA (DQ23 6078), M (DQ236084), and NS (DQ236083). The sequences obtained from the pigeon (H5N1) (A/Pigeon/ Thailand/KU-03/04) were submitted to GenBank under accession numbers HA (DQ236085), NA (DQ236086), and PB2 (DQ236087). Sequencing and phylogenetic analysis of the HA (Figure 2A) and NA (Figure 2B) genes of HPAI isolates (cat and pigeon) showed that the HA and NA genes of the viruses were similar to each other as well as to those of the viruses isolated from tigers, chickens, and humans in Thailand. Genetic comparisons of each gene of the cat isolate (KU-02) to those of the viruses isolated from chickens (January and July 2004) and tigers (January and October 2004) are shown in the Table. The analyses showed that the cat isolate (KU-02) was closely related to other H5N1 isolates collected from the region in 2004. This finding indicated that the H5N1 infection in the cat resulted from the virus circulating during the H5N1 outbreaks in early 2004. The HA gene of KU-02 and KU-03 contained multiple basic amino acid insertions at the HA cleavage site (SPQRERRRKKRR) as well as glutamine and glycine (Q222–G224) at the receptor binding site. The NA genes of KU-02 and KU-03 also had 20 amino acid deletions at positions 49–68 and contained histidine at position 274, indicating absence of antiviral drug resistant residues. The NS gene of the KU-02 isolate contained a 5–amino acid deletion (79–83), and the M2 gene of the KU-02 isolate contained an amino acid (asparagine) at position 31, conveying amantadine resistance. In summary, the viruses from the cat and the pigeon were similar to the H5N1 viruses isolated in Thailand and Vietnam in 2004, which had then been identified as genotype Z (*9*). A single amino acid substitution at position 627 of the PB2 gene (glutamic acid to lysine) was observed in the cat isolate (KU-02), as had previously been shown in the tiger isolates (*1*). In contrast, the PB2-627 amino acid residue of the pigeon isolate (KU-03) remained unchanged (glutamic acid).

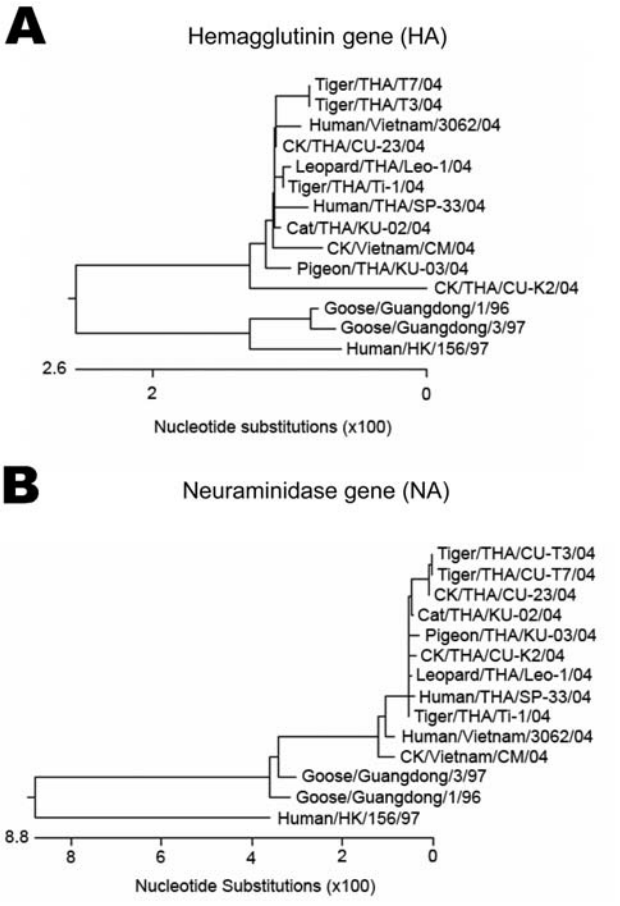

Figure 2. Phylogenetic analysis of the hemagglutinin (A) and neuraminidase (B) gene sequences of highly pathogenic avian influenza H5N1 from the cat in this study, compared with other sequences from GenBank database.

Table. Genetic comparison of the 8 gene segments of the cat isolate (KU-02) to those of H5N1 isolates from Thailand

Gene	Region of comparison	% nucleotide identity					
		KU-03 Pigeon (Jan 2004)	CU-T3 Tiger (Oct 2004)	CU-23 Chicken (Jul 2004)	Ti-1 Tiger (Jan 2004)	Leo-1 Leopard (Jan 2004)	CU-K2 Chicken (Jan 2004)
HA	46–1623	99.6	99.7	99.9	99.9	99.8	98.5
NA	25–1297	99.6	99.5	99.6	99.9	99.8	99.8
M	1–952	–	99.9	99.8	99.6	99.5	99.7
NS	36–824	–	99.5	99.9	99.9	99.7	99.5
NP	58–1474	–	99.7	99.7	99.8	99.9	99.9
PA	28–2132	–	99.6	99.8	99.6	99.6	99.5
PB1	62–2226	–	99.8	99.8	99.8	99.8	99.7
PB2	82–2220	99.5	99.6	99.7	99.6	99.7	99.6

Conclusions

This study is the first to report entire H5N1 genome sequences in a naturally infected domestic cat in Thailand, although experimental infection by H5N1 in domestic cats has been reported (*10*). The case of H5N1 in a cat was reported during the early H5N1 outbreaks in Thailand in February 2004. The likely route of infection was eating an infected pigeon carcass. Our study confirmed H5N1 infection in pigeon carcasses from the same area. In our study, both H5N1 isolates from the cat and the pigeon displayed characteristics identical to H5N1 isolates from the epidemic in Thailand. Moreover, genetic comparison indicated that the virus isolated from the cat (KU-02) was more similar to the H5N1 viruses from early 2004 (Ti-1 and Leo-1) than those from late 2004 (CU-T3 and CU-23).

Our results demonstrated that domestic cats are also at risk for H5N1 infection. Clinical signs and pathologic test results of the cat in this study are similar to those of an experimental study by Kuiken et al. conducted in 2004. Cats are companion animals and may live in very close contact with humans. Although no direct transmission of H5N1 from cats to humans has been reported, it is possible; therefore, cats in H5N1-endemic areas should be scrutinized. In *Felidae*, such as tigers and cats, probable horizontal transmission of H5N1 within the same species has been found (*4*). However, the risk for transmission from poultry to humans is probably much higher because poultry outnumber cats and excrete higher titers of the H5N1 virus (*10*). Hence, monitoring domestic animals for infection during H5N1 outbreak is recommended.

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Cryptosporidiosis Associated with Ozonated Apple Cider

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We linked an outbreak of cryptosporidiosis to ozonated apple cider by using molecular and epidemiologic methods. Because ozonation was insufficient in preventing this outbreak, its use in rendering apple cider safe for drinking is questioned.

Cryptosporidium spp. are protozoan parasites transmitted by the fecal-oral route that cause prolonged diarrhea. Only 2 reports describe outbreaks associated with apple cider (*1*,*2*), and none have been associated with ozonated cider. In October 2003, a northeast Ohio health department identified 12 local residents with laboratory-confirmed cryptosporidiosis; 11 had drunk a locally produced, ozonated apple cider (cider A) in the 2 weeks before illness. The cider was embargoed on October 24, and we initiated an investigation by using epidemiologic and molecular techniques to determine the cause and extent of the outbreak and the role played by the cider and ozonation.

The Study

We defined a probable case as a northeast Ohio resident with otherwise unexplained diarrhea for ≥3 days from September 1 to November 30, 2003, and a laboratory-confirmed case as a person with diarrhea and a positive *Cryptosporidium* laboratory result. Case finding encompassed interviewing persons with diarrhea who came to local health departments and emergency rooms and participants of school outings at which cider A was served. We then conducted 2 epidemiologic studies in which questionnaires showed exposures classically associated with *Cryptosporidium* transmission such as food, drinking and recreational water, person-to-person contact, animals, and travel.

Study 1 compared laboratory-confirmed case-patients and 2 controls (persons without diarrhea, abdominal pain, or vomiting) per case matched on age and county of residence and identified through random-digit dialing. Additionally, we conducted a retrospective cohort study of school children (study 2) who attended field trips at which cider A was served.

Stool samples from case-patients were screened by wet preparation and tested for *Cryptosporidium* by using an immunofluorescent assay (Meridian Merifluor *Cryptosporidium*/*Giardia* DFA kit, Meridian Bioscience, Cincinnati, OH, USA). Identification of *Cryptosporidium* in these samples was attempted by using 2 methods: 1) genotyping isolates by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis of the small subunit (SSU) rRNA gene (*3*,*4*) with subtyping by DNA sequence analysis of the GP60 gene (*5*), and 2) amplification of the SSU rRNA gene with genotype differentiation by a microsatellite marker (ML-1) (*6*,*7*).

Cider samples were concentrated by centrifugation, and water samples were concentrated by Environmental Protection Agency method 1623 (*8*). *Cryptosporidium* genotyping was performed by using the same methods described for stool samples (*4*). Newly designed GP60 primers were used to subtype cider samples (*9*).

We identified 23 laboratory-confirmed and 121 probable case-patients with onset dates from September 3 to November 19, 2003 (Figure); the first cider-related case occurred September 22. The median patient age was 20 years (range 1–80). The median incubation period was 7 days (range 1–21), and median period of diarrhea was 7 days (range 3–52). Two patients were hospitalized and none died.

In study 1, we enrolled 19 laboratory-confirmed case-patients and 38 age-matched, community-based controls. Twelve of 19 case-patients, but 0 of 38 controls, had drunk cider A. Although the matched odds ratio (OR) for this association was incalculably high, the lower limit of the 95% confidence interval (CI) was 5.6. Although 3 other exposures were also associated with illness by univariate analysis (Table), only drinking cider A was associated with illness in a conditional logistic regression model that included all of these exposures (estimated OR 14.0, 95% CI 1.8–167).

In study 2, we enrolled 402 persons who participated in outings at which cider A was served. Thirty-three (10%) of

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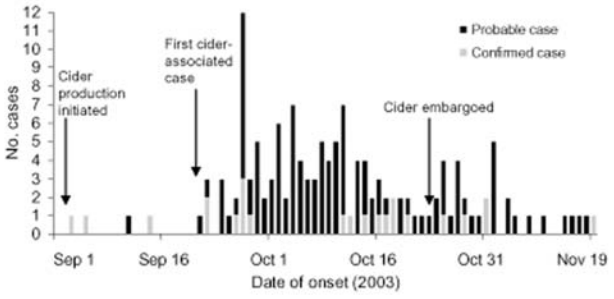

Figure. Laboratory-confirmed (n = 23) and probable (n = 121) cases of cryptosporidiosis from drinking ozonated apple cider, Ohio, 2003.

the 329 persons who drank cider A became ill, while only 2 (3%) of 73 who did not drink cider A became ill (adjusted relative risk 4.7, 95% CI 1.2–18.1). Only drinking cider A remained significantly associated with illness in a multivariate logistic regression model that included 4 other exposures that increased risk in univariate analysis (estimated OR 5.7, 95% CI 1.2–26.6).

No employees from the orchard or the separate cider pressing facility reported diarrhea from September 1 to November 7, 2003 (date of interview). The water supply for both was negative for *Cryptosporidium*; employees used "few" dropped apples for cider production. During production, an ozonating apparatus (Golden Buffalo Company, Orange, CA, USA) was used to treat the cider, which was then stored in refrigerated tanks. Most cider was ozonated a second time, then sold in plastic jugs onsite and at nearby grocery stores. Remaining cider was sold through a tap in the orchard's store. In contrast to jugged cider, this cider was not reozonated.

We performed a *Cryptosporidium* PCR on all 14 available samples from the laboratory-confirmed case-patients. Twelve (85.7%) of these were PCR positive; 11 of these 12 samples were identified as *Cryptosporidium parvum* and 1 as the cervine *Cryptosporidium* genotype (W4). Subtype identification of stool samples yielded 2 closely related *C. parvum* subtypes (IIaA15G2R1 and IIaA17G2R1).

The remaining contents of a jug of cider A that a laboratory-confirmed case-patient had partially drunk were also positive by PCR for *C. parvum* subtype IIaA17G2R1. This case-patient's stool sample yielded the same subtype of *C. parvum*, as did 4 other case-patients' stool samples; all of these persons drank cider A in the 2 weeks before illness onset.

Conclusions

Our investigation strongly implicates cider A as the cause of this outbreak. The timing of cider A production closely paralleled the outbreak, and drinking cider A was the only predictor significantly associated with illness in both univariate and multivariate analyses of both epidemiologic studies. Furthermore, detection of *C. parvum* subtype IIaA17G2R1 from the sample of partially drunk cider A from a laboratory-confirmed case-patient provided further evidence of this link.

The 2 *C. parvum* subtypes found in this outbreak likely represent a common contamination source; both are common in cattle, and multiple subtypes are commonly found on farms (*10*). This outbreak highlights the need for continued development of molecular biologic methods because these techniques will be useful to identify and define future *Cryptosporidium* outbreaks and supplement epidemiologic associations.

An issue raised by this outbreak is the role of ozonation in the treatment of apple cider. New regulations (Hazard Analysis and Critical Control Point [HACCP] standards) enacted by the US Food and Drug Administration (FDA) in 2001 (*11*) require juice manufacturers to demonstrate a

Table. Association of selected exposures to cryptosporidiosis from drinking of ozonated apple cider, Ohio, 2003*

Exposure	Cases (n = 19), No. exposed/total (%)	Controls (n = 38), No. exposed/total (%)	MOR	95% CI
Drinking cider A	**12/19 (63)**	**0/38 (0)**	–	Lower limit **5.6**†
Eating apples from orchard A	**4/19 (21)**	**0/38 (0)**	–	Lower limit **1.3**†
Eating green onions	0/19 (0)	6/37 (16)	0	–
Eating lettuce or garden salads	9/18 (50)	26/38 (68)	0.6	0.2–1.6
Eating raw berries	0/19 (0)	9/38 (24)	0	–
Drinking cider other than cider A	9/19 (47)	9/37 (24)	3.3	0.9–11.6
Drinking unfiltered tap water at home	12/19 (63)	17/37 (46)	2.3	0.7–8.1
Drinking well water at home	6/18 (33)	11/38 (29)	1.1	0.4–3.6
Swimming in recreational water	1/19 (5)	7/38 (18)	0.3	0.03–2.2
Household contact in day care	1/19 (5)	4/37 (11)	0.3	0.02–5.3
Household contact with diarrhea	**12/19 (63)**	**7/38 (18)**	**5.3**	**1.5–18.4**
Travel >50 miles from home	7/19 (37)	12/38 (32)	1.3	0.4–4.9
Contact with any animal	14/19 (74)	33/38 (87)	0.4	0.08–1.7
Contact with cattle	**6/19 (32)**	**3/38 (8)**	**5.5**	**1.1–28.4**

*MOR, matched odds ratio; CI, confidence interval. Significant values are in **boldface**.
†Exact one-sided 95% CI.

5-log reduction of "the most resistant microorganism of public health significance" in their production process.

Because this is the third reported *Cryptosporidium* outbreak related to unpasteurized apple cider (*1*,*2*), whatever sterilization procedure is used must be effective against *Cryptosporidium*. Although pasteurization kills *Cryptosporidium* oocysts (*12*), no data exist on the use of ozonation against *Cryptosporidium* in food or juice products, where turbidity and low temperature render ozonation less effective (*13*,*14*). Furthermore, ozonation is difficult to standardize because effectiveness depends on contact time and concentration.

The consideration of ozonation is important because effective disinfection would have prevented the outbreak unless contamination occurred at the final step before distribution. Furthermore, of the 12 ill persons in the case-control study who drank cider A, 6 drank once-ozonated cider and 6 drank twice-ozonated cider, suggesting that even repeated ozonation was inadequate to kill *Cryptosporidium*. The failure of ozone could have been due to an inherent inadequacy for killing *Cryptosporidium* in apple cider or improper use; either possibility emphasizes the problems with ozonation in this setting and the need for further testing before its use is accepted.

Given the paucity of evidence supporting ozonation for apple cider disinfection or for killing *Cryptosporidium* in this product, and its apparent failure in this outbreak, the FDA issued an addendum to its HACCP rule (*15*). This addendum advises that juice makers should not use ozone in their manufacturing process unless they can prove a 5-log pathogen reduction through ozonation. To our knowledge, no studies have established this reduction to date.

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Dr Blackburn is currently a clinical assistant professor of infectious diseases at Stanford University. He performed this work while an Epidemic Intelligence Service officer in the Division of Parasitic Diseases at the Centers for Disease Control and Prevention. His research interests include cryptosporidiosis, schistosomiasis, and strongyloidiasis in refugee populations; angiostrongyliasis; and use of insecticide-treated bed nets to control malaria and lymphatic filariasis.

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Long-term Follow-up for Multidrug-resistant Tuberculosis

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Patients treated in Peru for multidrug-resistant tuberculosis (MDR-TB) were followed-up for a median of 67 months. Among 86 patients considered cured after completion of treatment, 97% remain healthy; 1 patient relapsed. Employment increased from 34% before treatment to 71%. We observed favorable long-term outcomes among MDR-TB patients.

Increasing awareness of the rising global rates of multidrug-resistant tuberculosis (MDR-TB) has led to a concerted international effort to confront this disease, particularly in countries with a high incidence of TB (*1*–*3*). Nonetheless, despite cure rates >80% in some programs, MDR-TB patients tend to have chronic disease and require prolonged therapy.

Little is known about the long-term follow-up of patients treated for MDR-TB, including rates of relapse and chronic disability among cured persons. Among patients treated for pansusceptible TB, chronic disability caused by pulmonary sequelae and malnutrition can be substantial (*4*). Given the prolonged nature of MDR-TB, one might expect higher rates of chronic disability among patients with drug-resistant TB compared with those with pansusceptible TB. To explore these questions, we conducted long-term follow-up, defined as follow-up for a minimum of 4 years after treatment was initiated, of MDR-TB patients who received individualized therapy in Lima, Peru (*1*).

The Study

We performed a retrospective study among all patients who initiated individualized, community-based MDR-TB therapy from August 1, 1996, to March 1, 2000. The details of patient identification, enrollment, and treatment are described elsewhere (*5*). Patients were resistant to a median of 5 drugs (range 2–9). Regimens generally included at least 5 drugs to which the infecting isolate was susceptible, and treatment duration was 18–24 months. Routine follow-up after completion of MDR-TB therapy included 1) routine smear microscopy and culture on sputum samples 1 month after completion of treatment and then every 6 months for 1 year; 2) subsequent smear microscopy, culture, and clinical evaluation by a TB physician for any episode of potential TB symptoms, e.g., a respiratory illness lasting >14 days, hemoptysis, or weight loss of unclear cause; and 3) continued contact with Socios en Salud (the community-based organization working with the Ministry of Health on this MDR-TB treatment project) through the network of health promoters, patient group therapy sessions, and a social assistance program. Thus, loss to follow-up or undocumented medical attention for respiratory illness is rare.

When reporting cohort outcomes, the MDR-TB working group recommends follow-up for 2 years from the time of treatment initiation when reporting cohort outcomes (*6*); however no international definition of long-term follow-up for MDR-TB cohorts exists. Therefore, we defined long-term follow-up as twice the duration set forth by the MDR-TB working group.

We conducted a chart review to determine TB-related symptoms and employment status of persons recorded at baseline by the intake physician before they received MDR-TB therapy. In addition to data obtained through routine patient follow-up as per program norms described above, Socios en Salud staff involved in the social assistance program, members of group therapy sessions, and health promoters were interviewed to obtain additional follow-up information about the patients, including income, employment status, and household information. A community health worker conducted home visits to interview all patients; patients were questioned about current symptoms as well as their socioeconomic status. The study was reviewed and approved by the institutional review board at Harvard Medical School; local institutional review was not required.

Among 120 persons enrolled in this study, 23 patients died during treatment, and 1 person remained in culture-negative treatment at the time of analysis. Two patients (both of whom had defaulted from treatment) were lost to follow-up.

Data are reported on the remaining 96 (80%) patients who were alive at the time of stopping MDR-TB therapy. Patients were followed for a median (95% confidence interval) of 67 (47–88) months after initiation of treatment and a median of 46 (3–84) months after completion of treatment. As summarized in the Table, 86 (72%) patients were considered cured, 9 (8%) defaulted from treatment, and 1 (1%) had failed treatment.

Among those who were considered cured at the time of treatment completion, 83 (97%) are currently healthy. One patient relapsed 1 month after completion of treatment; this patient refused retreatment and subsequently died of TB.

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Table. Status of 96 multidrug-resistant tuberculosis patients after a median of 67 months of follow-up

Current status	Status at time of stopping treatment				Total
	Cured	Abandoned, culture positive	Abandoned, culture negative	Failed	
Alive, culture negative	83	0	2	0	85
Alive, culture positive	0	0	0	0	0
Died, culture positive	1	3	1	1	6
Died, culture negative	2	0	1	0	3
Unknown	0	0	2	0	2
Total	86	3	6	1	96

Two other cured (culture-negative) patients later died (1 of a narcotic overdose and the other of respiratory insufficiency).

Of the 9 patients who defaulted, 3 were culture-positive at the time they abandoned treatment. Among the 9 defaulters, 5 died (4 from TB and 1 by suspected suicide), 2 are currently culture negative, and 2 were lost to follow-up since the time of treatment default. One patient was considered a treatment failure and, despite further retreatment regimens, subsequently died of TB.

Among 96 patients who were alive at treatment completion, 85 (89%) currently remain healthy. Thus, among the entire cohort of 120 patients enrolled during the study period, favorable long-term outcome was observed among 71%. Four patients experienced long-term sequelae: hemoptysis caused by aspergilloma necessitating pulmonary resection (1 patient), bronchiectasis and recurrent respiratory infections (2 patients), and bronchopleural fistula after pneumonectomy (1 patient).

In addition to medical care, Socios en Salud provides social assistance with financial support to resume work and pursue studies. Of the 96 patients, 21 patients (22%) received financial aid to pursue work or study, and 13 (14%) have had children since they were cured. Employment improved from 34% before therapy to 71% after therapy. None of the persons employed before starting MDR-TB treatment have lost their jobs because of work disruption caused by their TB therapy.

Conclusions

Although MDR-TB presents a major challenge to TB control, effective treatment can result in cure. Long-term follow-up is important for understanding the long-term efficacy of treatment and the overall impact of this disease on patients' physical and socioeconomic well-being.

This study has several interesting findings. First, unlike previous reports of high death rates associated with MDR-TB, most of our patients met the definition of cure upon completion of treatment. Second, few patients who were cured at treatment completion had long-term sequelae or relapse. Third, most patients were able to resume work or studies and participate in family roles as parents and caretakers. Finally, the default rate was low (7.5%).

We recognize the limitations of this study. First, the cohort was small, and longer follow-up would be useful in determining if these indicators of physical and social recovery are sustained. Second, several patients were lost to follow-up; thus, the outcome of these patients is still not well characterized. Finally, these results may not be applicable to other situations, where the socioeconomic situation determines, in large part, the ability of a patient to resume work and studies.

With access to laboratory results to guide individualized therapy for persons with MDR-TB in the context of strong community-based support, the outcomes observed in Peru are encouraging. These outcomes favor the implementation of similar MDR-TB treatment programs globally.

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Immune Restoration Disease in HIV Patient

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We describe a severely immunosuppressed HIV-1–positive man in whom immune restoration disease associated with pulmonary infection caused by *Mycobacterium microti* developed after antiretroviral treatment. The diagnosis was made by using convenient spoligotyping techniques, but invasive investigations were required to exclude a tumor.

During the first few months of highly active antiretroviral treatment, immune restoration may be complicated by clinical events in which either previously subclinical infections are found or preexisting partially treated opportunistic infections deteriorate. This condition, termed immune restoration disease (IRD), is thought to be caused by the improvement in the host's immune response to pathogens. We report what we believe is the first recorded case of IRD with confirmed *Mycobacterium microti* infection. *M. microti* is an unusual infection associated with small rodents. Novel genetic techniques have confirmed it as a human pathogen, but its true incidence remains unclear.

Case Report

A 33-year-old man was admitted to the hospital in 2002 with a 3-month history of intermittent hemoptysis. He had previously visited hematology outpatient clinics for many years for hemophilia A (3%–5% factor VIII) and HIV-1 had been diagnosed in 1996 (a stored sample from 1989 retrospectively tested HIV-1–antibody positive). CD4 cell counts had been recorded as 434×10^6/L in 1996 and 260×10^6/L in 1998. After this time, he failed to attend any further clinic appointments.

In addition to worsening hemoptysis, he had minor weight loss but no night sweats. He appeared moderately unhealthy, with oral thrush and mild splenomegaly. A chest radiograph showed left upper lobe shadowing (Figure 1, panel A), sputum smears contained numerous acid-fast bacilli, and sputum was sent for culture, speciation, and sensitivity testing. Blood tests showed a CD4 cell count of 6×10^6/L and HIV viral load of 272,000 (log 5.4) copies/mL. His hemoglobin was 12.1 g/dL, leukocyte count 4.9×10^9/L, and platelets 367×10^9/L. Renal and liver function test results were normal, apart from a γ-glutamyltransferase level of 200 U/L (normal <35 U/L). An ultrasound scan of his abdomen showed a diffuse increase in liver echogenicity and an enlarged (13.6-cm) spleen. He was naturally immune to hepatitis B, and tests for hepatitis C antibody and viremia were negative.

He was given broad-spectrum antimycobacterial therapy (rifampin, isoniazid, pyrazinamide, ethambutol, and azithromycin) as treatment for both *M. tuberculosis* complex (MTB complex) and opportunistic mycobacteria. He improved throughout a 2-week hospital stay and highly active antiretroviral therapy (HAART) was begun. It consisted of zidovudine and lamivudine and an increased dose of efavirenz (800 mg daily) because of concomitant rifampicin therapy, together with cotrimoxazole for prophylaxis against *Pneumocystis jirovecii.*

At a 3-week follow-up appointment, the patient had continued to improve. No further hemoptysis had occurred, and he had gained 2 kg body weight. After that, by telephone, he complained of occasional fevers and malaise but declined to be seen. Nine weeks after his initial visit, he came to the ward and with symptoms of breathlessness, nausea, and diarrhea. He had fevers spiking to >38°C, and newly palpable lymph nodes were evident in the right axilla and over the left parotid gland. A chest radiograph showed increased shadowing (Figure 1, panel B) and a computed tomographic (CT) scan of the chest showed a 6×3-cm solid mass in the upper left chest with visible air bronchograms and minor volume loss, reported as "almost certainly solid neoplasm" (Figure 2). A bronchoscopy, before which the patient received factor VIII, showed an area of irregular nodular tissue in the posterior segment of the left upper lobe. His CD4 count was 26×10^6/L.

While we awaited histologic examination results, the patient's medication was withdrawn, and he improved within days. The lymph nodes reduced in size, and the spiking temperature resolved. Eight weeks after admission, the results of IS*6110* polymerase chain reaction (PCR) testing on the slow-growing, positive mycobacterial culture confirmed the presence of MTB complex. The bronchial biopsy specimen showed necrotizing granulomatous bronchitis; acid-fast bacilli were not visible, and this specimen did not grow any mycobacteria.

The *Mycobacterium* species was not growing well in the laboratory, but *M. microti* was identified in the culture with spoligotyping methods (*1,2*). On extension of culture, the isolate was confirmed as *M. microti* phenotypically and found to be sensitive in vitro to rifampin, isoniazid, ethambutol, and clarithromycin but resistant to pyrazinamide.

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The antimycobacterial drugs were reintroduced without problems 4 weeks later, as was HAART after an additional 8 weeks. Thirty months after restarting antimycobacterial therapy, the patient remained well with a total weight gain of 19 kg, complete radiologic resolution of the pulmonary mass, undetectable HIV viral load (<50 copies/mL), and a progressive rise in his CD4 count to 249 $\times 10^6$/L.

Conclusions

M. microti is a slow-growing member of the MTB complex. It has most commonly been described in association with small rodents (*2*,*3*). Its role as a pathogen in humans has been proposed by a handful of case reports involving both immunocompetent and immunodeficient persons (*4*–*6*). Because of the slow growth of the bacterium, confirmatory diagnosis is difficult, and cultures may be wrongly discarded as negative after a routine 8- to 12-week culture period. In such cases several commercial tests can identify the organism to the level of the MTB complex. The members of the tuberculosis complex are not reliably distinguished on biochemical grounds. Restriction fragment length polymorphism, DNA fingerprinting, is a time-consuming, slow, and expensive method of distinguishing members. Spoligotyping (spacer oligotyping) is a PCR-based test that can be quickly performed, even on the small amounts of DNA in poorly growing cultures. Strains differ in the size of spacer regions that intersperse direct repeat regions. These polymorphisms create differences in the PCR products produced, a process that allows strain identification (*1*,*7*). Other PCR-based tests are being developed that may have advantages over spoligotyping for identifying some members of the complex (*8*). Culture remains the standard and allows drug sensitivity testing. We suggest that such cultures be extended for up to 6 months if acid-fast bacilli are noted in specimens from a patient with consistent pulmonary pathologic findings.

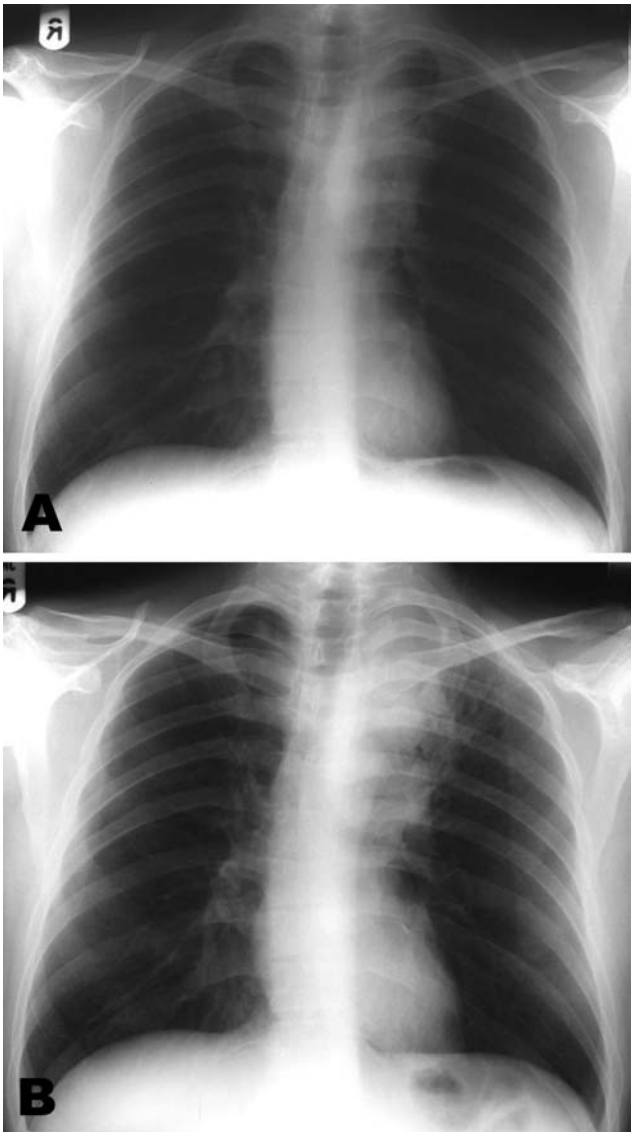

Figure 1. Chest radiographs at initiation of A) highly active antiretroviral therapy (HAART) showing left hilar mass; and B) after 9 weeks of HAART and antituberculosis treatment, suggesting enlargement of the hilar mass consistent with immune restoration disease. (The radiograph has been flipped horizontally to aid comparison.)

Severe immunosuppression associated with HIV-1 can allow coinfections to remain subclinical. After HAART is introduced, IRD can lead to the development of opportunistic infections with atypical and severe clinical signs and symptoms. In this case, necrotizing granulomatous inflammation occurred without isolation of mycobacteria, which is atypical in an immunodeficient HIV patient. These clinical manifestations have been described in many diseases, including MTB and other opportunistic mycobacterial infections (*9*), herpesvirus infections, and hepatitis B and C (*10*). Similar granulomatous endobrochial lesions have been described during immune restoration with *M. avium* complex in HIV-infected patients (*11*). IRD has been described as occurring in 30% to 40% of those with very low CD4 counts when they begin HAART, developing within the first few months of treatment as the CD4 count rises. IRD is a diagnosis of exclusion: other processes such as drug fever, resistance, treatment failure, or other infections must be excluded. IRD may be a life-threatening condition, and clinical interventions, such as stopping medications, steroids, or both, may be indicated. An associated increase in markers of immune activation occurs, which seems to vary depending on the pathogen involved (*12*,*13*) .

In MTB, the major risk factor for IRD is beginning HAART within 2 months of antituberculosis treatment. This fact has led many experts to recommend delaying HAART for 2 months, a strategy that may also reduce the

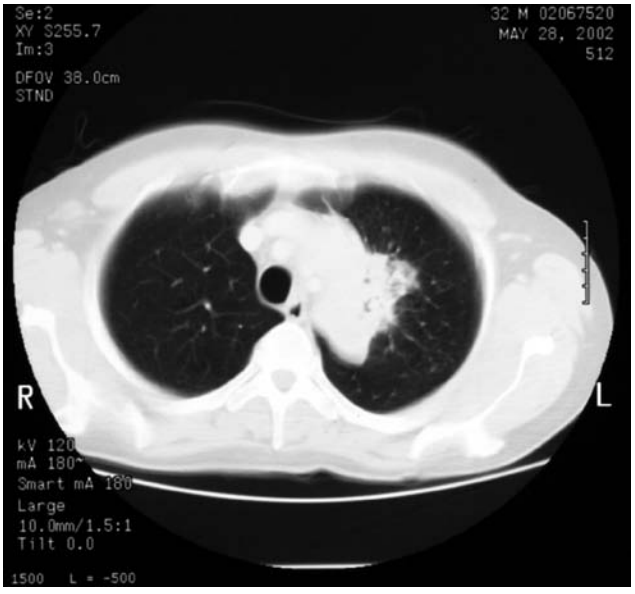

Figure 2. Computed tomographic scan of thorax at patient's initial visit. Results are highly suggestive of tumor.

incidence of adverse events. However, a recent retrospective review suggested that delaying HAART would, particularly in those with CD4 cell counts<100 cells $\times$ 10^6/L, lead to significant increase in risk for death and new opportunistic infections (*14*). IRD should always be considered along with treatment failure if a patient's clinical condition deteriorates after HAART is introduced.

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MassTag Polymerase Chain Reaction for Differential Diagnosis of Viral Hemorrhagic Fevers

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Viral hemorrhagic fevers are associated with high rates of illness and death. Although therapeutic options are limited, early differential diagnosis has implications for containment and may aid in clinical management. We describe a diagnostic system for rapid, multiplex polymerase chain reaction identification of 10 different causes of viral hemorrhagic fevers.

Increasing international travel, trafficking in wildlife, political instability, and terrorism have made emerging infectious diseases a global concern. Viral hemorrhagic fevers (VHF) warrant specific emphasis because of their high rates of illness and death, and the potential for rapid dissemination by human-to-human transmission. The term "viral hemorrhagic fever" characterizes a severe multisystem syndrome associated with fever, shock, and bleeding diathesis caused by infection with any of several RNA viruses, including Ebola virus and Marburg virus (MARV) (family *Filoviridae*); Lassa virus (LASV) and the South American hemorrhagic fever viruses Guanarito virus, Junín virus, Machupo virus, and Sabiá virus (*Arenaviridae*); Rift Valley fever virus (RVFV), Crimean-Congo hemorrhagic fever virus (CCHFV), and hantaviruses (*Bunyaviridae*); and Kyasanur Forest disease virus (KFDV), Omsk hemorrhagic fever virus, yellow fever virus (YFV), and dengue viruses (*Flaviviridae*) (*1*,*2*). Although clinical management of VHF is primarily supportive, early diagnosis is needed to contain the contagion and implement public health measures, especially if agents are encountered out of their natural geographic context.

Vaccines have been developed for YFV, RVFV, Junín virus, KFDV, and hantaviruses (*3*–*7*), but only YFV vaccine is widely available. Early treatment with immune plasma was effective in Junín virus infection (*8*). The nucleoside analog ribavirin may be helpful if given early in the course of Lassa fever (*9*), Crimean-Congo hemorrhagic fever (*10*), or hemorrhagic fever with renal syndrome (*11*) and is recommended in postexposure prophylaxis and early treatment of arenavirus and bunyavirus infections (*12*).

Methods for direct detection of nucleic acids of microbial pathogens in clinical specimens are rapid, sensitive, and obviate the need for high-level biocontainment. Numerous systems are described for nucleic acid detection of VHF agents; however, none are multiplex (*13*). Although geographic location or travel history of suspected patients usually restricts the number of agents to be considered, diagnosis of VHF may be difficult in case of an intentional release (*12*). Symptoms of VHF are initially nonspecific and may include fever, headache, myalgia, and gastrointestinal or upper respiratory tract complaints (*1*); thus, assays that allow simultaneous consideration of multiple agents are needed.

We recently described the application of MassTag polymerase chain reaction (PCR) in the context of differential diagnosis of respiratory disease (*14*). MassTag PCR is a multiplex assay in which microbial gene targets are coded by a library of 64 distinct mass tags. Nucleic acids (RNA or DNA) are amplified by multiplex (reverse transcription–) PCR using up to 64 primers, each labeled by a photo-cleavable link with a different molecular weight tag. After separation of the amplification products from unincorporated primers and release of the mass tags from the amplicons by UV irradiation, tag identity is analyzed by mass spectrometry. The identity of the microbe in the clinical sample is determined by the presence of its 2 cognate tags, 1 from each primer.

The Study

To facilitate rapid differential diagnosis of VHF agents, we established the Greene MassTag Panel VHF version 1.0, which comprises the following targets: Ebola Zaire virus

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(ZEBOV), Ebola Sudan virus (SEBOV), MARV, LASV, RVFV, CCHFV, Hantaan virus (HNTV), Seoul virus (SEOV), YFV, and KFDV. Oligonucleotide primers were designed in conserved genomic regions to detect the broadest number of members for a given pathogen species. We developed a software program that culls sequence information from GenBank, performs multiple alignments with ClustalW, and designs primers optimized for multiplex PCR. The program uses a greedy algorithm to identify conserved sequences and create the minimum set of primers for amplification of all sequences in the alignment. Primers are selected within standard design constraints whenever possible (melting temperature 55°C–65°C, guanine-cytosine content 40%–60%, no hairpins); degenerate positions are introduced in cases where template divergence requires more flexibility. Although degeneracy is not tolerated in the five 3′ nucleotides, MassTag PCR allows up to 4 nonneighboring variable positions per primer. Primers are checked by the basic local alignment search tool for potential hybridization to sequenced vertebrate genomes (Table 1).

Because only released mass tags are analyzed, staggering the size of amplification products created in multiplex reactions is unnecessary; thus, primers are selected for efficient and consistent performance irrespective of amplicon size (typically 80–200 bp). Before committing to synthesis of tagged primers, the functionality of candidate multiplex primer panels is examined in a series of amplification reactions that use prototype templates representing individual microbial targets. Primers that fail to yield a single, specific product band in agarose gel analysis are replaced. Target sequence standards for evaluation are cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) by using PCR amplification of cDNA templates obtained by reverse transcription (RT) of extracts from infected, cultured cells or by assembly of overlapping synthetic polynucleotides.

The agents assayed in the VHF panel have RNA genomes; thus, assay sensitivity was determined by using synthetic RNA standards. Synthetic RNA standards were generated from linearized target sequence plasmids by using T7 polymerase (mMessage mMachine, Invitrogen). After quantitation by UV spectrometry, RNA was serially diluted in 2.5 μg/mL yeast tRNA (Sigma, St. Louis, MO, USA), reverse transcribed with random hexamers by using Superscript II (Invitrogen), and analyzed by MassTag PCR as previously described (*14*). QIAquick 96 PCR purification cartridges (Qiagen, Hilden, Germany, with modified binding and wash buffers) were used to remove unincorporated primers before tags were decoupled from amplification products by UV photolysis in a flow cell and analyzed in a quadrapole mass spectrometer by using positive-mode atmospheric pressure chemical ionization (APCI-MS, Agilent Technologies, Palo Alto, CA, USA). The sensitivity of the 10-plex VHF panel with synthetic RNA standards was ≤50 RNA copies per assay (Table 2). Sensitivity and specificity of multiplex primer panels is assessed empirically by using calibrated synthetic standards as well as tissue culture–derived viral nucleic acid for each assembled panel.

Tissue culture extracts were used to examine assay specificity. Random primed cDNA obtained from cultures

Table 1. Greene MassTag panel VHF version 1.0*

Target	MassTag	Name	Sequence	Gene
ZEBOV	718 (fwd)	EboZA-U234	AACACCGGGTCTTAATTCTTATATCAA	L
	646 (rev)	EboZA-L319	GGTGGTAAAATTCCCATAGTAGTTCTTT	
SEBOV	503 (fwd)	EboSU-U416	CGAGCCTAACGTTTTGGGC	L
	630 (rev)	EboSU-L489	GCTCCAGGAATTGTTCGGGTA	
MARV	654 (fwd)	MARV-U12816C	CCCTCCATATCTTAGACAACATATTGTG	L
	395 (rev)	MARV-L12994	CCCAACACTCCTGGTTCACAGC	
LASV†	558 (fwd)	Las4-U92	ACTGCATTYTCATACTTYCTRGAATC	NP
	686 (rev)	Las4-L257	CCRGGYTTGACCAGTGCTGT	
RVFV	658 (fwd)	RVF-U578	GGATTGACCTGTGCCTGTTGC	N
	495 (rev)	RVF-L660	GCATTAGAAATGTCCTCTTTTGCTGC	
CCHFV	499 (fwd)	CCHV-U4	AGAAACACGTGCCGCTTACGCCCA	N
	710 (rev)	CCHV-L120	CCATTTCCYTTYTTRAACTCYTCAAACCA	
HNTV	479 (fwd)	HAN-U179	AYACAGCAGCAGTTAGCCTCCT	N
	702 (rev)	HAN-L245	GCT GCC GTA RGT AGT CCC TGTT	
SEOV	455 (fwd)	SEO-U243	CAGGATTGCAGCAGGGAAGA	N
	602 (rev)	SEOUL-L309	ATGATCACCAGGYTCTACCCC	
YFV	467 (fwd)	YF-U186	GCTGGGAGCGCGGTATC	NS5
	670 (rev)	YF-L249	GGAAGCCCAATGGTCCTCAT	
KFDV	483 (fwd)	KYF-U170	TGGAAGCCTGGCTGAAAGAG	NS5
	614 (rev)	KYF-L233	TCATCCCCACTGACCAGCAT	

*ZEBOV, Ebola Zaire virus; SEBOV, Ebola Sudan virus; MARV, Marburg virus; LASV, Lassa virus; RVFV, Rift Valley fever virus; CCHV, Crimean-Congo hemorrhagic fever virus; HNTV, Hantaan virus; SEOV, Seoul virus; YFV, yellow fever virus; KFDV, Kyasanur Forest disease virus; fwd, forward; rev, reverse.
†Primers were designed on Lassa lineage IV sequences (*15*) and the recently identified outlier sequence Lassa AV (AF256121).

Table 2. Sensitivity of detection with synthetic RNA standards

Pathogen*	Detection threshold (RNA copies)†
ZEBOV	20
SEBOV	20
MARV	20
LASV	20
RVFV	20
CCHFV	50
HNTV	20
SEOV	50
YFV	20
KFDV	20

*ZEBOV, Ebola Zaire virus; SEBOV, Ebola Sudan virus; MARV, Marburg virus; LASV, Lassa virus; RVFV, Rift Valley fever virus; CCHV, Crimean-Congo hemorrhagic fever virus; HNTV, Hantaan virus; SEOV, Seoul virus; YFV, yellow fever virus; KFDV, Kyasanur Forest disease virus.
†RNA copies refers to the number of molecules subjected to reverse transcription; half of the reverse transcription reaction was then used for polymerase chain reaction amplification.

of ZEBOV, SEBOV, MARV, YFV isolates from the Gambia and Côte d'Ivoire, RVFV, CCHFV, HTNV, SEOV, and LASV strains Josiah, NL, and AV were subjected to mass tag analysis. In all instances, only the appropriate cognate mass tags were detected (data not shown). No spurious signal was identified in assays with water or RNA controls.

Performance with clinical materials was tested by using blood, sera, or oral swabs from 24 human patients of VHF previously diagnosed through virus isolation, RT-PCR, or antigen detection enzyme-linked immunosorbent assay. Differential diagnosis by blinded MassTag PCR analysis was accurate in all cases (Table 3). For the samples from the 2005 Angola Marburg outbreak the result of MassTag PCR was similar to that of diagnostic single-plex PCR. ZEBOV sample 5004, obtained on day 17 of illness when serologic test results were positive for immunoglobulin M (IgM) and IgG, was negative by viral culture but positive in MassTag PCR.

Conclusions

These results confirm earlier work in respiratory diseases that show that MassTag PCR offers a rapid, sensitive, specific, and economic approach to differential diagnosis of infectious diseases. Small, low-cost, or mobile APCI-MS units extend the applicability of this technique beyond selected reference laboratories. Given the capacity of the method to code for up to 32 genetic targets, we are expanding the hemorrhagic fever panel to include additional viruses (dengue and South American hemorrhagic fever viruses) and are exploring the inclusion of bacterial and parasitic agents that may result in similar clinical signs and symptoms and, thus, have to be considered in differential diagnosis.

Table 3. MassTag polymerase chain reaction analysis of clinical specimens from viral hemorrhagic fever patients*

Previous diagnosis	Sample identification	Sample type	Year/origin	MassTag result†
ZEBOV	5015	Serum	1995/Kikwit, DRC	+++, ZEBOV
ZEBOV	5014	Serum	1995/Kikwit, DRC	+++, ZEBOV
ZEBOV	5004	Serum	1995/Kikwit, DRC	+++, ZEBOV
ZEBOV	6317	Serum	1995/Kikwit, DRC	+++, ZEBOV
ZEBOV	6313	Serum	1995/Kikwit, DRC	+++, ZEBOV
MARV	246-00-5	Hemolyzed whole blood	2000/Durba, DRC	+, MARV
MARV	226-00-4	Hemolyzed whole blood	2000/Durba, DRC	++, MARV
MARV	246-00-7	Hemolyzed whole blood	2000/Durba, DRC	+, MARV
MARV	98-00-2	Hemolyzed whole blood	2000/Durba, DRC	+++, MARV
MARV	461	Blood	2005/Uige, Angola	+++, MARV
MARV	462	Oral swab	2005/Uige, Angola	+++, MARV
MARV	475	Blood	2005/Uige, Angola	++, MARV
MARV	476	Oral swab	2005/Uige, Angola	+, MARV
LASV	98-04-1	Serum	2004/Sierra Leone	+++, LASV
LASV	98-04	Serum	2004/Sierra Leone	++, LASV
LASV	98-04-5	Serum	2004/Sierra Leone	+, LASV
LASV	80-04-1	Serum	2004/Sierra Leone	+++, LASV
RVFV	98002009	Serum	1998/Kenya	+, RVFV
RVFV	H6061989	Serum	1998/Kenya	+, RVFV
RVFV	98002019	Serum	1998/Kenya	++, RVFV
RVFV	77-04	Serum	2004/Namibia	++, RVFV
CCHFV	187-86	Serum	1986/South Africa	+, CCHFV
CCHFV	30-93	Serum	1993/South Africa	+++, CCHFV
CCHFV	465-88	Serum	1988/South Africa	+++, CCHFV
CCHFV	407-89	Serum	1989/South Africa	+++, CCHFV
CCHFV	215-90	Serum	1990/South Africa	++, CCHFV

*ZEBOV, Ebola Zaire virus; MARV, Marburg virus; LASV, Lassa virus; RVFV, Rift Valley fever virus; CCHV, Crimean-Congo hemorrhagic fever virus; DRC, Democratic Republic of Congo.
†Relative ranking of results: +, signal-to-noise ratio ≤4; ++, signal-to-noise ratio >4 and <8; +++, signal-to-noise ratio ≥8.

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Atypical Enteropathogenic *Escherichia coli*: Typical Pathogens?

James P. Nataro*

Escherichia coli is both the most abundant facultative commensal of the human gastrointestinal tract and the most common bacterial cause of human diarrhea (*1*). However, precise recognition of *E. coli* pathotypes remains problematic. Enteropathogenic *E. coli* (EPEC), classically associated with outbreaks of infant diarrhea, harbors distinctive chromosomal (the locus of enterocyte effacement, or LEE island) and plasmidborne (residing on the EPEC adherence factor, or EAF, plasmid) virulence factors, which are linked by common gene regulators (*1*). At the Second International Conference on EPEC, held in São Paulo, Brazil, in 1995, the foremost authorities in the field proclaimed the global importance of such "typical" EPEC (tEPEC) but pondered the clinical relevance of strains carrying only the LEE island (dubbed at that conference "atypical EPEC," or aEPEC) (*2*). Had aEPEC lost the EAF plasmid? Had it incidentally acquired only fragments or incomplete packages of virulence-associated genes? Or were some aEPEC true pathogens of humans or animals?

In this issue of Emerging Infectious Diseases, Nguyen et al. propose a distinct role for aEPEC in human infection (*3*). Previously, these investigators reported a high prevalence of aEPEC among pediatric diarrhea patients in Melbourne, including both infants and older children (in contrast to the strong tendency for infants to be infected with tEPEC) (*4*). Now these authors show that, in contrast to patients infected with other pathogens, patients infected with aEPEC are far more likely to experience diarrhea past 14 days, the point long recognized as a clinical watershed that heralds increased risk for illness and death. aEPEC's prevalence among diarrhea patients, the pathogen's strong association with diarrheal symptoms, and the infection's distinctively persistent nature argue for a high disease burden in Melbourne. Although the authors define aEPEC strictly on the basis of positivity for the LEE *eae* gene and failure to amplify a *bfpA* pilin gene (not assessing additional plasmid loci), the absence of tEPEC serotypes and the occurrence of disease in children older than infants suggest that these are indeed aEPEC.

This communication also illustrates 2 principles that should be recognized more generally with regard to diarrheogenic *E. coli*: 1) one can authoritatively implicate a particular strain as a pathogen by (only) outbreak implication or by volunteer studies, but one cannot definitively prove by any data that a putative pathotype is not a human pathogen; and 2) the implication of pathogenicity for 1 strain is not sufficient to implicate any similar strain as a pathogen. Thus, once definitive evidence is presented that a particular strain is pathogenic, the challenge is to determine the genotypic or phenotypic fingerprints that permit extrapolation to other strains. This obstacle has long proved daunting for the enteroaggregative pathotype (shown to be pathogenic in both volunteer studies and outbreaks), and it may prove similarly frustrating for aEPEC.

Diarrheal epidemiology studies today are able to identify a likely etiologic agent for most patients, in contrast to studies of 20 years ago, because of improved diagnostic tests and the identification of new pathogens, but the task is not finished. Additional studies are needed in many places to refine our understanding of putative and emerging pathogens and to determine their full epidemiologic roles.

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Computer-assisted Telephone Interview Techniques

To the Editor: Fox et al. used computer-assisted telephone interview (CATI) techniques in an outbreak of cryptosporidiosis (*1*). Australian health agencies have used CATI for several years. A case-control study during an outbreak of *Salmonella* Mbandaka in 1996 employed CATI to interview 15 case-patients and 45 controls; contaminated peanut butter was implicated (*2*). Foodborne disease outbreaks are often geographically widespread and suited to using CATI.

Australian health authorities investigate ≈100 outbreaks of foodborne disease each year, with 3–4 using CATI-based case-control studies. Some jurisdictions investigate outbreaks by using CATI interviews of controls sampled from a bank of potential study participants (*3*). Potential study participants are recruited at the conclusion of rolling risk factor survey interviews, similar to the Behavioral Risk Factor Surveillance System.

A "control bank" allows investigators to rapidly obtain contact details for appropriately matched controls because age and sex of all household members are recorded in a database. Using control banks with CATI allows completion of studies quicker than CATI or traditional methods alone (*4*). South Australia has used CATI during 11 case-control studies of salmonellosis, legionellosis, Q fever, campylobacteriosis, Shiga toxin–producing *Escherichia coli*, and cryptosporidiosis (http://www.dh.sa.gov.au/pehs/notifiable-diseases-summary/current-outbreak-table.htm).

During an Australian CATI survey about gastroenteritis, 5,123 (84%) of 6,087 households agreed to be in a control bank (*5*). This bank of 14,024 potential controls was used in 4 case-control studies of sporadic salmonellosis and campylobacteriosis. This system avoided randomly dialing thousands of households to enroll controls in young age groups. The control bank was used for 3 years after initial collection, although many jurisdictions update banks annually.

Investigators may find CATI useful, although it can be costly and introduce biases (*4*). Programming questionnaires can delay investigations, which makes paper-based collection better in small outbreaks (*4*). CATI cannot be used in areas where a small proportion of the population has telephones. Despite limitations, CATI, when combined with control banks, may improve outbreak investigations.

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In Response: We appreciate the comments of Martyn Kirk and colleagues, who describe their experience using computer-assisted telephone interview (CATI) techniques in Australia with geographically widespread foodborne outbreaks (*1*). The intent of our article was to illustrate 1 example of the use of the CATI infrastructure in investigating a large communitywide cryptosporidiosis outbreak (*2*); yet we recognize the applicability of this infrastructure to multiple acute infectious disease outbreak scenarios.

In our article, we comment that the use of existing CATI systems, like the Behavioral Risk Factor Surveillance System (BRFSS), can provide a practical means for obtaining controls in case-control studies, and the letter by Kirk and colleagues describes their use of the CATI infrastructure to create a "control bank" for acute infectious disease outbreak investigations. This control bank includes participants of longitudinal risk factor surveys, like BRFSS, who are subsequently recruited as controls for outbreak investigations. We acknowledge that a "bank" of these readily accessible controls could permit more rapid recruitment of participants in numerous age strata and obviate the need for extensive random digit dialing to recruit an adequate age-matched control population in many investigations. Nevertheless, in most epidemiologic investigations, controls need to be selected from the same geographic area as the case-patients, and even in large telephone surveys, the number of respondents in any

given region can be small. This would make it difficult to recruit enough controls within the small areas affected by most outbreaks, particularly within specific age strata. A control bank may therefore be more practical for use in large communitywide outbreaks, outbreaks that occur over large regions (i.e., an entire state), or in densely populated areas. Additionally, the lengthy start-up time required for questionnaire programming with a CATI system also supports the view that the CATI method may be maximally applicable in large-scale investigations.

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Lack of Transmission of Vaccinia Virus

To the Editor: Recently, the US government completed a targeted vaccination strategy limited to healthcare workers, first responders, and the military because of concern that variola virus, the etiologic agent of smallpox, might be used as a biowarfare agent (*1*). A concern in such programs is the potential for unintended spread of the vaccine virus (vaccinia) from the primary vaccinee to contacts who may be at the greatest risk of having adverse reactions resulting from secondary transmission (*2*,*3*).

Contact spread of the live attenuated vaccinia virus is considered the predominant method of secondary transmission. The conventional methods of preventing a secondary transmission event in the household of a smallpox vaccine recipient include the use of bandages and long sleeves to limit direct contact with the lesion and immediate hand-washing when contact occurs (*4*).

Several recent reports have measured the presence of vaccinia virus on the dressings or hands of vaccinated persons; however, the recovery of vaccinia virus in the environment has not been evaluated after vaccination in a controlled setting (*5*–*7*). We present the first reported attempt to recover live vaccinia virus from the homes of recently vaccinated persons. This study was approved by the St. Louis University Institutional Review Board. We hypothesized that live vaccinia virus shed from the skin reaction could not be recovered in the natural environment, and as a result, constitutes a limited risk for contact transmission.

Three hundred eighty-seven environmental swab samples were collected on 3 different study days from 43 persons (mean age 24 years) with major cutaneous reactions. Persons who participated in this study were selected from a randomized, double-blind, single-center study that compared the safety, tolerability, and immunogenicity of 3 smallpox vaccines (*8*,*9*). Following vaccination and after each study visit, the vaccination site was covered with an OpSite Post-Op dressing (Smith and Nephew, Massilon, OH, USA). On postvaccination days 7, 10, and 15, a sterile Calgiswab type 2 applicator (Harwood Products Co., Guilford, ME, USA), moistened in sterile water, was rotated over the linen from the study participant's bed (approximate location of sleeping area), the middle of his or her bath towel, and the inside area of a shirt sleeve adjacent to the vaccination bandage (before laundering). These sampling areas were chosen on the basis of the likelihood of exposure to the semipermeable bandage and the potential for another person to come in contact with the vaccinia virus in these areas. An additional 129 samples from the palm of the study participant's hand used to take the environmental samples were taken to serve as a control mechanism.

After sampling, the tip of the swab was stored in a 15-mL conical tube containing 3 mL multimicrobe transport media (Remel, Lenexa, KS, USA). The 15-mL conical tubes were returned to the clinic in a cooler on cold packs the same day. Recovery of vaccinia virus was determined by infectivity assay. Samples were tested for infectious vaccinia virus by inoculation of fluid cultures of Vero cells grown in 12-well plates. A sample was defined as positive if cytopathic effects were observed (*10*).

Concurrent with the environmental sampling, the lesion and the outside of the bandage covering the inoculation site for each study participant were swabbed with a Calgiswab Type 2 sterile applicator, and the samples were analyzed by infectivity assay. These samples served as a positive control, indicating that the method

used to sample the environment was appropriate and sensitive.

All 516 environmental samples from designated sampling areas in the homes of recently vaccinated vaccinia-naive persons were negative for live virus as determined by plaque infectivity assay (Table). Only 1 (0.78%) of 129 dressing samples tested on day 7 had measurable titers of vaccinia.

Contact with live vaccinia virus from the lesion at the site of vaccination is the underlying cause of secondary transmission. Common mechanisms for transmission include contact with contaminated bandages and intimate sexual contact. Recent studies have compared a variety of bandages used to cover the vaccination site to determine which class of bandage provides the greatest protection against disseminated virus. Talbot et al. observed that <1% (N = 918) of dressing samples were positive for vaccinia (an initial semipermeable OpSite Post-Op dressing and an outer semipermeable Tegaderm bandage) (*5*). In a single-blind randomized trial design, Waibel et al. compared the presence of vaccinia virus on the external surface of 3 different types of bandages and noted that the semipermeable membrane with gauze had the smallest proportion of recoverable virus compared with the groups that used a Band-Aid or double gauze with adherent tape (*6*). Despite the difference in types of bandages from these studies, the results were remarkably consistent with regard to the limited dissemination of vaccinia virus outside the dressing. In concordance with our results, the semipermeable bandage provided significant protection from exposure to the virus on the outside of the bandage.

This study presented many challenges regarding the sampling and collection of specimens. Collection times, sampling technique, and environmental conditions may have contributed to the absence of viral recovery. In addition, we chose to measure live vaccinia virus as opposed to noninfectious viral genomes (by polymerase chain reaction) because we were concerned with transmission and infectivity. If we had chosen to measure noninfectious viral genomes, a positive outcome may have been likely. However, from a public health standpoint, such information would have been less valuable. Further studies will need to examine the viability of vaccinia virus in the environment to evaluate the possibility of contact transmission.

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Table. Sampling outcomes of 43 study participants who received 1 of 3 smallpox vaccines

Variable	All participants (N = 43)	Vaccine group		
		ACAM1000 (n = 14)	ACAM2000 (n = 15)	Dryvax (n = 14)
Mean ± SD titer from lesion*†‡				
Day 7	3.84 ± 0.98	3.77 ± 1.15	3.85 ± 1.06	3.91 ± 0.74
Day 10	3.75 ± 0.89	3.62 ± 0.54	3.58 ± 1.21	4.07 ± 0.74
Day 15	4.19 ± 1.40	4.08 ± 1.24	3.67 ± 1.57	4.86 ± 1.18
Mean ± SD titer from dressing§				
Day 7	0.63 ± 0.85	0.9 ± 1.50	0	0
Day 10	0	0	0	0
Day 15	0	0	0	0

*Viral shedding results reported as <X were converted to X/2 before calculating.
†Titers are reported as $\log_{10}$ PFU/mL.
‡There were no significant differences among the groups ($p>0.05$).
§Limit of detection was 1.0 $\log_{10}$ PFU/mL.

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Discrimination between Highly Pathogenic and Low Pathogenic H5 Avian Influenza A Viruses

To the Editor: To thoroughly investigate avian influenza outbreaks, identifying highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) is essential. Currently, determination of inserted basic amino acids within the hemagglutinin cleavage site of HPAI relies on nucleotide sequencing (*1–3*). Direct sequencing is relatively time-consuming and laborious and thus is not suitable for local and regional diagnostic laboratories that receive large numbers of samples that may contain HPAI or LPAI subtype H5N1. Therefore, a rapid diagnostic assay was developed to discriminate between HPAI and LPAI subtype H5 viruses by 1-step real-time reverse transcription–polymerase chain reaction (RT-PCR) with melting curve analysis.

H5 primers flanking the cleavage site were designed from conserved regions among HPAI and LPAI strains by using nucleotide sequences obtained from GenBank and our previous studies (*4–6*). The primers consisted of a forward primer H5F3+ (nucleotides 1001–1021: 5′-AACAGATTAGTCCTTGCGACTG-3′) and a reverse primer H5R2+ (nucleotides 1124–1103: 5′-CATCTACCATTCCCTGCCATCC-3′), which yielded products of ≈124 bp and ≈112 bp, corresponding to HPAI and LPAI, respectively. Consequently, the sizes of the amplicons and percentage guanine-cytosine content were different, allowing discrimination between HPAI and LPAI by melting curve analysis (*7*).

One-step, real-time RT-PCR with melting curve analysis was performed in an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). In each reaction, 3.0 μL of RNA sample was combined with a reaction mixture containing 10 μL 2× SYBR Green PCR Master Mix (Applied Biosystems), 0.5 μL 40× MultiScribe (Applied Biosystems) and RNase inhibitor, each primer (at final concentration of 0.5 μmol/L), 1.5 mmol/L $MgCl_2$, and RNase-free water in a final volume of 20 μL. The thermal profile began with incubation at 48°C for 45 min (reverse transcription), then incubation at 95°C for 10 min (predenaturation), followed by 40 cycles of amplification alternating between 94°C for 15 s (denaturation) and 68°C for 40 s (annealing/extension). The SYBR Green I fluorescent signal was obtained once per cycle at the end of the extension step. After amplification, melting curve analysis was performed by heating the sample to 95°C for 15 s, then cooling it to 70°C for 1 min, followed by a linear temperature increase to 95°C at a rate of 0.5°C/s, while continuously monitoring the fluorescent signal. Data were analyzed by the 7500 System SDS Software version 1.2 (Applied Biosystems).

To develop the assay, samples previously identified as influenza A/chicken/Nakorn-Patom/Thailand/CU-K2/04 (H5N1) served as a control for HPAI, and A/duck/Hong Kong Special Administrative Region, People's Republic of China/308/78 (H5N3) served as a control for LPAI. The H5 genes (nucleotides 914–1728) of each strain were inserted into pGEM-T Easy Vector and then transcribed in vitro by using RiboMAX Large Scale RNA Production System-T7 (Promega, Madison, WI, USA). Serial 10-fold dilutions of the standard H5 RNA were subjected to a sensitivity test (*8*). The fluorescent signal can be detected at RNA dilutions as low as 10^2 copies/μL. To assess the specificity, viral RNA extracted from other subtypes of influenza A viruses (H1–H4 and H6–H15) was tested. The assay was specific for the H5 subtype, since no amplification was detected from other subtypes.

Three preliminary melting curve analyses showed that this assay was effective in discriminating between the melting peaks of HPAI and LPAI (Figure). The variations of melting temperature (T_m) between runs were experimentally determined. The mean (standard deviation) of T_m values for HPAI and LPAI were 77.43°C (0.21°C) and 79.57°C (0.23°C), respectively. This assay provided high reliability and reproducibility, since the coefficients of variation were <0.30.

Seventy-eight specimens of influenza A virus were used to validate the assay. The 75 HPAI samples were isolated during the 2004 outbreak in Thailand; 3 LPAI samples, including A/avian/NY/01 (H5N2), A/Chicken/Mexico/31381–3/94 (H5N2), and A/shoveler/Egypt/03 (H5N2), were provided by the Centers for Disease Control and Prevention. The viruses were isolated in embryonated chicken

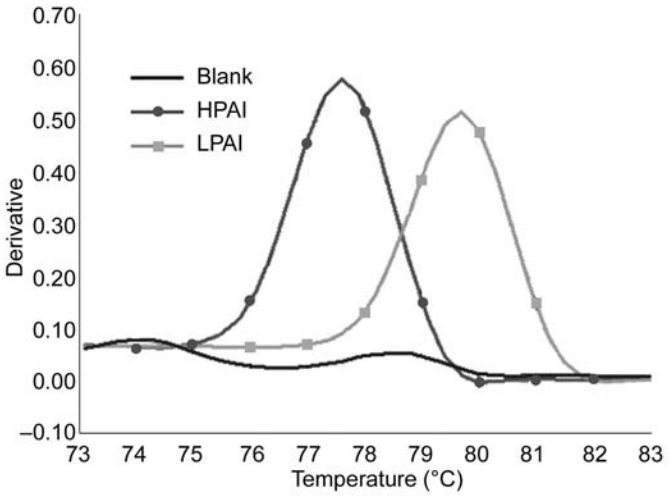

Figure. Discrimination between highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) by melting curve analysis based on real-time reverse transcription–polymerase chain reaction of the H5 HA gene with SYBR Green I fluorescent dye. The melting peaks of HPAI and LPAI were clearly separated. The cutoff value was set at 78.50°C (midpoint between HPAI and LPAI) and used to interpret the pathogenicity of unknown samples.

eggs as described previously (*9*). RNA was extracted from 140 µL of allantoic fluid, and RT-PCR was performed as described above. Melting curve analysis showed that all H5N1 isolates from the 2004 outbreak in Thailand were interpreted as HPAI, whereas the 3 samples of H5N2 subtype were classified as LPAI. The T_m varied from 77.2°C to 78.1°C for HPAI and 78.75°C to 79.5°C for LPAI. The melting curve analysis results were completely concordant with the results of direct sequence analysis of the H5 gene for all samples tested.

The melting curve analysis described here provides a rapid, accurate, and high-throughput approach to discriminate between HPAI and LPAI. This assay is particularly attractive for large-scale screening of suspected subtype H5 influenza A virus during outbreaks to identify candidate LPAI that could be used as vaccine strains.

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Rift Valley Fever in Goats, Cameroon

To the Editor: Rift Valley fever (RVF) is caused by an RNA virus (*Phlebovirus*, *Bunyaviridae*), which is carried by mosquito vectors (*1*). In nature, it is only known in Africa and the Arabian Peninsula. In the central African region, RVF has been reported in humans and livestock in the savanna of northern Cameroon and Chad (*2*,*3*) and from forests in the Central African Republic (*4*). Human epidemics are sometimes preceded by an increase in RVF virus (RVFV) prevalence in domestic ruminants, which manifests as increased abortions and high neonatal deaths (*3*). Human epidemics can have serious health implications, as demonstrated by the most recent outbreaks in Kenya in 1997 and 1998 (*5*) and Saudi Arabia in 2001 (*6*).

In June 2003, 14 goats from an urban market in Yaoundé (3.9°N, 11.5ºE), Cameroon, and 36 goats from 3 adjacent villages in tropical lowland rainforest ≈80 km south of Mvangan (2.4ºN, 11.8ºE), Cameroon, were sampled. The goats in the rural villages were bred locally and allowed to roam freely throughout the villages. No vaccinations had been given to goats in the rural sites. Goats in the urban market in Yaoundé generally originated in northern Cameroon and had been transported by train to Yaoundé. Owners did not report high levels of abortion or high neonatal deaths.

Jugular blood was collected in a 5-mL dry Vacutainer tube and centrifuged within 48 hours of collection. The frozen sera were shipped on ice to the Onderstepoort Veterinary Institute, the United Nations Food and Agriculture Organization reference laboratory for RVFV. An indirect enzyme-linked immunosorbent assay (I-ELISA) was used to detect RVFV immunoglobulin G (IgG) antibodies in 26 samples. In this assay, optical density readings were converted to a percentage of high-positive control serum (PP) and positive samples were those with PP ≥10. These samples were further tested with RVFV hemagglutination inhibition (HI), and samples with titers ≥20 were considered seropositive (*7*). Positive I-ELISA and HI samples were confirmed by using a serum neutralization (SN) assay (*7*,*8*). A sample was considered seropositive when it had an SN titer of ≥4, determined during experiments on laboratory-injected sheep (*7*–*9*) and testing of wild and domestic African ruminants (*7*). The SN assay has been shown to be highly specific; cross-reactivity with other viruses from the family *Bunyaviridae* is not likely (*9*).

Of the 26 goat samples tested for RVFV, 6 tested positive to RVFV (Table). Samples from 5 goats from the rural villages and 1 from the urban market had RVFV IgG PP ≥10. Samples from 2 goats from the rural villages had high RVFV HI titers (320 and 5,120). Three of the 6 samples from the rural villages with high IgG PP and HI titers had elevated neutralization titers (≥4).

The results indicate for the first time that RVFV is present in forests of southern Cameroon. Given the ages of the seropositive goats (2, 3, and 4.5 years), transmission of the virus occurred recently.

In savanna goat herds in northern Cameroon, RVFV IgG prevalence has been reported at 9% to 20% (*2*). To determine prevalence of RVFV in goats in southern Cameroon, more animals need to be sampled; the small sample size and isolation of the few rural villages are unrepresentative.

The presence of RVFV antibodies in domestic animals suggests that this virus may also be circulating in human populations, despite the absence of reports. A study of 21 persons in Ngoïla in southern Cameroon found no RVFV antibodies during a bloody diarrhea epidemic in 1998 (*10*,*4*); however, testing facilities for RVFV are not available in Cameroon, and the general population and healthcare providers have limited awareness of the virus and associated disease.

Central African forests have a high diversity of forest ungulates (>10 species), and RVFV has been reported from a number of wild African ungulates (*7*). The potential for exchange of RVFV and other pathogens between goats and wild ungulates could have substantial effects on animal production and on the conservation of wild species, some of which are considered near-threatened. Livestock disease surveillance can play a role not only in assessing the distribution of livestock pathogens but also in monitoring disease emergence.

Table. Site, age, and sex of goat samples positive for Rift Valley fever virus*

Site	Age, y	Sex	I-ELISA PP	HI titer	SN titer
Market	1.5	F	10	–	/
Rural	3	M	14	–	/
Rural	3.5	F	15	–	/
Rural	4	F	16	–	/
Rural	4.5	M	35	–	4
Rural	3	F	/	320	8
Rural	2	F	106	5,120	12

*By indirect enzyme-linked immunosorbent assay (I-ELISA), hemagglutination inhibition (HI), and serum neutralization (SN); PP, optical density expressed as a percentage of high-positive control serum. /, not tested; –, negative.

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HIV-1 CRF07_BC Infections, Injecting Drug Users, Taiwan

To the Editor: To date, Taiwan's human immunodeficiency virus type 1 (HIV-1) epidemic has primarily spread via sexual contact. The subtype B and circulating recombinant form (CRF) 01_AE account for >95% of all infections (*1*). However, since 2003 Taiwan has experienced a major outbreak of CRF07_BC among injecting drug users (IDUs).

The first wave of HIV-1 infections in Taiwan can be traced to the early 1980s, when a group of hemophilia patients received imported HIV-1-contaminated antihemophilia medications. By the time these medications had been replaced by heat-treated factor VIII concentrates, at least 53 patients had contracted HIV-1 infections (*2*). According to Taiwan's Center for Disease Control (CDC), HIV infections have been diagnosed in 9,229 persons (including 523 foreigners) as of July 31, 2005 (*3*). The number of persons living with HIV-1/AIDS has increased rapidly in the past few years, with a 77% increase in 2004, compared to 11% in 2003 (online Table, available at http://www.cdc.gov/ncidod/EID/vol12no04/05-0762.htm#table). According to the results of a risk factor analysis of people living with HIV-1/AIDS reported to the Taiwan CDC, the proportion of IDUs increased from 1.7% (13/773) in 2002 to 8.1% (70/861) in 2003 to 30.3% (462/1,521) in 2004 (online Table). The Taiwan CDC received reports of 1,241 IDUs diagnosed with HIV-1 infections from January 1 to July 31, 2005; these account for >75% of all reported HIV-1 infections in 2005 (*3*). The evidence points to an explosive epidemic of HIV-1 infections among IDUs in Taiwan since 2003, with no indication of a slowdown.

Taiwan has ≈60,000 IDUs (*1*). According to the Republic of China Ministry of Justice, the number of incarcerated drug offenders increased from 5,988 in 2003 to 9,303 in 2004; the rate of HIV-1 seropositive inmates increased from 13.3/100,000 in 2002 to 56.8/100,000 in 2004 (Y-M. Wu, Ministry of Justice, pers. comm.). Since all inmates are routinely tested for HIV-1 in detention centers, and all infected inmates are separated from HIV-1–seronegative inmates, the

potential of HIV-1 transmission in prisons is remote. We therefore suggest that the Taiwanese IDU population and its HIV-1 seropositive rate have both increased rapidly in the past few years.

To identify the primary HIV-1 strains in the current epidemic, we collected blood specimens from HIV-1-infected inmates in 3 detention centers (1 each located in the northern, central and southern regions of Taiwan). HIV-1 subtypes were determined by polymerase chain reaction, DNA sequencing, and phylogenetic analyses of *pol* or *env* genes. Our results indicate that 145 (96%) of 151 IDUs were infected with CRF07_BC and 6 (4%) were infected with subtype B; 97% of the CRF07_BC cases were diagnosed in 2003 or 2004. According to our phylogenetic analysis of the *env* gene, the Taiwanese CRF07_BC strains clustered with CRF07_BC strains drawn from IDUs in China (Figure).

CRF07_BC is a recombinant of the B′ and C subtypes. Several studies have suggested that CRF07_BC originated in China's Yunnan Province, with subtype B′ from Thailand mixing with subtype C from India before moving northwestward to Xinjiang Province along a major Chinese heroin trafficking route (*4*–*6*). To our knowledge, this is the first report of a large group of IDUs in northeastern Asia having CRF07_BC infections. It may have followed another drug trafficking route from Yunnan Province to southeast China, moving through Guangxi Province and Hong Kong to Taiwan (*7*–*9*). In a bid to combat skyrocketing HIV/AIDS infection rates among IDUs, the Taiwan CDC has proposed a 5-year harm reduction program to the Republic of China Executive Yuan.

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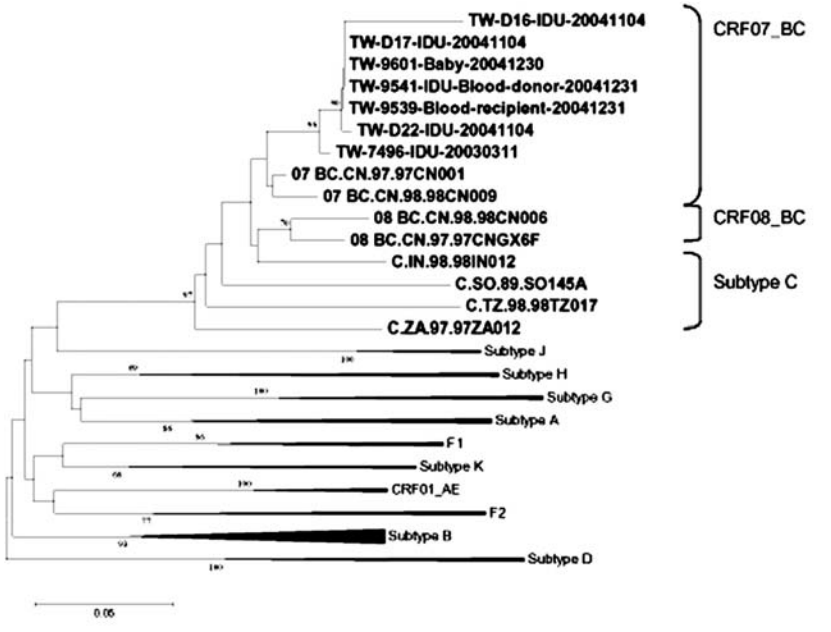

Figure. Phylogenetic analyses of 7 HIV-1 isolates identified in Taiwan. TW-7496, TW-D16, TW-D17, and TW-D22 were collected from detention center inmates; TW-9541 and TW-9539 were collected from a blood donor and 1 of his donation recipients. This neighbor-joining tree was created from 100 bootstrap samples of aligned *env* sequences corresponding to the 7077–7340 nucleotide residues of HIV-1-HXB2 from different isolates. Bootstrap values are shown on branch nodes. Reference isolates from the GenBank HIV database are indicated by subtype.

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Chlamydialike Organisms and Atherosclerosis

To the Editor: *Chlamydophila pneumoniae* causes pneumonia, but its role in the pathogenesis of atherosclerosis is controversial (*1–4*). The role of *C. pneumoniae* in atherosclerosis is supported by seroepidemiologic studies and detection in atherosclerotic lesions by polymerase chain reaction (PCR), immunohistologic analysis, culture, and electron microscopy (*2*,*3*). However, these results were not confirmed by other serologic or PCR-based studies (*4*). Meijer et al. evaluated abdominal aortic aneurysm biopsy specimens and detected *C. pneumoniae* membrane antigen more frequently than lipopolysaccharide antigens but did not detect heat shock protein 60 (*1*). In addition, they could not amplify or detect specific *C. pneumoniae* DNA by PCR and fluorescence in situ hybridization (*1*). They hypothesized that this discrepancy may result from a chlamydialike organism present in aortic samples that has surface antigens similar to those of *C. pneumoniae*.

Parachlamydia acanthamoebae and *Neochlamydia hartmanellae* are chlamydialike organisms that share ≈86% 16S rRNA sequence similarity with *C. pneumoniae* (*5*). Like *C. pneumoniae*, they have elementary and reticulate bodies visible by electron microscopy (*6*). *Neochlamydia*-related DNA (GenBank accession no. AF097191) has been amplified from 5 different arterial samples, including 1 aortic aneurysm (*7*), and a relationship (p = 0.009) between cerebral hemorrhage and serologic evidence of *Parachlamydia* infection has been reported (*8*). Therefore, we investigated the role of *Parachlamydia* in pathogenesis of atherosclerosis by using a molecular approach.

We analyzed 78 surgical samples from 27 patients undergoing aortic or carotid surgery for atherosclerotic disease at Hôpital Nord in Marseille from June 1, 2003, to December 31, 2003. The study was approved by the local ethics committee, and written informed consent was obtained from all participants. Demographic and clinical data were prospectively recorded.

DNA was extracted from aortic or carotid samples with atherosclerotic lesions by using the QIAamp DNA tissue kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. A nested PCR was performed by using external primers 16SIGF (5′-CGGCGTGGATGAGGCAT-3′) and 16SIGR (5′-TCAGTCCCAGTGTTGGC-3′) (*9*) and internal primers CHL16SFOR2 (5′-CGTGGATGAGGCATGCAAGTCGA-3′) and CHL16SREV2 (5′-CAATCTCTCAATCCGCCTAGACGTCTTAG-3′) (*7*). PCR included negative controls from the DNA extraction step. DNA extractions and PCR amplifications were conducted in a laboratory in which parachlamydial DNA had not been extracted or amplified. PCR products were purified by using the QIAquick PCR purification kit (Qiagen) and sequenced by using the d-rhodamine terminator cycle sequencing reaction kit (Perkin-Elmer Biosystems, Warrington, UK) and a 3100 ABI Prism automated sequencer (Applied Biosystems, Courtaboeuf, France).

Sequences were analyzed with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) using gap existence and extension penalties of 5 and 2, respectively. Results were considered positive only when the sequence of the amplified product exhibited a best BLAST hit with a chlamydialike organism. Statistical analyses were performed with STATA software (Stata Corporation, College Station, TX, USA).

A positive PCR result was obtained with samples from 5 (18.5%) of 27 patients (Table). Three sequences had a best BLAST hit with the sequence of *Parachlamydia* sp. UV7 (GenBank accession no. AJ715410), with a sequence similarity ranging from 99% to 100%. The other 2 sequences had 98% sequence similarity with *Neochlamydia*-related symbiont TUME-1 (GenBank accession no. AF098330). PCR positivity was not associated with age, sex, or location of the atherosclerotic lesion.

All patients with positive PCR results were ≥68 years of age. Patients without cardiovascular risk factors were more likely than those with ≥1 risk factor to have positive PCR results (p = 0.023). Despite the small number of patients in this study, this association was also confirmed in a multivariate logistic regression model adjusted for sex and previous cardiovascular disease (odds ratio 0.035, 95% confidence interval 0.001–0.94).

These findings suggest that *Parachlamydia* and *Neochlamydia* are associated with atherosclerosis. In addition, these obligate intracellular

Table. Comparison of patients with and without parachlamydial DNA by polymerase chain reaction (PCR) in surgical aortic or carotid specimens

Characteristic	Positive PCR result (n = 5)	Negative PCR result (n = 22)	p value
Males, no. (%)	4 (80)	16 (73)	0.74
Median age (interquartile range)	72 (71–77)	66 (61–74)	0.11
Any cardiovascular risk factor, no. (%)	3 (60)	21 (95)	0.02
Hypertension	3 (60)	10 (45)	0.56
Hypercholesterolemia	0	5 (23)	0.24
Tobacco use	3 (60)	17 (77)	0.42
Diabetes	1 (20)	6 (27)	0.74
Aortic surgery, no. (%)	2 (40)	13 (59)	0.44
Previous cardiovascular disease, no. (%)	2 (40)	8 (38)	0.94

bacteria may be present in both carotid and aortic atherosclerotic lesions of elderly patients.

Chlamydialike organisms in atherosclerotic lesions may explain controversies about the role of *C. pneumoniae* in pathogenesis of atherosclerosis (*4*). Some PCRs might amplify both *Chlamydia* and chlamydialike organisms, leading to erroneous conclusions, especially when the specificity of the product is not confirmed by sequencing. Chlamydialike organisms in atherosclerotic lesions might also explain discrepancies of serologic studies (*4*). *Parachlamydiaceae* likely cross-react with *C. pneumoniae* (*1*). Such cross-reactivity will not be recognized if patients with positive serologic results for *C. pneumoniae* are not tested for antibodies to chlamydialike organisms. This cross-reactivity may result in false-positive serologic results for *C. pneumoniae*, especially when low antibody titers (8–16) are considered positive.

Since elementary and reticulate bodies are similar in both *Chlamydiaceae* and *Parachlamydiaceae* (*6*), Chlamydiales cannot be identified in a specimen solely by electron microscopy. Consequently, elementary and reticulate bodies in atherosclerotic lesions (*10*) might be chlamydialike organisms and not *C. pneumoniae*. If chlamydialike organisms are involved in the pathogenesis of atherosclerosis, this finding would have public health implications, given their presence in free-living amebae that are widespread in water.

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Maculopathy in Dengue Fever

To the Editor: A recent article by Chlebicki et al (*1*) described 4 patients hospitalized for dengue fever who were found to have retinal hemorrhages. These patients reported reduced visual acuity and metamorphopsia, i.e., distorted visual images attributable to intrinsic retinal disease involving the macula; macular hemorrhages and exudates were found on retinal examination. The authors concluded that the retinal hemorrhages were responsible for the patients' visual symptoms.

This conclusion is misleading because retinal hemorrhages alone cause scotomas. Rather, the accumulation of subretinal fluid in the macula results in metamorphopsia and blurring of vision. In previous reports of patients in whom macular changes developed from dengue fever, some were found to have macular hemorrhages (*2–4*). In addition, clinical examination and investigation of these patients showed vasculopathologic changes in the macular region that affected the retinal and choroidal blood vessels (*5*), although the tissues of the periphery tended to be spared. A fluorescein angiograph of the retina showed knobby hyperfluorescence of the retinal arterioles with minimal leakage, as well as some spots of leakage at the level of the retinal pigment epithelium. An indocyanine green angiograph showed diffuse hyperfluorescence of the choroid. These pathologic changes in the macula were the most likely cause of the blurring of vision in such patients, which has been the case in our experience.

The article by Chlebicki et al. did not state whether these procedures had been performed on their patients to confirm or exclude retinal or choroidal vasculopathy in the macula. Therefore, these authors would have had difficulty concluding that retinal hemorrhages caused blurring of vision and metamorphopsia in patients with dengue maculopathy.

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Pulmonary Tuberculosis and SARS, China

To the Editor: As part of a cohort study of 83 patients with severe acute respiratory syndrome (SARS) in Beijing, China, we conducted a follow-up study of all the patients by routine medical examination. During the process, 3 patients with chest radiographs consistent with active disease were identified as having pulmonary tuberculosis (TB). Here we describe the 1-year clinical outcome and immune response in these patients.

Demographic details and coexisting conditions are shown in the Table. Patient 1 was a healthcare worker who became infected with SARS-associated coronavirus (CoV) while on duty with SARS patients. After he was transferred to a hospital dedicated to SARS management, pulmonary TB was diagnosed (positive acid-fast bacilli smear on sputum samples). Patients 2 and 3 were known to have cases of pulmonary TB and became infected with SARS-CoV after contact with other patients hospitalized for SARS. These 2 patients were sputum smear–negative for acid-fast bacilli, and diagnosis was made on the basis of previous exposure to TB, relevant symptoms of typical pulmonary TB, chest radiographs consistent with active disease, a positive tuberculin skin test result, and the finding of cavity regression on chest radiographs after anti-TB treatment was initiated. No cultures were obtained for isolation and comparison of *Mycobacterium tuberculosis* strains (*1*). All 3 patients had confirmed SARS based on amplification of SARS-CoV RNA by reverse transcriptase–polymerase chain reaction (RT-PCR) from sputum and stool specimens (*2*). Patients 2 and 3 recovered without complications; patient 1 had the most severe disease and required mechanical ventilation in an intensive care unit before recovering.

Both cellular and humoral immunity were evaluated during the follow-up of these patients. T-lymphocyte subsets were measured 6 months after disease onset by flow cytometry using fluorescein isothiocyanate–labeled specific monoclonal antibodies. Compared to other SARS patients (n = 47), the 3 patients with TB had lower mean CD4+ T cells (368.4/μL vs. 656.6/μL, respectively; $p = 0.05$) and lower mean CD8+ T cells (371.0/μL vs. 490.1/μL, respectively; $p = 0.39$). SARS-CoV immunoglobu-

Table. Demographic and clinical information on severe acute respiratory syndrome (SARS) patients with pulmonary tuberculosis (TB), Beijing, China, 2003

Demographic/clinical characteristic	Patient 1	Patient 2	Patient 3
Sex	M	M	M
Age, y	48	18	20
Other coexisting conditions	*Pseudomonas aeruginosa* infection	None	None
Date of SARS onset	Apr 5, 2003	Apr 7, 2003	Apr 4, 2003
Date of TB diagnosis	Jun 12, 2003	Jan 24, 2003	Mar 5, 2003
Date of hospitalization or transfer to SARS ward	Apr 7, 2003	Apr 7, 2003	Apr 5, 2003
Leukocyte count at admission, /μL	12,500	6,800	2,500
Total steroid dose used, mg	25,280	2,600	3,600
Intensive care unit admission	Yes	No	No
CD4/CD8 cell ratio 6 mo after disease	0.63	1.56	1.23
Absolute CD4 cell count 6 mo after disease, /μL	368	431	306
Absolute lymphocyte count 6 mo after disease, /μL	2,098	1,115	1,666

lin G (IgG) antibody titers were measured by enzyme-linked immunosorbent assay kit (Huada Company, Beijing, China) at months 1, 2, 3, 4, 7, 10, and 16 after disease onset. (Titers were not measured for the 3 TB patients at month l.) Compared to most (26 [78.8%] of 33) other SARS patients whose antibodies remained detectable throughout follow-up, 2 of the 3 TB patients (patients 1 and 3) had undetectable antibody titers as of months 7 and 16, respectively. In patient 1, antibody titers, when detectable, were unusually low (40). Both patients 1 and 3 had prolonged viral excretion in stools, sputum, or both. While the median (range) duration of virus excretion in stools and sputa for the entire measurable cohort (n = 56) was 27 (16–127) and 21 (14–52) days, respectively (*3*), it was 125 and 16 days for patient 1, and 109 and 52 days for patient 3 (viral excretion data could not be obtained from patient 2 because sequential specimens for detection were unavailable).

TB in SARS patients has been reported on rare occasions (*4*,*5*). In a cohort of 236 patients in Singapore, it was diagnosed in 2 patients after recovery from SARS (*4*). As with patient 1 in this study, TB had developed after the patient acquired SARS, most likely as the result of reactivation of past infection or new infection with *M. tuberculosis*, while temporarily immunosuppressed because of SARS (*6*) and corticoid therapy. Such phenomena have been described with other viral infections such as measles and HIV (*7*,*8*). By contrast, patients 2 and 3 were known TB patients who acquired SARS through exposure to SARS patients in the same hospital wards. Both diseases are known to be transiently immunosuppressive (*6*,*9*), and their combined effect resulted in more pronounced CD4+ cell decreases in coinfected SARS patients than others. Such immunosuppression also resulted in poorer IgG antibody response in coinfected SARS patients than in others and delayed viral clearance, as shown by longer viral excretion in sputum and stools. While viral excretion could be prolonged in coinfected patients, no virus could be isolated from any RT-PCR–positive specimen collected after 6 weeks of illness, which suggests that excreted viruses were no longer infectious (*3*).

These case reports remind us of the importance of strict isolation of SARS patients, careful use of steroids for their case management, and the possibility of coinfection with TB in SARS patients with incomplete recovery.

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Tetanus in Injecting Drug Users, United Kingdom

To the Editor: The epidemiology of tetanus in the United Kingdom changed in 2003 when a cluster of cases in injecting drug users (IDUs) occurred (*1*,*2*). Before 2003, the incidence of tetanus was low in the United Kingdom, with occasional cases predominantly in unvaccinated elderly persons (*3*). The situation contrasted with the United States where injecting drug use is commonly reported among persons with tetanus (*4*).

We investigated the UK cluster to identify the source of infection and opportunities for prevention. We ascertained cases through statutory and nonstatutory reporting to the Health Protection Agency and collected additional information on IDUs for all reported cases of tetanus since January 1, 2003, by adapting the existing enhanced tetanus surveillance. A case was defined as mild-to-moderate trismus and at least 1 of the following: spasticity, dysphagia, respiratory embarrassment, spasms, autonomic dysfunction, in a person who injected drugs in the month before symptom onset.

Twenty-five cases were reported from July 2003 to September 2004 (Figure). Thirteen (50%) were women; the median age of male and female patients was 39 and 32 years of age, respectively (range 20–53, p = 0.1). Twenty patients were white, and 1 was Chinese (information was missing for 4). None reported travel overseas before becoming sick. Seventeen of 21 patients with information reported having injected heroin intramuscularly or subcutaneously (popping) or having missed veins. Most patients (16/25) came to the hospital with severe generalized tetanus. Injection site infections were common (17/19).

Two patients died (case fatality 8%). Of 23 survivors, 2 had mild disease and 21 required intensive treatment for a median of 40 days (range 24–65 in 15 cases with complete information). Tetanus immunization status available for 20 case-patients (based on medical records or patient and parental recall) indicated that only 1 patient (with severe disease) had received the 5 doses necessary for complete coverage. Nine patients were never vaccinated. Twelve of 14 patients tested for tetanus immunity on admission by a standard indirect enzyme-linked immunosorbent assay had antibody levels lower than the cutoff value for protection (<0.1 IU/mL). One patient with severe disease had a level just above the cutoff value and 1 patient with mild disease had a protective antibody level. *Clostridium tetani* was isolated from 2 patients; tetanus toxin was detected in serum from 1 and also from another patient. Other anaerobes, including *C. novyi*, *C. histolyticum*, and *C. perfringens*, were

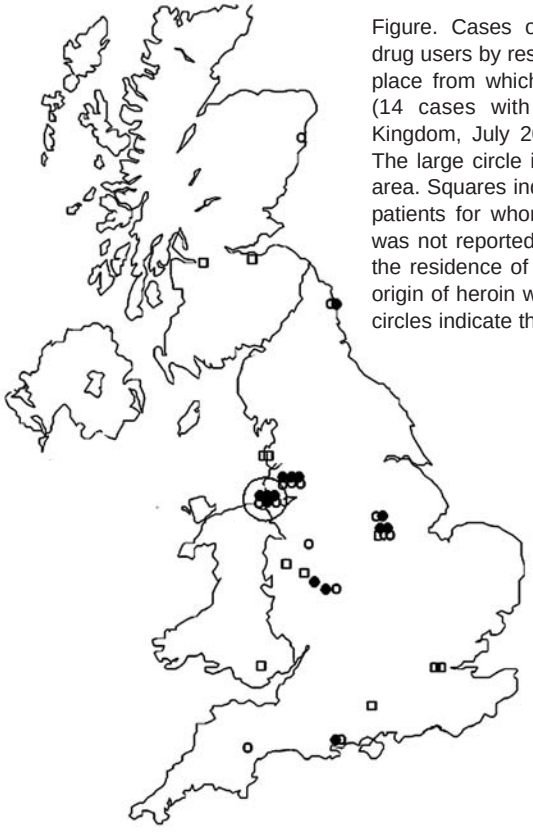

Figure. Cases of tetanus in injecting drug users by residence (25 cases) and place from which heroin was supplied (14 cases with information), United Kingdom, July 2003–September 2004. The large circle indicates the Liverpool area. Squares indicate the residence of patients for whom the origin of heroin was not reported, open circles indicate the residence of patients for whom the origin of heroin was reported, and solid circles indicate the origin of heroin.

isolated from injection site wounds of 3 patients. A heroin sample from 1 patient was tested by polymerase chain reaction, but no evidence of tetanus contamination was found (J. McLauchlin, pers. comm.). One (fatal) case of tetanus was reported from the Netherlands through the European Monitoring Centre for Drugs and Drug Addiction (L. Wiessing, pers. comm.).

Tetanus, arguably the oldest infection associated with IDU (*5*), can be caused by spore contamination during production, distribution, storage, cutting, reconstitution, and injection of drugs. The widespread distribution and temporal clustering (*1*) of cases in the United Kingdom suggest that its cause was contamination of heroin rather than changes in injecting practices. This finding is consistent with results of a similar investigation of a cluster of *C. botulinum* in IDUs in California (*6*). Only 1 case was reported outside the United Kingdom, which suggests that contamination occurred within the United Kingdom. The pronounced clustering of the place from which the heroin was supplied, compared with the residence of IDUs (Figure), is consistent with contaminated heroin having been distributed from Liverpool.

Intramuscular or subcutaneous injection of heroin was common among case-patients. This was also found in a large international outbreak of *C. novyi* in IDUs (*7*) and a botulism outbreak in IDUs in California (*6*), and is consistent with the obligate anaerobe characteristic of *Clostridium* spp. In our cluster and in other outbreaks, women and older injectors were overrepresented compared with demographic estimates of IDUs (*8*). Women and long-term IDUs may have difficulty accessing veins and frequently inject intramuscularly or subcutaneously. Furthermore, tetanus immunity is more likely to be inadequate or have waned with age.

The reasons for emergence of *Clostridium* infections in IDUs in the United Kingdom remain speculative (*9*). They include an increase in contamination of heroin and an aging cohort of heroin users who are more likely to use popping as the mode of injection.

In the United Kingdom, 5 doses of tetanus toxoid–containing vaccine at appropriate intervals are considered to provide lifelong protection, as long as tetanus-prone wounds are treated with tetanus immunoglobulin (*10*). Only 1 case in the present cluster received the recommended 5 doses of tetanus vaccine. This coverage is lower than what might be expected. During the period in which most of the patients were born (1964–1984), primary immunization coverage increased from 75% to 85% (www.hpa.org.uk). Since IDUs are at risk for *Clostridium* infections (*9*), drug action teams, needle exchange programs, prison staff, and clinicians should ensure that IDUs are vaccinated against tetanus and educated about signs and symptoms of soft tissue infections that require prompt medical intervention.

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Henipavirus in *Pteropus vampyrus* Bats, Indonesia

To the Editor: The emergence of Nipah virus (NiV) in Malaysia in 1999 resulted in 265 known human infections (105 fatal), widespread infection in pigs (with >1 million culled to control the outbreak), and the collapse of the Malaysian pig export market (*1*). As with the closely related Hendra virus (HeV) that emerged in Australia in 1994 and caused fatal disease in horses and humans (*2*), bats of the genus *Pteropus* (commonly known as flying foxes) were identified as the major reservoir of Nipah virus in Malaysia (*3*,*4*). This report describes a serologic survey of *Pteropus vampyrus* in neighboring Indonesia.

We nonrandomly sampled 106 *P. vampyrus* bats from market sellers on the Indonesian islands of Java and Sumatra during a 12-day period from July 23 to August 3, 2002 (Figure). Bats were typically caught locally by sellers. Screening by indirect enzyme-linked immunosorbent assay with inactivated NiV antigen was done at the Research Institute for Veterinary Science in Bogor, Indonesia. Virus neutralization tests (VNT) with NiV and HeV were performed under biosafety level 4 conditions at the Commonwealth Scientific and Research Organization (CSIRO) Australian Animal Health Laboratory in Geelong, Australia. The gold-standard (*6*) VNT results are presented here; a neutralizing titer ≥5 was considered positive.

Serum samples from 32 bats neutralized NiV (median titer 20, range 5–160), samples from 52 bats did not, and samples from 20 bats caused toxic reactions in the cell sheet at dilutions <10 (n = 7), <20 (n = 9), or <40 (n = 4), precluding a definitive test outcome. Two bats had inadequate samples for NiV VNT. Samples from 19 bats neutralized HeV (median titer 10, range 5–80), samples from 60 bats did not, and samples from 27 bats caused toxic reactions at dilutions <10 (n = 18), <20 (n = 7), or <40 (n = 2), precluding a definitive test outcome. Of the 70 bats whose samples had a definitive outcome in both tests, 11 neutralized NiV only, 1 neutralized HeV only, and 17 neutralized both viruses. Of these 17 bats, 14 samples had a higher titer to NiV than to HeV, 2 had identical titers to each virus (5 and 10), and 1 had a higher titer to HeV (40) than to NiV (20). Infection was attributed to NiV in 25 bats (11 whose samples neutralized only NiV and 14 whose sera neutralized both viruses but had a higher titer to NiV), a prevalence of 35.7% (95% confidence interval [CI] 24.6%–48.1%). Infection was attributed to HeV in 2 bats (1 had a HeV titer of 5 and no NiV titer, and the second had a HeV titer of 40 and a NiV titer of 20), a prevalence of 2.9% (95% CI 0.3%–9.9%).

The detection of antibodies that neutralized NiV at all 3 sampling locations indicates that infection with NiV (or a cross-neutralizing virus other than HeV) is widespread in *P. vampyrus* in Sumatra and Java. These findings, in conjunction with earlier findings in peninsular Malaysia, suggest that NiV infection is likely to be found in *P. vampyrus* across its entire range (Figure). Recent satellite telemetry studies showing regular *P. vampyrus* movements from Malaysia to Sumatra and Thailand also support this contention (*7*). Additionally, experience with HeV in Australian flying fox populations suggests that where susceptible flying fox species share communal roosts, evidence of infection is seen in in-contact species (*8*). Therefore, NiV (or a Nipah-like virus) infection probably occurs in other *Pteropus* species whose geographic distributions overlap or abut that of *P. vampyrus*. This contention is supported by the positive NiV serologic findings in *P. lylei* in Cambodia

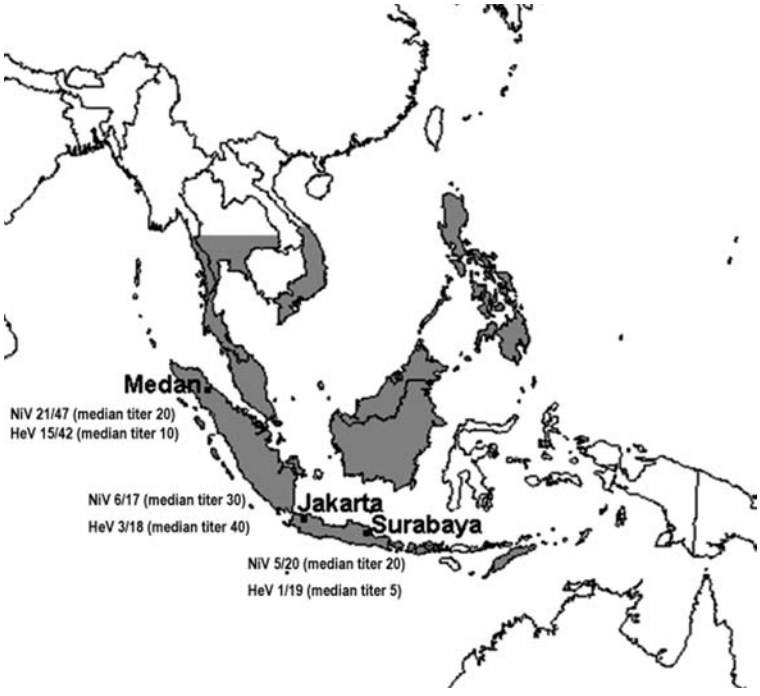

Figure. Geographic range of *Pteropus vampyrus* (*5*) and proportion of bats whose sera neutralized Nipah virus (NiV) and Hendra virus (HeV) at each location. Numbers are given as the ratio of the number of positive samples to the total number of positive and negative samples (excluding bats in which a toxic reaction precluded a definitive test outcome and bats that had inadequate samples for neutralization testing).

in 2002 (*9*) and *P. giganteus* in India (J. Epstein et al., unpub. data) and Bangladesh (*10*).

Infection was attributed to HeV in only 2 bats. The finding of 2 true HeV-positive bats in Medan and Jakarta would require sporadic HeV infection in a population in which NiV infection predominates or, alternatively, nomadic movement of animals from a population in which HeV circulates. Given the equivocal HeV titers in the 2 bats, these results are likely false positives.

The findings indicate that NiV or an unidentified Nipah-like virus is endemic in *P. vampyrus* in Indonesia. Further interpretation is limited by the nonrandom sample, the <100% specificity of the VNT, and the inability to corroborate serologic results by virus isolation or polymerase chain reaction (tissue collection was not permitted by Indonesian wildlife authorities).

Similar serologic findings are likely in overlapping *P. vampyrus* populations and possible in overlapping populations of other *Pteropus* species. Further research is needed to explain the geographic extent of NiV infection in flying foxes and the nature and stability of the interface between HeV and NiV, and to investigate the possible presence of other cross-neutralizing henipaviruses.

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We dedicate this article to our colleague and friend David Banks, who died on May 7, 2005, in an aviation accident while returning from Cape York in northern Australia after a field survey for quarantine pests and diseases.

Financial support was provided by Biosecurity Australia and facilitated by David Banks.

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AIDS in Asia: A Continent in Peril

Susan S. Hunter
Palgrave Macmillan,
New York, 2005

ISBN: 1403967741
Pages: 288; Price: US $30.00

Susan Hunter's book, AIDS in Asia: A Continent in Peril, tackles an important subject. The subheading leaves no doubt that this volume belongs squarely in the "next wave" school—Africa has been devastated by the AIDS epidemic, and Asia must be next. Hunter's book paints a picture of a continent about to be engulfed in social, economic, and political chaos, all because of 1 small retrovirus.

Unfortunately, Hunter presents no data that support this hypothesis. Her chapter on the epidemiology of HIV in Asia, The Looming Mushroom Clouds of Infection, relies heavily on a single report by Nicholas Eberstadt, a demographer and foreign policy analyst who has little experience in the modeling of sexually transmitted and infectious disease. She draws occasionally on advocacy reports by other international organizations such as The Joint United Nations Programme on HIV/AIDS. The book is poorly referenced and shows no evidence that Hunter has attempted to examine surveillance data (much of which is in public domain) or other original data sources. For example, she provides no source for the claim that "HIV… typically infects 50%–90% of a developing country's sex workers." Five years of systematic review of primary surveillance data from thousands of sentinel sites throughout Asia has shown only 1 site with rates >50% among sex workers, Mumbai in India. Virtually all the sex worker sites in the Philippines, Indonesia, China, Bangladesh, Pakistan, the most populous countries in the region other than India, report prevalence rates <5%.

The book is consistently structured. Each chapter begins with conversations between the downtrodden victims of the HIV epidemic in Asia, persons whose clothing, gestures, and thoughts Hunter describes in heartrending detail, although they are not, in fact, real persons. Next comes a homily based on the life of Emily Pankhurst, the 19th century English feminist whose relevance to the current HIV epidemic in Asia is uncertain. Then comes a bit of Asian history, a nicely written précis of the Mongol advance or the opium wars, for example, and some observations on historical and cultural injustices of life, injustices that ultimately lead people to have unprotected sex or share needles. Each chapter closes with an apocalyptic statement, along the lines of "More and more countries in Asia are teetering on the brink of a violent explosion… and the outcome may be an epidemic the likes of which has never been seen in world history."

Hunter may perhaps be forgiven for absorbing the alarmist tone of the advocacy reports she has read, but more attention to the facts would show that this sort of doom mongering is contradicted by every reliable measure of HIV prevalence and sexual behavior in Asia. These data suggest that in Asia (as in the industrial world), HIV will largely be confined to populations with well-defined risk of exposure to the virus. Hunter is absolutely correct in taking Asian governments to task for not doing enough to confront HIV. However, suggesting that HIV is an impending catastrophe that requires turning Asian societies upside down is unhelpful and outdated. This may be true in Africa, where Hunter has more experience, but had she looked beyond the hype at any real data, Hunter would have found that HIV infection does not, in fact, threaten to engulf Asia in social and economic chaos. It is a relatively well-contained infectious disease that, for prevention and control, requires clean needles, condoms, lubricant, screening for sexually transmitted infections, and treatment for a small proportion of the population. Those goals can, and should, be achieved shortly.

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Biological Weapons Defense: Infectious Diseases and Counterbioterrorism

Luther E. Lindler, Frank J. Lebeda, and George W. Korch, editors
Humana Press, Totowa,
New Jersey, 2004

ISBN: 1588291847
Pages: 597; Price: US $145.00

This insightful text, complete with an eBook version on CD-ROM, is edited by 3 scientists from the Department of Defense (DOD) and includes 58 contributors and forewords by David Franz and Mathew Meselson. The 25 chapters are divided into 4 sections: 1) Preparation and Military Support for a Possible Bioterrorism Incident, 2) Medical Countermeasures and Decontamination, 3) Emerging Threats and Future Preparation, and 4) Diagnostic Development for Biowarfare Agents.

Threats discussed include plague, glanders, Q fever, filoviruses (as a

specific example of "a world aswarm with viral zoonoses"), anthrax, smallpox, brucellosis, botulism, and ricin. Information is also provided on genetically-engineered protein toxins, as well as genetic fingerprinting for forensic studies and the use of genomics for the agents of tularemia, brucellosis, and clostridial gas gangrene.

Notably, several chapters are devoted to critical topics that are often not found in other books. For example, 2 chapters on aerosol pathogenesis and "Biological Weapons Defense: Effect Levels" are particularly relevant given the US Cities Readiness Initiative that involves planning for an aerosol attack with anthrax or another agent in US metropolitan areas. Similarly valuable is the chapter on decontamination because it provides insight on how to respond to the challenge of recreating a safe environment in which to live and work after a biological attack.

The 25-page chapter on the Global Emerging Infections System (GEIS) of the DOD as it applies to biodefense is well written. Surveillance systems used by DOD-GEIS are described, from the Electronic Surveillance System for the Early Notification of Community-based Epidemics to newer systems, along with ways to integrate DOD and civilian surveillance systems. Other particularly useful chapters that encompass multiple present and potential future biological threats include those on diagnostics. These 3 chapters focus on explaining biological threat identification systems, DNA-based pathogen identification, and immune response–based assays. Taken together, the 25 chapters of this book are a welcome addition to the growing field of counterbioterrorism and complement well the mostly clinical publications already in print.

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Correction: Vol. 12, No. 2

In "Detecting Emerging Diseases in Farm Animals through Clinical Observations" by G. Vourc'h et al., acknowledgment of contributions by Sandia National Laboratories, New Mexico State University, New Mexico Department of Agriculture, and Kansas State University were omitted. Acknowledgments should include the following:

Sandia National Laboratories designed and developed the original RSVP surveillance system, a system with applications in both human and animal disease surveillance.

Sandia National Laboratories and New Mexico State University/New Mexico Department of Agriculture are primary collaborators, along with Kansas State University, on the RSVP-A project that has been jointly pursued since 2003. The opinions on RSVP-A in this article do not necessary reflect all of the project's collaborating parties.

The corrected article appears online at http://www.cdc.gov/ncidod/EID/vol12no02/05-0498.htm

We regret the omission and any confusion it may have caused.

Alexis Rockman (b. 1962). Manifest Destiny (2003–2004)
Oil and acrylic on 4 wood panels (2.44 m × 7.32 m). Brooklyn Museum, New York. Courtesy of the artist

Manifesting Ecologic and Microbial Connections

Polyxeni Potter*

"I'm a pop artist using natural history as my iconography," Alexis Rockman has said of himself (*1*). Pop art, a movement that coincided with the youth and music phenomena of the 1950s and 1960s, draws its subject matter from the modern urban consumer experience, adopting popular culture icons and introducing them to the art world, much as American artist Andy Warhol incorporated and immortalized in his work canned soup and actress Marilyn Monroe (*2*). A man of his times, Rockman lives and breathes the culture of his native New York City, drawing inspiration from it, absorbing its trends, obsessions, conflicts, and fears, which he then brings to life in fantastic images created from nature.

Apart from a brief stay in a remote area of Peru, where his mother, anthropologist Diana Wall, did field work, Rockman grew up an urban child in an Upper East Side apartment oddly populated with newts, cats, boa constrictors, iguanas, tortoises, and lizards. He collected specimens and kept poisonous dart frogs, which he drew from a very young age, and planned to be a scientist. His playground was the American Museum of Natural History, where his mother worked with anthropologist Margaret Mead. The museum's extensive collections and dioramas became an influential part of his childhood. The artists who created the dioramas, he later said, were guided by the same painters who inspired him, Thomas Cole, Frederic Church, Albert Bierstadt (*3*).

His interest in zoology and botany was rivaled by other interests, among them film making and animation. He studied at the Rhode Island School of Design and graduated from New York's School of Visual Arts with a major in illustration. He worked as columnist and illustrator for Natural History magazine, while he gradually moved toward fine arts and started to show his work in solo and group exhibitions. A major influence was artist Ross Bleckner, whom he served as assistant for a time and who advised him to move toward modernism (*4*).

A leader among contemporary artists returning to figurative content, Rockman wants to paint what he sees (*5*). Taken with the natural world, he studies not only nature's creatures but also the puzzles surrounding them: their origins, survival, adaptability, evolution. Plants and animals are photographed and researched in libraries, their native habitats, or the Bronx Zoo. He delves into taxonomy and molecular biology and has enlisted the help and gained the following of paleontologists, biologists, ecologists, ichthyologists, and other scientists, who provide him clues to the accuracy of his exacting images. He has traveled to the rainforests of Brazil and Guyana in search of authentic

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specimens and to the South Pacific to sketch the extinct Tasmanian tiger in a local laboratory (*6*). He counts Charles Darwin as a mentor.

A combination of natural science and fantasy, his work explores the predatory relationship between nature and culture. Inspired equally by scientific curiosity and artistic compulsion, his startling images are at once literal, naturalistic, and entirely imaginary. Challenging the way we see and categorize the world, he questions human-animal-nature interaction by creating "in your face" scenarios based on vital popular culture dilemmas, among them genetic engineering and global warming.

"He tweaks my cerebrum," late professor Stephen Jay Gould said of Rockman (*7*). His snakes grow legs and chickens sport multiple sets of wings. Kangaroo-sized rats stroll across futuristic landscapes. A pig harbors human organs for harvest, and grossly oversized parasites, ticks, ants, and viruses populate his large surreal scenes. Botanical compositions, swarming with nature's less appreciated creatures and extinct or mutant forms feature aquatic or tree-sized dandelions. Humans are rarely present, though human handiwork always is. Riddles and humor are mixed in with actual soil, mud, sand, vegetation, and other collage materials, adding tactile interest to rich layers of color and varnish, which create a highly finished, luminous effect.

For nearly two decades, Rockman has worked from his studio in TriBeCa (Triangle Below Canal, between the Hudson River and Broadway), transforming historical culture into naturalistic images. Referring to himself as a "paleogeek," he favors large prehistoric landscapes reinterpreting the ecologic past and still lifes exploring the evolutionary future.

In Manifest Destiny, on this month's cover, Rockman imagines Brooklyn 3,000 years in the future. Fueled by exhaustive research, his artistic imagination produces a panoramic view of Brooklyn Bridge and environs. The polar ice caps have melted and the borough is under water. An eerie orange glow permeates layers of underwater ruins covered with slime and inhabited by weird creatures. In what the painter has referred to as "democratic space," prehistoric beasts paddle with newfangled mutants and everyday pests.

"I'm dying to see what scientists will think," Rockman said, while still working on the painting, transforming technical information from his research into visual language (*3*). In this restructured environment, geology is turned on its ear, along with the food chain. Large fish with snake heads or oversized whiskers swim by a tirelike cell infected with giant HIV. Jellyfish tentacles stretch halfway across the seascape, past a two-tailed salmon. Flocks of wild birds hover above the waterline. Minute life forms, enlarged against the ruins, signal the survival of the unexpected. A galleon rests near the wreckage of a nuclear submarine. And the grand bridge lies broken, a fossil amidst decaying structures and vegetation.

Rockman's haunting vision of the future is rife with cultural and evolutionary undertones. The geologic, botanical, and zoologic clues to the future, rooted in the past and buried in the lurid reds of rust and pollution, are well understood by scientists. For ecologic disaster and disease emergence evolve along the same path, guided by the same factors: human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, microbial adaptation and change, and the breakdown of public health measures (*8*).

And while short-term risk for epidemics after geophysical disasters may be low (*9*), long-term effects of ecologic change on disease emergence, aptly shown in the exaggerated size of viruses (e.g., HIV), are huge. Rockman's meticulously drawn mutants, alluding to genetic engineering or environmental pollution, also articulate dilemmas inherent in disease control: because of microbes' evolutionary potential, our very drugs or pesticides, may contribute to selection of mutations, adaptations, and migrations that enable pathogens to proliferate and nonpathogens to become virulent. Manifest or not, the destiny of humans, animals, and the natural environment is inextricably interlinked.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the May issue for the following topics:

Universal Genotyping and Tuberculosis Control Program, New York City, 2001–2003

Tuberculin Skin Testing in Children

Tuberculosis Transmission and HIV, Malawi

Isoniazid Preventive Therapy and Risk for Resistant Tuberculosis

Worldwide Distribution of Beijing/W Genotype *Mycobacterium tuberculosis* and Drug Resistance

Tuberculosis-HIV Co-infection, Ukraine

Mycobacterium tuberculosis and Rifampin Resistance, United Kingdom

Evidence of Coronavirus HKU1 Infection in the United States

The Trojan Chicken Study, Minnesota

Aedes aegypti Larval Indices and Risk for Dengue Epidemics

Shiga-toxigenic *Escherichia coli* O157 in Agricultural Fair Livestock, United States

Complete list of articles in the May issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

May 19–23, 2006
Council of Science Editors 49th Annual Meeting
Hyatt Regency Tampa
Tampa, FL, USA
http://www.councilscienceeditors.org

June 17–22, 2006
Negative Strand Viruses 2006: Thirteenth International Conference on Negative Strand Viruses
Salamanca, Spain
Contact: 404-728-0564 or meeting@nsv2006.org
http://www.nsv2006.org

June 25–29, 2006
ISHAM 2006 (International Society for Human and Animal Mycology)
Palais des Congrès
Paris, France
Contact: 770-751-7332 or c.chase@imedex.com
http://www.imedex.com/calendars/infectiousdisease.htm

EMERGING INFECTIOUS DISEASES

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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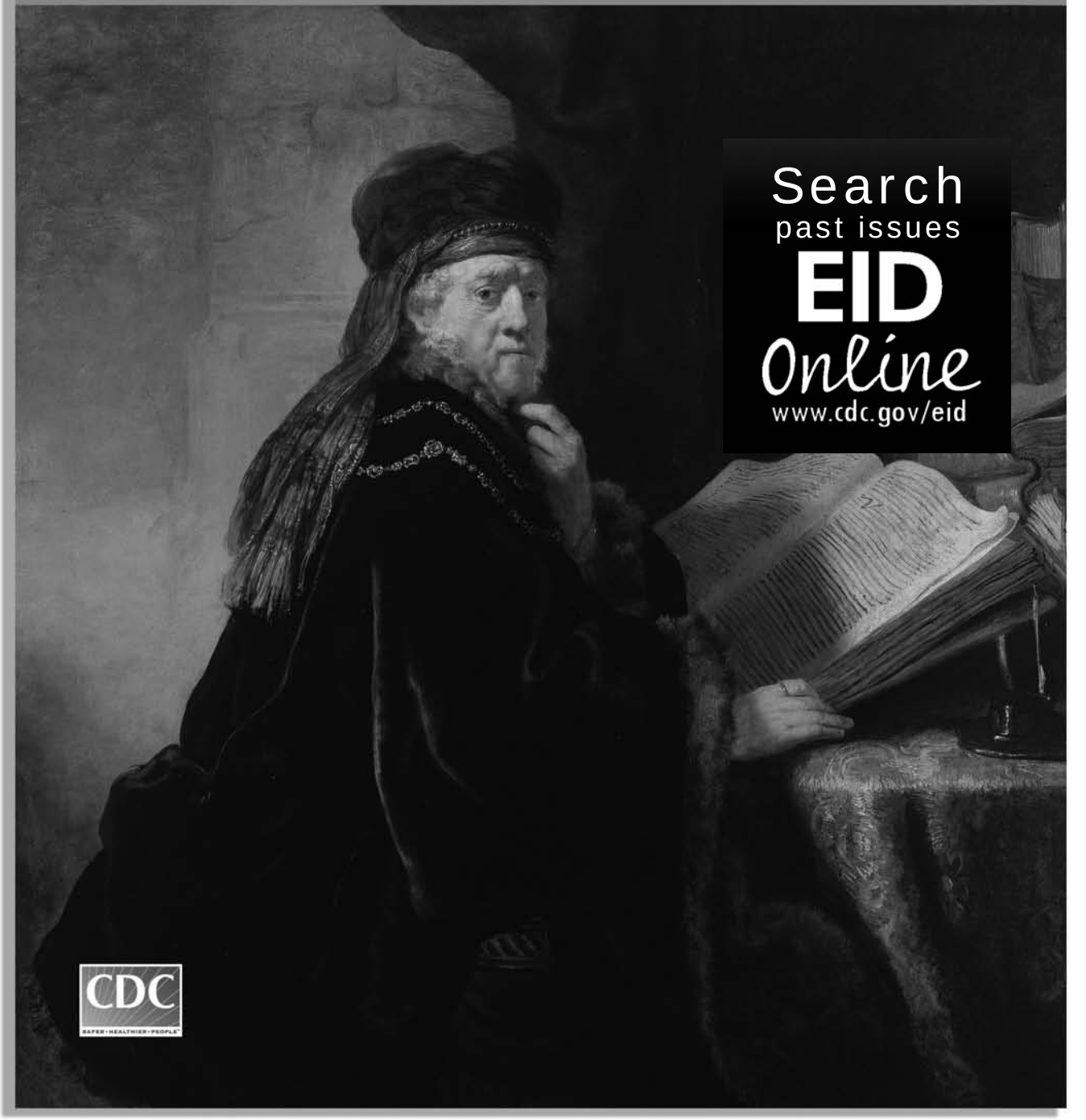

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Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

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).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

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.