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EMERGING INFECTIOUS DISEASES

March 2007

On the Cover

Francisco Roa (b. 1963)
Sands Flowers (1994)
Oil on panel (45 cm × 36 cm)
Courtesy of Oglethorpe University Museum, Atlanta, Georgia, USA
Photographed by James Gathany

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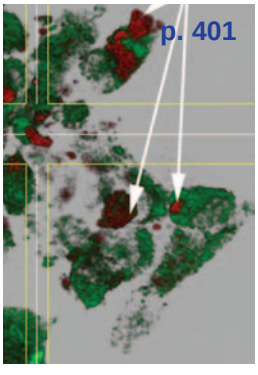

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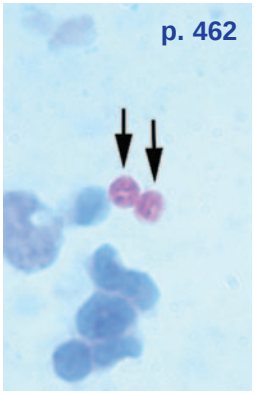
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Bird Migration Routes and Risk for Pathogen Dispersion into Western Mediterranean Wetlands

Elsa Jourdain,*† Michel Gauthier-Clerc,* Dominique J. Bicout,† and Philippe Sabatier†

Wild birds share with humans the capacity for moving fast over large distances. During migratory movements, birds carry pathogens that can be transmitted between species at breeding, wintering, and stopover places where numerous birds of various species are concentrated. We consider the area of the Camargue (southern France) as an example to highlight how ad hoc information already available on birds' movements, abundance, and diversity can help assess the introduction and transmission risk for birdborne diseases in the western Mediterranean wetlands. Avian influenza and West Nile viruses are used as examples because birds are central to the epidemiology of these viruses.

Birds are the only terrestrial vertebrates that share with humans the peculiarity of traveling in a few hours across national and intercontinental borders. The record for distance covered in a single year belongs to the arctic tern (*Sterna paradisaea*), which travels ≈50,000 km between Antarctica and northern Scandinavia. As a whole, billions of birds travel between continents twice a year in only a few weeks (*1*). During these yearly migrations, birds have the potential of dispersing microorganisms that can be dangerous for public as well as animal health (*2*,*3*). For instance, birds are believed to be responsible for the wide geographic distribution of various pathogens, including viruses (e.g., West Nile, Sindbis, influenza A, Newcastle), bacteria (e.g., borrelia, mycobacteria, salmonellae), and protozoa (e.g., cryptosporidia). Insight into the ecology of bird populations is necessary to understand the epidemiology of bird-associated diseases. Furthermore, data about avian movements might be used to improve disease surveillance schemes or to adapt preventive measures. However, solid bridges between ecology and human medicine are still lacking.

We explored the bird sector, in an attempt to provide general ideas on bird abundance, migration, geographic origin, and interspecies mingling. We focused on the Camargue area, an alluvial lowland covering some 140,000 ha in the Rhône Delta. As other Mediterranean wetlands (Figure 1), the Camargue is a major rallying point for Palearctic birds that are migrating between the great continental masses of Eurasia and Africa. This area is the current focus of intense sampling to study 2 pathogens closely associated with wild birds: avian influenza (AI) virus and West Nile virus (WNV). These 2 viruses have very different transmission cycles and ecology: AI viruses have a waterborne transmission, and ducks are their main natural reservoirs (*4*–*8*); WNV has a vectorborne transmission, and passerines are believed to play a major role in the

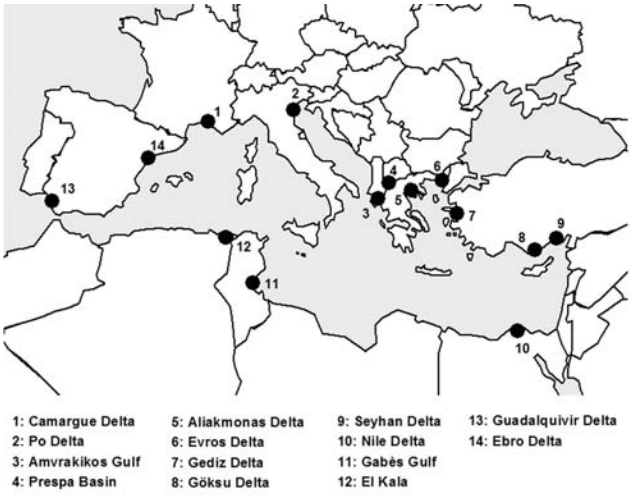

Figure 1. Map of the main Mediterranean wetlands (sites 1, 2, 11, 12, 13, and 14 are considered western Mediterranean wetlands).

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amplification cycle (*9*–*11*). However, both viruses are known to be carried by reservoir birds during migration and have been associated with emerging disease transmission risk for humans and domestic animals (*2*,*5*,*7*,*11*,*12*). For both of them, the avifauna abundance, diversity, and departure origin may be of key importance in terms of disease transmission. We use these 2 viruses as examples in our discussion of the risk for dispersion of bird-carried pathogens into Mediterranean wetlands.

We address the following questions: 1) What are the main geographic origins of birds observed in western Mediterranean wetlands? 2) How abundant and diverse in species are they during the year cycle? 3) When are interspecies contacts between birds from different origins most likely to occur? To address these issues, we used crude empiric indexes, which are known to have biases yet prove valuable within the scope of our objectives. Readers interested in modern ecologic methods used to study wildlife diseases in natural populations may refer to general publications on host-parasite systems (*13*–*15*).

Methods

Bird Origins from Individual Ringing

Migration research is constantly changing, and new methods are always emerging. Historically, information about the movements of individual birds was first acquired through ringing studies. Bird ringing (also known as bird banding) consists of catching birds and attaching a small individually numbered metal or plastic ring to their legs or wings. Ring-recovery data are obtained when ringed birds are resighted, recaptured, hunted, or found dead. In Europe, large-scale ringing projects have been conducted, mostly between the 1950s and 1980s, and they represent a wealth of information that has not yet been fully exploited.

Data recovered from birds ringed from 1950 to 1975 at the Station Biologique de la Tour du Valat in the Camargue were collected from annual reports. Seven species of waterbirds were chosen to illustrate various migratory patterns. We selected 4 species of the Anatidae family, known to have different geographic origins, including 3 dabbling ducks, i.e., ducks that search for their food primarily in surface water (mallard, *Anas platyrhynchos*, n recovered = 434; green-winged teal, *A. crecca*, n = 3,903; garganey, *A. querquedula*, n = 181) and 1 diving duck, i.e., a species that mostly searches for its food under water (tufted duck, *Aythya fuligula*, n = 313). We also took the example of the common coot (*Fulica atra*, n = 99), a diving bird of the Rallidae family that frequently shares ponds with ducks. The common snipe (*Gallinago gallinago*, n = 54) is an example among waders, i.e., shorebirds that feed in muddy swamps and coastlines. Finally, the purple heron (*Ardea purpurea*, n = 39) is an ardeid species that lives in reed beds and marshes. All these species are large or hunted, which explains the high number of rings recovered. We only considered data recovered from birds ringed in the Camargue area and later reported outside France.

Migratory Bird Abundance and Diversity

Since the 1950s, a large amount of data have been collected at the Station Biologique de la Tour du Valat thanks to bird counts, netting records, and field ornithologists' observations (see supplemental, online Technical Appendix Table l, indicating the methods used for each bird genus; available from www.cdc.gov/EID/content/13/3/365-appT1.htm). This information was used to create a database with a row for each of the 289 avian species regularly observed in the Camargue (*16*). Strictly pelagic birds were not taken into account as they do not have any contact with terrestrial vertebrate species. Quantitative data were completed on the number of birds (abundance) and number of bird species (diversity) observed monthly in the Camargue. Three categories of migrating birds were considered, depending on the area from which they come: incoming birds from sub-Saharan Africa in spring and those arriving in autumn either from continental Europe or from Scandinavian and the Siberian tundra and taiga. Analyses were performed for all species and separately for species of the Anatidae family (ducks, swans, geese) and waders (shorebirds of the families Scolopacidae and Charadriidae), which are essentially associated with wetlands or coastlines.

Interspecies Bird Cohabitation

Regular bird counts provide information on bird populations for the studied area and therefore give an idea of potential contacts between species that share similar biotopes. Since September 1964, the Camargue duck and coot populations have been estimated every winter (*17*). The count was made monthly by the same observer from a plane flying at an altitude of 200 feet. One hundred brackish lakes and marshes used by waterbirds as resting places were counted. The arrival of the plane made dabbling ducks fly off, which is necessary for detecting them and identifying their species. To count diving ducks, it was necessary to turn the group of birds around by using the plane. Results of the winter 2004–05 counts were used as examples.

Results

Bird Origins from Individual Ringing

Ringing recoveries provide a valuable insight into the origins and dispersion areas of bird species. Figure 2 illustrates that western Mediterranean wetlands provide habitat for birds from a wide geographic range: all European

countries but also other areas in the Mediterranean Basin, central and northern Asia, and sub-Saharan Africa. Ringed common coots and common snipes were mostly reported from continental Europe and Mediterranean areas, whereas mallards and common teals were also found in more northern places, including the former Soviet Union and Scandinavia. The pattern was slightly different for tufted ducks, for which >40% of recoveries were located in areas of taiga and tundra. Garganeys were recaptured in very distant places far north (Siberia, Finland), far east (Kazakhstan, Altai), and far south (Senegal, Mali) of the Camargue. In contrast to the previously described species, purple heron rings were recovered only from areas located south, including 4 countries in the Guinea Gulf in Africa (Benin, Côte d'Ivoire, Ghana, and Sierra Leone). As a whole, we discerned 3 broad areas from which Mediterranean waterbirds come and potentially disperse pathogens: continental Europe, northern Siberia and Scandinavia, and sub-Saharan Africa.

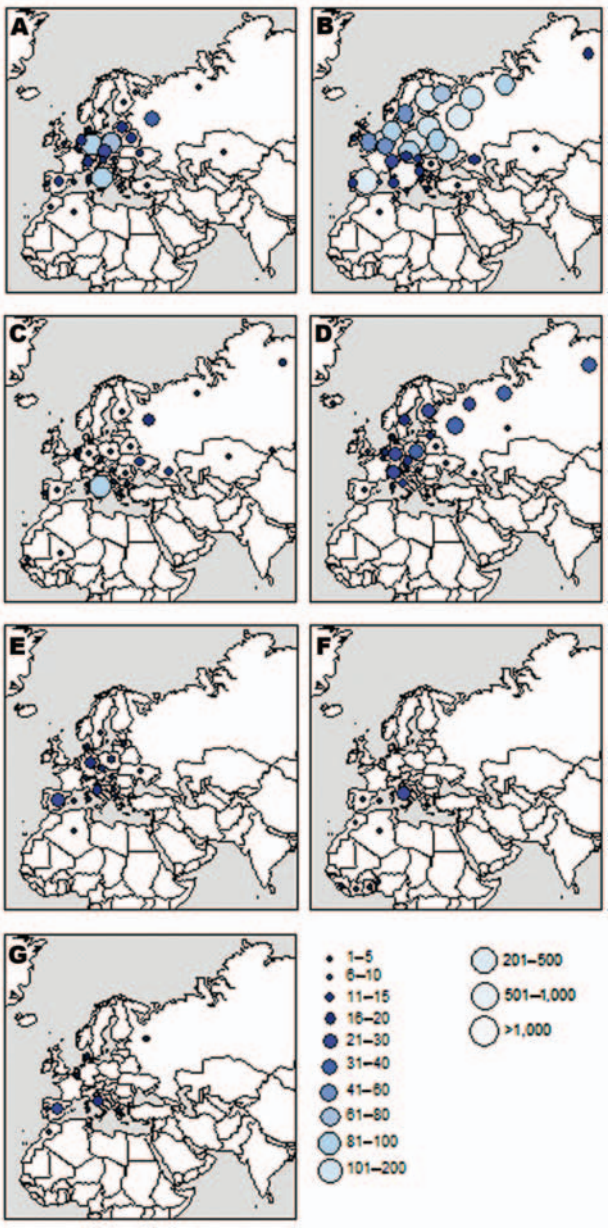

Figure 2. Countries and regions of the former USSR where birds ringed in the Camargue were recaptured for 7 species (n = number of ring recoveries and m = number of marked individual birds): A) mallard (*Anas platyrhynchos*), n = 434, m = 13,176; B) green-winged teal (*A. crecca*), n = 3,903, m = 58,347; C) garganey (*A. querquedula*), n = 181, m = 2,436; D) tufted duck (*Aythya fuligula*), n = 313, m = 3,845; E) common coot (*Fulica atra*), n = 99, m = 7,866; F) purple heron (*Ardea purpurea*), n = 39, m = 5,017; G) common snipe (*Gallinago gallinago*), n = 54, m = 2,445. These maps provide an insight into the potential origins and dispersion areas of birdborne pathogens.

Migratory Bird Abundance and Diversity

Monthly abundance (number of individual birds) and diversity (number of species) in the Camargue are presented respectively in Figures 3 and 4 for birds originating from the 3 major areas of provenance described above. These figures show how many birds are in the Camargue, just as monthly photographs of bird populations do. A corresponding table indicates monthly abundance of each species (online Technical Appendix Table 2, available from www.cdc.gov/EID/content/13/3/365-appT2.htm.).

Birds Coming from sub-Saharan Africa

As many as 111 bird species might disperse pathogens from sub-Saharan Africa into the Camargue. Broadly speaking, birds coming from sub-Saharan Africa become rapidly and simultaneously abundant and diverse in spring, are still numerous in summer, and decrease in winter. The pattern is different if one considers solely ducks, as only 3 duck species fly south to tropical Africa, namely, the northern pintail (*Anas acuta*), the garganey, and the northern shoveler (*Anas clypeata*). Conversely, numbers and species diversity are high for waders, which are mainly passage visitors, especially in spring and late summer.

Birds Coming from Northern Areas of Tundra and Taiga

A total of 53 species might introduce pathogens from northern areas into the Camargue. Abundance is highest in April and October–November with a higher peak in autumn, notably because of juvenile birds. Species diversity is high during winter and low from May to July. The opposite pattern was observed for sub-Saharan species. This pattern is even clearer for birds of the Anatidae family: They are abundant from October to January and in very small numbers from March to September. In contrast to ducks, waders are mainly transient visitors, and only a few individual birds spend the winter in the Camargue. Their number is greatest in spring and autumn.

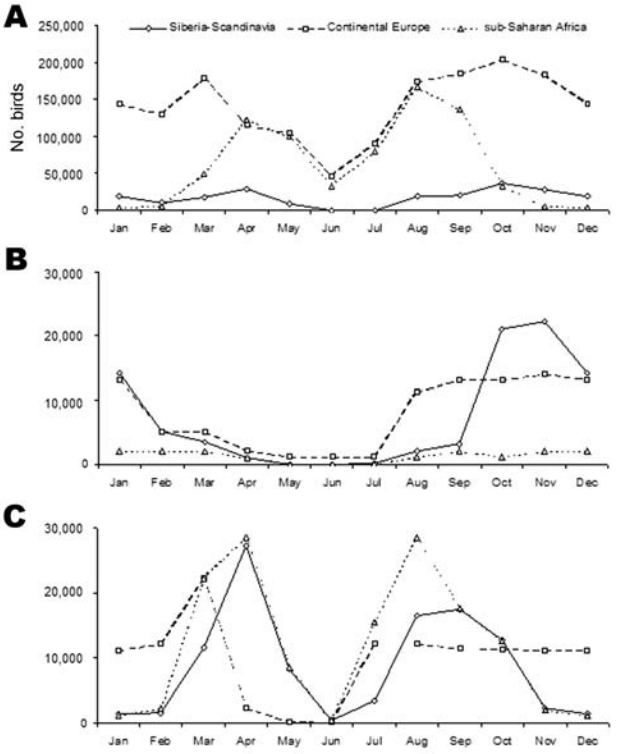

Figure 3. Monthly abundance in the Camargue of birds coming from Siberia/Scandinavia, continental Europe, and sub-Saharan Africa for A) all species, B) species of the Anatidae family, and C) waders, respectively. Peaks in bird abundance are expected to be associated with a higher probability of dispersing birdborne pathogens into the Camargue.

Birds Coming from Continental Europe

Up to 135 species could be involved in pathogen dispersion from continental Europe. Their abundance is highest from February to April and later from September to November. Species diversity remains high year-round with peaks in spring and autumn due to migrating passage visitors. The pattern observed for Anatidae species is the same as the 1 we described for Arctic species: birds are abundant in autumn and winter and in very small numbers in spring and early summer. However, the number of duck species remains stable year-round. Indeed, in species such as the mallard or the red-crested pochard (*Netta rufina*), some birds are sedentary whereas others are migratory. Waders show a constant level of species diversity because migration staggers over several months, but numbers are highly variable throughout the year.

Interspecies Bird Cohabitation

The results of the winter 2004–05 waterfowl counts are presented in Figure 5 for the species mentioned in Methods. Other species are also present, such as the northern pintail or the common shelduck (*Tadorna tadorna*). Garganeys are present in small numbers in September and February–March, but from an airplane they cannot be distinguished from common teals. These counts show that numerous species, with various migratory patterns, congregate on the same wetlands during the long winter period and therefore easily transmit waterborne pathogens such as AI virus. Most wintering birds are still present in March, when the first African migratory birds have already returned to breed in the Camargue or make a stop for refueling before flying further north. For instance, as many as 11,550 black-tailed godwits (*Limosa limosa*) were counted in the Camargue in March 2005.

Moreover, the movement and abundance of birds vary greatly from 1 year to another because of movements influenced by weather conditions. For example, the duck population in the Camargue was estimated at ≈60,000 ducks in March 2005 compared with only ≈40,000 the previous year, when climatic conditions in Europe were warmer.

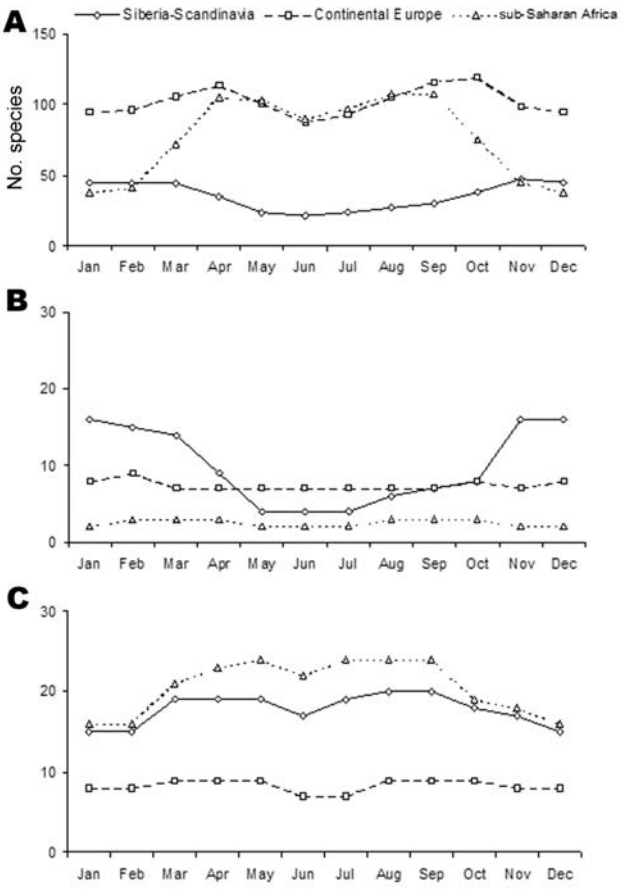

Figure 4. Monthly diversity in the Camargue of birds coming from Siberia/Scandinavia, continental Europe, and sub-Saharan Africa for A) all species, B) species of the Anatidae family, and C) waders, respectively. Peaks in bird species diversity are expected to be associated with a higher diversity of birdborne pathogens.

Discussion

Maps of ring-recovery data and graphs of monthly variations in bird abundance and diversity show that western Mediterranean wetlands such as the Camargue are a hub for birds from all origins (Central Asia, Siberia, northern and Eastern Europe, western Africa, and the Mediterranean basin) and that numerous birds of various species are seasonally aggregated in similar habitats. Under the hypothesis that risk for dispersion of pathogens into the Camargue is correlated with the number of birds and bird species encountered in a given area, these indices are helpful to determine periods at higher risk for introduction and emergence of birdborne diseases. We recall that these empiric estimates are skewed, which is briefly discussed with the perspectives below.

Periods of Higher Risk for Pathogen Introduction

Birds Coming from sub-Saharan Africa

The risk for introduction of African pathogens in Mediterranean wetlands would be highest from March to July, which corresponds with spring migration and breeding for birds. Conversely, in autumn, birds return to Africa and are more likely to introduce pathogens originating from the north than from the south (Table). Of the 111 species that come every year to the Camargue from different countries of sub-Saharan Africa, most are insectivorous passerines that spend winter in Africa and breed in Europe; among aquatic birds, waders are the most numerous.

Up to now, no evidence exists that birds migrating from sub-Saharan regions play a major part in the epidemiology of AI viruses. However, under the assumption that this area became an important epicenter for AI viruses, ducks would likely have the highest probability of introducing AI viruses in Mediterranean wetlands, even if they are less numerous than waders. Indeed, recent studies in Europe showed that overall AI virus prevalence in waders is really low compared with that in dabbling ducks (*7*). WNV, which is transmitted by arthropod vectors, could potentially be introduced by any species of bird that comes from disease-endemic areas in Africa, is exposed to mosquito or tick bites (*18*), and sustains high viremia levels. Insectivorous passerines are the most numerous and thus may be particularly suspected. WNV dispersion by birds migrating from sub-Saharan Africa might explain why an outbreak occurred in 2000 in the Camargue, even though the virus had not been observed there since the 1960s (*19*).

Birds Coming from Northern Areas of Tundra and Taiga

Pathogens may be introduced into Mediterranean wetlands by birds coming from northern areas of Scandinavia and Siberia. The risk would be higher from September to December when Arctic bird abundance reaches its peak (Table). In spring, the northern birds observed in the Camargue have recently spent a long time in southern lands so that their associated probability of introducing pathogens originating from Scandinavia or Siberia is rather low. Waterbirds and granivorous passerines, which do not need to fly further south to find food supplies throughout the cold season, could introduce pathogenic microorganisms that could be transmitted later between wintering birds when densities are high. Waders, which migrate from Siberia and stop in the Mediterranean wetlands in autumn before crossing the Mediterranean Sea, could contaminate other bird species before pursuing their flight. As a whole, 53 species seen in the Camargue come from Arctic areas, which is half the number of species that come from sub-Saharan Africa or continental Europe. As a result, the probability of pathogens being introduced from Arctic areas should be lower than from birds of these 2 other areas. Another scenario can nevertheless be considered: if birds coming from northern areas disseminate a pathogen all along their migration route, then this pathogen would also infect continental European species and the probability of its being introduced into the Mediterranean wetlands would depend on the arrival of both Arctic and continental birds.

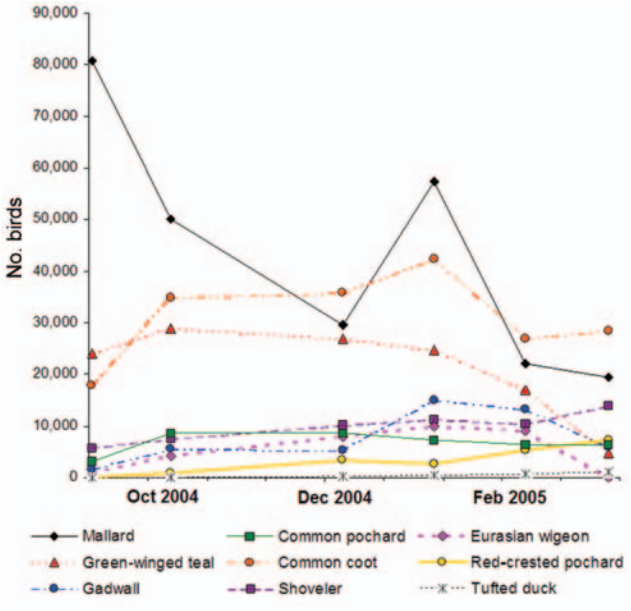

Figure 5. Cumulative number of the most abundant waterfowl species recorded in the Camargue during winter 2004–05: mallard (*Anas platyrhynchos*), northern shoveler (*A. clypeata*), green-winged teal (*A. crecca*), Eurasian wigeon (*A. penelope*), gadwall (*A. strepera*), red-crested pochard (*Netta rufina*), common pochard (*Aythya ferina*), tufted duck (*Aythya fuligula*), and common coot (*Fulica atra*). This figure shows that numerous birds and bird species are congregated in the same wetlands during winter and can therefore easily transmit pathogens to each other.

Table. Periods of major risk for pathogen introduction in the Camargue from sub-Saharan Africa, Siberia-Scandinavia, or continental Europe for all species, Anatidae species, and waders*

Origin	Species	Spring	Summer	Autumn	Winter
Sub-Saharan Africa	All	++	+	(±)	(–)
	Waterfowl	+/–	–	(±)	(–)
	Waders	++	+	(+)	(–)
Siberia-Scandinavia	All	(±)	(–)	++	+
	Waterfowl	(–)	(–)	++	++
	Waders	(+)	(±)	++	±
Continental Europe	All	(±)	(–)	++	+
	Waterfowl	(–)	(–)	++	++
	Waders	(+)	(–)	++	+

*The risk is supposed to increase both with the number of species and the number of individual birds present in the Camargue (++, very high; +, high; ±, medium; –, low). In addition, the timing of migration matters since the introduction of pathogens from Africa (Eurasia) during the autumn (spring) migration is less likely because birds do not come directly from these areas. The corresponding risks are therefore in parentheses.

AI viruses are likely to be introduced in autumn by ducks that breed in northern Europe and Siberia, especially since numbers are high because of the presence of juveniles. Furthermore, surveillance studies of wild ducks showed that the prevalence of AI viruses is primarily high in juveniles (*5*,*7*,*20*). Conversely, WNV activity has never been reported in Scandinavia and Siberia, probably because the transmission cycle cannot be maintained in these northern biotopes.

Birds Coming from Continental Europe

Autumn and winter are the 2 seasons during which the transmission of bird pathogens originating from continental Europe would be most likely (Table). Indeed, in spring, the introduction of pathogens from continental Europe is less probable because birds have been absent from this area for 5 or 6 months. As previously seen, up to 135 species have the potential to introduce pathogenic agents in the Camargue. Granivorous passerines, birds of prey, and waterfowl are among the species that come in large numbers to take advantage of the Mediterranean wetlands' temperate climate during winter. Aquatic birds, which need unfrozen ponds to feed, show variations in their movements, depending on climatic conditions. For instance, if a cold spell occurs in eastern or northern Europe, the number of green-winged teals in the Camargue increases (*17*). These weather-associated movements might at certain times prove essential in pathogen dispersion within European and Mediterranean wetlands.

Surveys of wild waterbirds in Europe have shown that AI viruses are frequently found (*21–24*), which means that waterbirds arriving from continental Europe might often be carriers of AI viruses. Similarly, because WNV activity was recently reported in Romania (*25*) and the Czech Republic (*26*), wild birds migrating in autumn from these countries to the Mediterranean basin could introduce WNV, either because of a high viremia level or because they carry infected ectoparasites. If the virus managed to overwinter in a reservoir host or a vector, it could then be responsible for an outbreak the next summer, when mosquito vectors are abundant (*27*).

Risk for Bird-to-Bird Transmission of Pathogens

Several factors affect the risk for bird-to-bird transmission: bird abundance or density, bird diversity, species receptivity and sensitivity to pathogens, interspecies interactions, and environmental conditions (*14*). For water-transmitted pathogens such as AI viruses, risk for transmission may be associated with the number of ducks congregated in the same pond, particularly in autumn and winter (Figure 5). This crowding of wintering species, in addition to the permanent presence of a transient population of birds using wetlands to stop off during migration, could allow AI viruses to circulate and be maintained because of rapid dissemination on shared water. For vector-transmitted pathogens such as WNV, transmission possibilities depend both on the bird reservoir density and on the dispersion abilities and activity periods of the arthropod vectors.

The risk for interspecies transmission of disease is particularly problematic when wild and domestic species are involved. Ducks are aquatic birds that are most likely to come in contact with free-range poultry, especially because the presence of congeners can induce migrating wild ducks to make a stopover. Captive-bred mallards, used for hunting purposes and voluntarily put in the wild to attract other ducks, are particularly likely to share pathogens with their migratory congeners and facilitate the transmission of diseases to other domestic species. The risk is different for domestic chickens or turkeys, which are more likely to have contact with granivorous birds. Conversely, waders are rarely in direct contact with human-raised species.

Bird-carried pathogens are above all susceptible to being spread worldwide because of human activities such as legal or illegal trade of wild and domestic birds or bird products (*28*). The mechanism for the introduction of WNV into America in 1999 is not known with certainty,

but a plausible scenario is the importation of an infected bird (*29*,*30*). Similarly, the highly pathogenic AI strain H5N1 was isolated in Belgium from crested hawk-eagles (*Spizaetus nipalensis*) smuggled by air travel (*31*). In Asia, transmission of H5N1 influenza virus has mainly been the result of human activity such as live-poultry markets and the international trade of birds, bird products, or contaminated equipment (*32*–*35*).

Methodologic Concerns and Perspectives

The ornithologic data we have presented are merely crude estimates. Ring-recovery data, for instance, are subject to strong biases related to where and when the ringing was conducted but also to high variability in the probability of reporting marked animals among areas (*36*). Similarly, our estimates of bird abundance and diversity are basic indices associated with the number of birds heard, seen, or caught in the Camargue (see online Technical Appendix Table 1). These estimates do not take into account 2 important sources of error: detection error, related to the fact that the probability of detecting a bird is <1, and survey error, associated with spatial and temporal variability (*37*). Since our motivations were merely to show that information already available on birds may lead to better understanding of animal and human health issues associated with birdborne pathogens, these biases do not invalidate our objectives.

The results obtained were helpful to identify key groups of species likely to introduce pathogens from a given area at a given time of year. We voluntarily chose to focus on birds and leave pathogens aside, but studies of diseases in natural bird populations are obviously critically needed. Ecology, the science of interactions between living organisms and their physical environment, has been extended to include microorganisms. Understanding the relationships between organisms (such as hosts, pathogens, predators, competitors) and their environment is the aim of disease ecology. As studying the dynamics of systems with many hosts and pathogenic agents is complex, efforts should primarily focus on a few specific bird-pathogen models.

Mathematical modeling may help to predict specific bird-pathogen interactions and to identify key parameters that need to be better estimated through additional research. Long-term records enable establishment of databases, which would illustrate bird-pathogen relationships in natural conditions. These data would focus on hosts, their migration, population age, behavior, and so forth. Host-pathogen interactions should be described by using data such as antibody prevalence in different age classes, frequency of virus isolation, and characterization of the strains involved. Complementary laboratory and field experiments within a controlled environment might also provide relevant information. All these investigations should gradually make it possible to gather valuable baseline data to test specific hypotheses and gain new insights in bird-pathogen relationships in Mediterranean wetlands.

Acknowledgments

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Dr Jourdain is researching the role of wild birds in the epidemiology of emerging infectious diseases such as WNV infection and AI. She works in the Epidemiology Unit of the College of Veterinary Medicine (Lyon, France) and at the Station Biologique de la Tour du Valat, a private foundation specialized in bird ecology and located in the Camargue, France. She is particularly interested in wildlife diseases and zoonoses.

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Human and Animal Vaccination Delivery to Remote Nomadic Families, Chad

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Vaccination services for people and livestock often fail to achieve sufficient coverages in Africa's remote rural settings because of financial, logistic, and service delivery constraints. In Chad from 2000 through 2005, we demonstrated the feasibility of combining vaccination programs for nomadic pastoralists and their livestock. Sharing of transport logistics and equipment between physicians and veterinarians reduced total costs. Joint delivery of human and animal health services is adapted to and highly valued by hard-to-reach pastoralists. In intervention zones, for the first time ≈10% of nomadic children (>1–11 months of age) were fully immunized annually and more children and women were vaccinated per day during joint vaccination rounds than during vaccination of persons only and not their livestock (130 vs. 100, p<0.001). By optimizing use of limited logistical and human resources, public health and veterinary services both become more effective, especially at the district level.

Vaccinating persons and livestock in hard-to-reach rural communities remains a serious challenge and calls for innovative, specially designed strategies. In Africa, the ability of human and veterinary health systems to deliver services is constrained by decreasing public-sector budgets; loss of confidence due to unmet demand; a severe shortage of human resources, especially qualified personnel (*1*); inadequate infrastructure and equipment; and weak monitoring and information systems (*2*). Most governments rely heavily on complementary donor funding from bilateral and multilateral partners.

One of the 4 strategies of the World Health Organization (WHO) and the United Nations International Children's Emergency Fund to achieve their vision of equitable, sustainable, and high-coverage immunization among children and women by the year 2015 is the provision of vaccination services linked to other health interventions. To achieve common health goals, the cross-over benefits of integrating other interventions in public health such as distribution of vitamin A, selling of insecticide-treated mosquito nets, and deworming and malaria treatments of children are increasingly being acknowledged (*3*). Initial evaluations on cost-effectiveness of combined approaches (*4*) provide the needed data for more efficient planning.

For children and for women of childbearing age, immunization provided through the National Expanded Program on Immunization is considered one of the most cost-effective public health interventions and society's best healthcare investment, especially in developing countries (*5*,*6*). National and global efforts aim to substantially increase vaccination coverage in general, particularly for those who have so far not benefited. Global coverage of fully immunized children is 78%, but in the sub-Saharan Africa (WHO African region) it is only 67% (*7*). A setback for the global polio eradication program was direct and indirect spread of wild polio virus type 1 from Nigeria to 11 African countries from 2002 through 2004. Affected countries that did not experience sustained transmission of this virus type had an average polio vaccination coverage of 83%, in contrast to the others that did have sustained transmission and had a much lower mean coverage of 52% (*8*). The virus spread directly into neighboring Chad and was further disseminated from there. With vaccination

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coverage <50% (*7*) and a large disparity between urban and rural vaccination coverage (20% vs. 9% for fully immunized children in 1997 [*9*]), Chadian public health professionals expressed their need for strategies to better reach the remote pastoral communities in the border regions to establish a "cordon sanitaire."

The mobility and dispersion of mobile livestock owners in semiarid Africa and elsewhere lead to difficulties in reaching them with preventive and curative treatments as well as with information and education. Outreach human vaccination services rarely exist for rural communities living far (>15 km) from the nearest health facility, which in Chad accounts for 40% of the rural population (*9*). Especially difficult is providing health services to nomadic groups, which also represent substantial numbers. Although now out of date, the most recent Chadian census of 1993 registered 9.3% (83,500) nomads in a rural population of 900,000 in the Chari-Baguirmi and Kanem Districts of Chad (*10*); these figures are probably underestimated because during the census period (April), several nomadic groups would have been seasonally located across the border in neighboring countries. Evaluating effective coverage of such groups is particularly problematic; the records of rural health centers do not distinguish between the ways of life (settled or mobile) of vaccinated persons nor do health centers have appropriate stratified population numbers (numbers have simply been extrapolated annually since 1993) to permit calculating coverages in settled and mobile communities.

Strategies of vaccinating mobile pastoralists on market days have been tested; however, children and women tend to stay in their camps, resulting in very low participation (*11*). Mobile services cost more than stationary facility services (*12*), but preventive interventions in remote zones are hardly possible if not mobile. In 2000, the prevalence of fully immunized nomadic children and women in Chadian Chari-Baguirmi and Kanem was zero (*13*); in the same nomadic camps, however, livestock were compulsorily vaccinated by mobile veterinary teams.

Livestock are part of the livelihood of ≤70% of the world's rural poor (*14*). Vaccination of livestock is a central tool for control and elimination of contagious livestock diseases. Elimination of foot and mouth disease with vaccination strategies, for example, can provide a country or a region access to export markets and dramatically increase a country's benefits from veterinary intervention (*15*). Some veterinary vaccines prevent not only important production losses of livestock but also human disease (e.g., rabies of dogs and anthrax of ruminants). Anthrax spores remain dormant for years in soil until they infect other animals. Control of anthrax in a disease-endemic setting such as the Sahel includes educating persons about how handling and consuming infected livestock products influence risk of infection, meat inspection, and particularly yearly vaccination of livestock. In Africa during the 20th century, Rinderpest virus persisted between pandemics in nomadic or seminomadic pastoralists' herds and in wildlife (*16*), from which it could spread anew to neighboring zones, as occurred in Ethiopia in the 1990s (*17*). Although a thermostable, efficacious, and safe vaccine exists, inaccessibility of cattle populations (because of remote locations, political crises, and insecurity) postponed the successful elimination of Rinderpest virus in livestock by the Pan-African Rinderpest Campaign.

Professionals from WHO and the Food and Agriculture Organization of the United Nations and others have proposed sharing of resources by public health and veterinary services to deliver health interventions at lower costs, thereby allowing for economies of scale (*18*,*19*). Few examples of joint implementation exist and evaluations of these experiences are rare (*20*). We review field research on joint human and animal vaccination campaigns among nomadic pastoralists of Chad and advocate for intersectoral approaches for service delivery in similar remote rural settings.

Joint Delivery of Public Health and Veterinary Services for Nomadic Pastoralists

The Chadian Ministries of Health and of Livestock Production (hosting the veterinary services), together with the nomadic communities, recommended testing the feasibility of joint human and livestock vaccination campaigns. The goals of these joint campaigns were to make best use of visits by professionals in nomadic communities, reduce costs of services by sharing of infrastructure, and increase vaccination coverage.

Organization of Campaigns and Vaccinated Children, Women, and Livestock

The Swiss Tropical Institute supported the implementation of several joint campaigns. From 2000 through 2001 and from 2003 through 2005, 14 vaccination campaigns for nomadic children, women, and the camps' livestock were conducted among the 3 principal nomadic ethnic groups (Fulani, Arabs, and Dazagada) in the Chari-Baguirmi and Kanem of western Chad. Five campaigns were conducted in the zone of Gredaya and 3 campaigns each in Dourbali, AmDobak, and Chaddra (Table 1). Each campaign was composed of 3 vaccination rounds to ensure a complete course of vaccination of children in 1 year. The capacity of existing mobile veterinary teams was extended for simultaneous vaccination of people and animals during at least 1 round for 10 of the 14 campaigns (Table 1).

The joint campaigns were organized in consultation with local health and veterinary personnel to avoid duplication of efforts and to make use of all existing personnel

Table 1. Overview of 14 vaccination campaigns among mobile pastoralists in 4 zones of Chari-Baguirmi and Kanem Prefectures, Chad*

Zone	Campaign no.	Beginning–end of campaign	No. vaccination days	Vaccination contacts		% Dropout	
				Children and women	Livestock	Children	Women
Gredaya†	1	Jul 7, 2000–Feb 11, 2001	46	11,731	31,721‡	56	21
Chaddra†	2	Jan 6–Apr 21, 2001	26	1,961	2,182	68	7
Gredaya†	3	Jun 21–Nov 11, 2001	37	3,855	22,760	–	–
AmDobak†	4	Aug 3–Dec 5, 2001	34	3,079	0	59	42
Gredaya§	5	Apr 2–Jul 1, 2003	46	5,595	16,138	45	19
Dourbali§	6	Mar 8–Jun 7, 2003	47	6,715	887	72	41
Chaddra	7	Sep 27, 2003–Jan 3, 2004	43	3,031	215	–	–
AmDobak	8	Oct 19–Dec 6, 2003	29	3,049¶	0	–	–
Gredaya§	9	Mar 5–Jul 5, 2004	54	6,522	24,514	68	33
Dourbali§	10	Apr 29–Jul 25, 2004	54	3,370	5,104	89	57
Gredaya	11	Jan 10–Mar 30, 2005	42	3,883	13,217	–	–
Dourbali	12	May 18–Aug 17, 2005	41	3,477	32,517	–	–
AmDobak	13	Oct 23–Dec 7, 2005	21	2,705¶	0	–	–
Chaddra	14	Oct 16, 2005–Jan 9, 2006	31	1,886	0	–	–

*Dropout rates were calculated for the first campaign in each zone (vaccination in a naive population) and for 2003 and 2004 after omission of persons with previous vaccinations and not considering those entering the campaign during the second and third rounds. –, not applicable.
†Costing study.
‡Two rounds with veterinarians.
§Estimation of achieved vaccination coverage during campaign.
¶Only 2 human vaccination rounds carried out.

and infrastructure (cold chain and transportation means). Before vaccination campaigns, trained community-based facilitators showed to and discussed with nomadic community members pictograms and short movies with health and veterinary messages.. The National Expanded Program on Immunization provided human vaccines and consumables such as syringes through the regional health administration, assessed continuously the number of vaccinated persons, and was involved in evaluation of the achieved vaccination coverage (*20*).

Veterinarians vaccinated 149,255 livestock against anthrax, pasteurellosis, blackleg, and contagious bovine pleuropneumonia. After 3 visits from the vaccination team, 4,653 children ≤5 years of age were fully immunized against diphtheria, whooping cough (pertussis), and tetanus (DPT) and against polio; 7,703 women received at least 2 doses of tetanus vaccine (TT2+). The average dropout rate within a given campaign was 64% for children ≤5 years from the first to third vaccination for polio and DPT within a given campaign and 32% for women from the first to second dose of tetanus vaccine (Table 1). Dropping out was caused rarely by refusal of revaccination, more often by high mobility of nomadic families. Achieving smaller dropout rates remains a critical need, although DPT and polio vaccination can be continued in subsequent years, and 1 vaccination contact is already useful for immunizing children against measles and yellow fever.

Assessment of Costs

An assessment of costs for the vaccination years 2000 and 2001 (*21*) used unit costs based on detailed data (e.g., replacement costs, maintenance, and insurance costs) and local prices. Purchase prices of human vaccines obtained from the Pan American Health Organization (*22*) were adjusted to include supply, transport, and storage costs and a wastage rate of 10%. The purchase costs of livestock vaccines at the national veterinary laboratory were adjusted for a wastage rate of 5%. Private costs incurred by households to participate in the vaccination campaign were not included in this study. Results are given in Euros (1€ = 656 Francs de la Communauté Financière d'Afrique, September 2006). To allocate the costs of resources used jointly by the 2 sectors, the costs of the vehicle(s), fuel, and guides have been distributed proportionally to the number of personnel present during joint campaigns. The costs of the cold chain, program coordination and administration, information campaigns, and the vehicle and fuel during preparative missions have been distributed according to the number of vaccination rounds by sector. Cold-chain costs were shared with the livestock sector only when veterinarians used thermosensitive contagious bovine pleuropneumonia vaccines. The proportion of public health costs saved by cost-sharing was calculated on the basis of independent public health sector campaigns (*23*).

Most costs of vaccination campaigns for both sectors were the variable and fixed costs of supplying vaccines and vaccine-related costs such as syringes and needles (46% for public health and 53% for veterinary sector) (Table 2). Livestock owners were charged €0.15 per veterinary vaccine dose administered. This covered the costs in Gredaya but not in AmDobak/Chaddra (Table 3). In

Table 2. Variable and fixed costs of vaccinations in the veterinary and public health sectors in Gredaya and AmDobak/Chaddra, Chad*

	Veterinary sector		Public health sector			
			Gredaya		Chaddra/AmDobak	
Cost	Gredaya, Euros (% Fixed)	Chaddra/AmDobak, Euros (% Fixed)	Euros (% Fixed)	% Euros shared	Euros (% Fixed)	% Euros shared
Personnel/administration	2,559 (0)	475 (0)	3,627 (0)	10.6	3,376 (0)	2.7
Transportation	2,835 (80)	345 (75)	4,004 (82)	19.3	3,797 (79)	3.3
Cold chain	62 (36)	45 (56)	1,185 (37)	6.2	531 (36)	10.1
Vaccines and vaccines-related	7,541 (29)	214 (21)	12,146 (12)	0	4,072 (12)	0
Other (buildings, supplies)	480 (95)	152 (100)	938 (98)	25.4	938 (98)	9.1
Total costs	13,476	1,231	21,900	6.7	12,712	2.8
Total costs without vaccines	5,935	1,025	9,754	15.1	8641	4.1

*In Gredaya, 3 vaccination rounds were conducted jointly between veterinarians and public health professionals and another 3 rounds were conducted by the public health sector alone to fully immunize children, whereas in Chaddra/AmDobak, only 1 of 6 rounds was conducted jointly with the veterinarians. The cost-sharing scheme and the proportion of reduced costs due to the joint approach are described in the text.

Gredaya, a higher proportion of personnel and transportation could be shared than in Chaddra/AmDobak because of more joint campaigns (3 of 6 rounds) than in Chaddra/AmDobak (1 of 6 rounds). Of total costs, the proportion shared across the veterinary and public health sectors in the 2 districts was 6.7% and 2.8%, respectively. These proportions were considerably higher when vaccine costs that cannot be shared between sectors were excluded: 15.1% and 4.1%, respectively (Table 2). Costs per fully immunized child and tetanus-vaccinated (TT2+) woman were €11.9 and €6.8, respectively, in Gredaya, but comparatively higher in Chaddra/AmDobak at €30.3 and €18.7, respectively (Table 2). Loss to follow-up (dropout rates) was crucial for the calculated costs per outcome because each incompletely immunized child and woman added to the total costs but did not appear in the denominator. The cost-effectiveness and running costs are currently being evaluated by combining vaccination coverage and cost data of both sectors and including household costs.

Achieved Vaccination Coverage

Sizes of the pastoralist communities were largely unknown. To calculate the proportion of children and women reached in Gredaya and Dourbali in 2003 and 2004, initial population sizes have been estimated by the mark-recapture method. Vaccination cards were used as marks; then during transect studies 1 year after the vaccination, the vaccination status of randomly selected persons was recorded. A Bayesian model enabled us to combine different population estimates of nomadic groups and recapture probabilities to obtain estimates of population dynamics and population sizes at time of vaccination. Estimated coverages of fully immunized children 0–11 months of age (DPT3/polio3) were 8% in 2003 and 14% in 2004 in Gredaya, and 8% in 2003 and 7% in 2004 in Dourbali. Proportions of women who received TT2+ were 16% in 2003 and 36% in 2004 in Gredaya, and 13% in 2003 and 11% in 2004 in Dourbali. No cumulative coverage could be calculated because the total population sizes in the vaccination zones varied between years. A trend toward saturation of fully immunized children (12–36 months of age) and women could not be shown. Further complicating evaluation, new nomadic families enter the vaccination zones because of flexible routes and schedules. This dynamic is reflected in high estimated emigration rates (on average involving 70% of the total population).

Perception of Campaigns

Pastoralist communities highly value the combined approach that considers the health of their family members and of their livestock (Figure) (*20*). In addition to anecdotal positive feedback from pastoralists and health and veterinary personnel, our data collected during the 14

Table 3. Cost per vaccinated livestock and cost and marginal cost per fully immunized child and woman in Gredaya and Chaddra/AmDobak, Chad*

	Veterinary sector		Public health sector			
			Gredaya		Chaddra/AmDobak	
Cost per outcome	Gredaya, livestock	Chaddra/AmDobak, livestock	FIC	TT2+	FIC	TT2+
Total no. vaccinated	54,185	2,182	1,697	1,679	405	488
Cost per vaccine dose administered	0.11	0.56	0.6	2.0	1.1	5.6
Cost per vaccinated livestock† or fully immunized child or woman	0.25	0.56	11.9	6.8	30.3	18.7
Marginal cost per FIC/TT2+‡			8.7	4.6	13.6	5.0

*FIC, fully immunized child; TT2+, woman with at least 2 antitetanus vaccinations.
†Cost per vaccinated animal: either vaccination against anthrax, blackleg and pasteurellosis, or against contagious bovine pleuropneumonia at 1 encounter.
‡Marginal cost per FIC and per TT2+ at a capacity limit of 200 vaccinated children and 100 women per day.

campaigns showed a higher mean of 131 (95% confidence interval [CI] 115–148) persons vaccinated per day for 176 combined vaccination days compared with 100 (95% CI 94–106) for 377 days when only persons, and not livestock, were vaccinated ($p<0.001$ by Poisson regression with random effect on zone and adjusted to number of vaccination rounds). Pastoralists no longer refused vaccination of their livestock as had sometimes been the case before veterinarians were accompanied by medical personnel. A key statement repeatedly made by nomadic parents was, "Measles and whooping cough have disappeared among nomads, although it remains at the market-sites we visit. And when we attend markets, we no longer contaminate our camps with these diseases."

Typically, the initial contact between nomadic families and health personnel is established during the vaccination program (*24*). The nomadic pastoralists perceive the high-quality services that are offered at the health center and start to trust the health providers. The public health sector was thus able to use the campaigns as a gateway to the pastoralists. Increasingly, nomadic parents are visiting health centers with their children to seek vaccination services. Based on the positive outcomes of these pilot campaigns, Chadian public health and veterinary officials are currently planning a common policy for child and livestock vaccination in pastoralist populations. Going to scale at district and national levels with combined public health and veterinary campaigns is sought in concert with other ministries such as education. This may become a model for other governments who face similar difficulties in reaching remote livestock keepers because of communities reluctant to comply with public or private officials or insufficient infrastructure and resources.

Figure. Polio vaccination of a nomadic child in Chad. While children and woman in the camp received vaccinations by public health workers, the livestock in the camp received vaccinations by veterinarians. Source: Project Santé des Nomades au Tchad.

Identifying Synergies in Face of Public Sector Financial Constraints

Privatization of veterinary services was initiated in many parts of Africa and Asia as part of a broader effort to improve delivery of animal healthcare in the face of decreasing governmental expenditure and poor public sector performance (*25*). Numerous incentive schemes were designed to stimulate the privatization process. Subcontracted veterinarians can be effective in the implementation of vaccination campaigns, given that the government subsidizes work in more remote zones. Our analysis on vaccination costs of private veterinarians showed that even with subsidies, income margins of private veterinarians in rural zones for vaccination services (and vaccination being their main activity) were narrow, and most private veterinarians who had started business in rural zones had since withdrawn. Community-based animal health service projects partly fill the gap in chronically underserved rural areas (*26*). However, to efficiently reach remote zones for mass vaccination campaigns, vehicles are needed to maintain the cold chain that cannot be achieved with other transportation means such as horses. In some instances, there is a lack of independent and regular quality control of veterinary vaccine production facilities in low-income countries (*27*). Insufficient vaccination coverage was commonly believed to be responsible for anthrax outbreaks in Chad. However, a survey showed that most pastoralists reported that vaccines were no longer efficacious. Subsequent, quality control of the anthrax vaccine confirmed the concerns of the livestock owners (*23*).

To ensure effective surveillance and vaccination coverage, which were once provided by the government, veterinary privatization policy is currently under review by the World Organization for Animal Health, the Food and Agriculture Organization of the United Nations, and the African Union (Regional Conference in N'Djaména, February 13–15, 2006). New policies should be judged on whether they adequately support the rural poor in continuing livestock-based livelihoods (*2*,*28*), which in arid and semiarid zones are often the only way to productively use the natural resource base.

Exploiting synergies for interventions and information dissemination becomes more and more important. Within countries, regular intersectoral exchange of disease occurrence information adds to better preparedness in both sectors, but such exchange of information does not exist in all countries. The more recently evaluated institutional collaborations between public health and veterinary services seek to identify appropriate control strategies for diseases where medical and veterinary cooperation for control is becoming a single continuum. Transsectoral economic analyses of livestock disease control may demonstrate increased

profitability and contribute to advocacy for improved control of zoonoses in developing countries (*29*).

Conclusions

Sustained vaccination programs are essential tools for both the public health and veterinary sectors. Combined human and livestock vaccination reduces operational costs of interventions requiring costly transportation and is adapted to livestock holders who highly value the approach that considers the health both of the family and of the animals that contribute importantly to their livelihood. In Chad, a common policy agreement between the 2 sectors on cooperation in rural zones should define a cost-sharing scheme. By optimizing the use of limited logistical and human resources, public health and veterinary services will be strengthened, especially at the district level, and, in turn, will be more prepared and operational in responding to endemic and epidemic diseases.

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Dr Schelling recently joined the International Livestock Research Institute (ILRI) in Nairobi, Kenya as a joint appointment scientist of ILRI and the Swiss Tropical Institute. Her current research interests include identification and testing of cost-effective control strategies for zoonoses to preserve the health and livelihoods of small-scale livestock producers and of consumers of livestock products.

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Worldwide Emergence of Extensively Drug-resistant Tuberculosis

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Mycobacterium tuberculosis strains that are resistant to an increasing number of second-line drugs used to treat multidrug-resistant tuberculosis (MDR TB) are becoming a threat to public health worldwide. We surveyed the Network of Supranational Reference Laboratories for *M. tuberculosis* isolates that were resistant to second-line anti-TB drugs during 2000–2004. We defined extensively drug-resistant TB (XDR TB) as MDR TB with further resistance to ≥3 of the 6 classes of second-line drugs. Of 23 eligible laboratories, 14 (61%) contributed data on 17,690 isolates, which reflected drug susceptibility results from 48 countries. Of 3,520 (19.9%) MDR TB isolates, 347 (9.9%) met criteria for XDR TB. Further investigation of population-based trends and expanded efforts to prevent drug resistance and effectively treat patients with MDR TB are crucial for protection of public health and control of TB.

Multidrug-resistant tuberculosis (MDR TB) has been documented in nearly 90 countries and regions worldwide (*1*); 424,203 cases of MDR TB were estimated to have occurred in 2004, which is 4.3% of all new and previously treated TB cases (*2*). Treatment for MDR TB patients requires use of second-line drugs for ≥24 months. These drugs are more costly, toxic, and less effective than first-line drugs used for routine treatment of TB (*3–6*). As with other diseases, resistance to TB drugs results primarily from nonadherence by patients, incorrect drug prescribing by providers, poor quality drugs, or erratic supply of drugs (*7*).

To facilitate treatment of MDR TB in resource-limited countries, where most TB cases occur (*1,2*), the World Health Organization (WHO) and its partners developed the Green Light Committee, which helps ensure proper use of second-line drugs, to prevent further drug resistance (*8*). Nonetheless, the Green Light Committee encountered numerous anecdotal reports of MDR TB cases with resistance to most second-line drugs. Once a strain has developed resistance to second-line drugs, these new TB strains are even more difficult to treat with existing drugs. Untreated or inadequately treated patients are at increased risk of spreading their disease in the community, which could lead to outbreaks in vulnerable populations and widespread emergence of a lethal, costly epidemic of drug-resistant TB, reminiscent of the MDR TB outbreaks in the early 1990s (*9–13*). Therefore, to determine whether these anecdotal reports were isolated events, early evidence of an emerging epidemic, or the occurrence of virtually

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untreatable forms of drug-resistant TB that had not been described previously in different parts of the world, we characterized and quantified the frequency of second-line–drug resistance in several geographic regions.

We sought to determine the extent to which highly resistant *Mycobacterium tuberculosis* strains have been identified by the international laboratories that participate in the Network of Supranational Reference Laboratories (SRLs). The SRL Network consists of 25 highly proficient TB laboratories on 6 continents. These laboratories collaborate with national reference laboratories to strengthen culture and drug-susceptibility testing capacity and to provide quality control for the WHO/International Union Against Tuberculosis and Lung Diseases Global Project on Anti-TB Drug Resistance (*14*).

Methods

Participants

From November 2004 through November 2005, we surveyed the global SRL Network. All SRL directors were invited to participate during the 2004 annual SRL directors meeting, by individual mailings, and by personal phone calls. Drug-susceptibility testing results were requested for *M. tuberculosis* isolates that had been tested for resistance to first-line drugs and second-line drugs during 2000–2004. Two SRLs were not eligible because they did not test for second-line drugs or tested for <3 classes of second-line drugs.

The 14 SRLs that provided data for this study support 112 TB laboratories in 80 countries worldwide (Figure 1). SRLs serve as international reference laboratories to a wide geographic area, performing drug-susceptibility testing that may not be available in a country (e.g., for second-line drugs) and providing quality assurance for first-line–drug testing. Most SRLs also serve as the national reference laboratory for the country in which they are located; they receive varying proportions of isolates from their own and other countries for surveillance, clinical diagnosis, and quality assurance. First-line–drug susceptibility testing is performed on all isolates; second-line–drug susceptibility testing is usually limited to isolates from patients known or suspected to have drug-resistant TB. Of the 14 participating SRLs, not all tested for all 6 classes of second-line drugs, and 4 did not submit data for the entire survey period.

In contrast, the SRL in the Republic of Korea serves as the national reference laboratory and routinely performs an extended diagnostic panel of drug-susceptibility testing on isolates from culture-positive TB patients referred from health centers, hospitals, and clinics in the Republic of Korea. This SRL tests all isolates for 6 classes of second-line drugs; thus, data from the Republic of Korea reflect most culture-positive cases and provide a close approximation to a population estimate of prevalence. Because of the large number of isolates received and because sampling for these isolates is systematically different from that at the other SRLs (testing of all TB patients in the Republic of Korea vs testing of patients more likely to have drug-resistant TB in other SRLs), resistance patterns for the Republic of Korea were analyzed separately from those for the other SRLs.

Laboratory Methods

Among participating SRLs, different but internationally accepted methods were used to test for second-line drug resistance (details available upon request). Validation of drug-susceptibility testing results for second-line drugs was not performed as part of this survey, but as part of their role as global reference laboratories, all SRLs participate in international proficiency testing for first-line drugs. Quality assurance procedures for second-line–drug susceptibility testing have not been developed; as a proxy for quality assurance, we examined the accuracy of second-line–drug susceptibility testing among isolates susceptible to the 4 main first-line drugs (isoniazid [INH], rifampin [RIF], ethambutol, and streptomycin). On the basis of known mechanisms of drug resistance, finding an isolate that is susceptible to all first-line drugs and resistant to second-line drugs is unlikely (*7*).

Procedures and Definitions

A standardized reporting form requested anonymous data for all isolates tested for resistance to ≥3 second-line drug classes during 2000–2004. Data were abstracted from the records, electronic or paper, depending on laboratory practices for data management. Results were submitted for 1 isolate per patient. Because SRLs rarely receive multiple isolates from the same patient, reporting of the same patient more than once was unlikely (B. Metchock and

Figure 1. Shading indicates 48 countries that submitted at least 1 isolate to participating Supranational Reference Laboratories, 2000–2004. See Table 4 for complete list of participating countries.

G.H. Bai, pers. comm.). No specimens were collected for this study; we used only data from records of isolates that had already been tested. Limited clinical information about the patient was available with each isolate. Consistent data were available for country of origin and date of drug-susceptibility testing. Data about age and TB treatment history were available for <10% of patients, so analysis was not considered reliable for these variables.

To best compare data for the study samples with data from the Global Drug Resistance Survey and other population-based drug-resistance surveillance, we analyzed first-line–drug resistance patterns according to standard methods used in anti-TB–drug resistance surveys (*1*). These patterns included any drug resistance, monoresistance (resistance to only the 1 specified drug), polyresistance (resistance to ≥2 first-line drugs, but which drugs not specified), and multidrug resistance (resistance to at least INH and RIF, with or without other drugs).

We defined 6 classes of second-line drugs as follows: aminoglycosides other than streptomycin (e.g., kanamycin and amikacin), cyclic polypeptides (e.g., capreomycin), fluoroquinolones (e.g., ofloxacin, ciprofloxacin, levofloxacin, and moxifloxacin), thioamides (e.g., prothionamide and ethionamide), serine analogs (e.g., cycloserine and terizidone), and salicylic acid derivatives (e.g., para-aminosalicyclic acid).

For this survey we created a consensus definition that incorporates second-line–drug susceptibility results and is based on international guidelines for management of drug-resistant TB (*15*). The mainstay of an MDR TB treatment regimen consists of 1 injectible drug (e.g., aminoglycoside or cyclic polypeptide) and a fluoroquinolone; additional drugs from the remaining classes are added until the total reaches 4–6 drugs to which the organism is susceptible. If the infecting organism is resistant to ≥3 second-line drug classes, designing a treatment regimen with sufficient drugs that are known to be effective against TB is difficult. Thus, we defined extensively drug-resistant TB (XDR TB) isolates as those meeting the criteria established for MDR TB plus resistance to ≥3 of the 6 classes of second-line drugs.

Second-line–drug resistance patterns were analyzed by geographic region from which the isolate was submitted to the SRL. Regions were grouped into epidemiologically meaningful categories on the basis of prevalence of TB and MDR TB (*1,16*). This retrospective survey was evaluated and approved as public health surveillance by the US Centers for Disease Control and Prevention (CDC).

Results

We received data for 18,462 patients from 14 (61%) of 23 eligible SRLs. We excluded those patients tested before 2000 (n = 223), tested after 2004 (n = 14), or tested for resistance to <3 classes of second-line drugs (n = 535). Our final study sample consisted of 17,690 patients whose isolates were tested for resistance to ≥3 second-line drugs during 2000–2004 (Figure 2). Of these, 11,939 (67.5%) patients were from the Republic of Korea and 5,751 (32.5%) were from the remaining SRLs.

First-line–Drug Susceptibility

Among isolates from patients from the 13 SRLs other than the Republic of Korea, 3,765 (65.5%) were resistant to ≥1 first-line TB drug (Table 1). Of these, 3,305 (58.5%) were resistant to at least INH and 2,345 (41.5%) were resistant to at least RIF. Among isolates from the Republic of Korea patients, 2,508 (21%) had resistance to any drug; most (n = 2,196; 18.4%) were resistant to INH.

Single-drug resistance was found for isolates from 884 (15.4%) patients from the 13 SRLs; 456 (8.1%) of these were resistant to INH and 99 (1.8%) to RIF. Among isolates from patients from the Republic of Korea, 952 (8%) displayed single-drug resistance, 666 (5.6%) to INH and 148 (1.2%) to RIF.

Polyresistance other than MDR TB was seen for isolates from 651 (11.5%) patients from the 13 SRLs and 258 (2.2%) from the Republic of Korea SRL. Not all SRLs routinely tested for resistance to pyrazinamide.

Multidrug resistance (i.e., MDR TB) was present in isolates from 2,222 (39.4%) patients from the 13 SRLs and 1,298 (10.9%) from the Republic of Korea. Resistance to

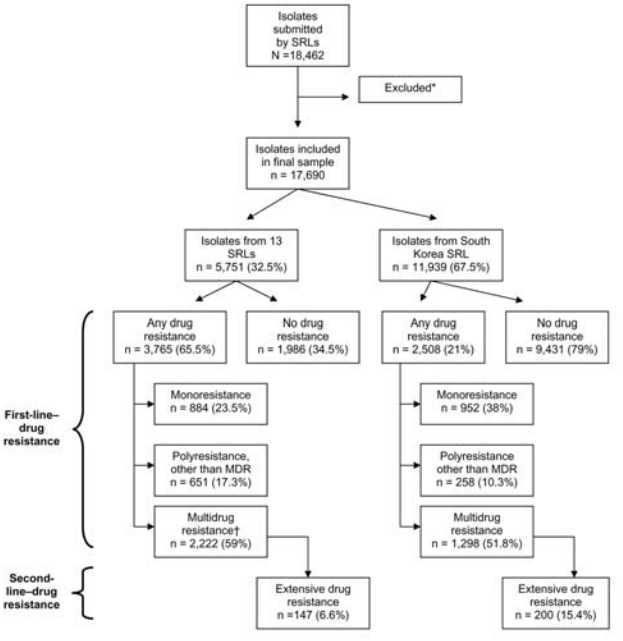

Figure 2. Selection of study sample and summary of drug-resistance patterns of isolates. SRL, Supranational Reference Laboratory. *Tested before 2000 or after 2004 (n = 247) or tested for resistance to <3 classes of second-line drugs (n = 535). †Data for ethambutol resistance missing for 5 isolates.

Table 1. First-line–drug resistance patterns for *Mycobacterium tuberculosis* isolates, 2000–2004 (N = 17,690)*

Pattern	Other 13 SRLs (n = 5,751)		Republic of Korea SRL (n = 11,939)	
	No. tested	No. (%) resistant	No. tested	No. (%) resistant
Any resistance (total)*†‡	5,751	3,765 (65.5)	11,939	2,508 (21.0)
INH	5,645	3,305 (58.5)	11,939	2,196 (18.4)
RIF	5,649	2,345 (41.5)	11,939	1,469 (12.3)
EMB	5,508	1,356 (24.6)	11,939	988 (8.3)
SM	5,618	2,581 (45.9)	11,939	578 (4.8)
Monoresistance (total)§¶	5,751	884 (15.4)	11,939	952 (8.0)
INH	5,645	456 (8.1)	11,939	666 (5.6)
RIF	5,649	99 (1.8)	11,939	148 (1.2)
EMB	5,508	8 (0.1)	11,939	25 (0.2)
SM	5,618	321 (5.7)	11,939	113 (0.9)
Polyresistance, non-MDR (total)¶	5,644	651 (11.5)	11,939	258 (2.2)
INH + other drugs (except RIF)	5,645	627 (11.1)	11,939	232 (1.9)
RIF + other drugs (except INH)	5,649	24 (0.4)	11,939	23 (0.2)
Multidrug resistance (total)¶#	5,644	2,222 (39.4)	11,939	1,298 (10.9)
INH + RIF, only	5,644**	399 (7.1)	11,939	392(3.3)
INH + RIF + EMB, only	5,508**	182 (3.3)	11,939	584 (4.9)
INH + RIF + SM, only	5,618**	619 (11.0)	11,939	89 (0.7)
INH + RIF + EMB + SM	5,476**	1,017 (18.6)	11,939	233 (2.0)

*SRLs, Supranational Reference Laboratories; INH, isoniazid; RIF, rifampin; EMB, ethambutol; SM, streptomycin.
†Missing data for INH (106 isolates), RIF (102 isolates), EMB (243 isolates), SM (133 isolates).
‡Cells are not mutually exclusive.
§Numerator is isolates with resistance to the specified drug and no known resistance to other first-line drugs. Denominator is isolates tested to at least the specified drug in the numerator.
¶Each cell is mutually exclusive.
#Denominator is isolates tested for at least INH + RIF.
**Denominator is isolates tested for at least the drugs in the specified combination.

all first-line drugs tested (i.e., MDR TB with additional resistance to ethambutol and streptomycin) was found in isolates from 1,017 (18.6%) patients from the 13 SRLs and 233 (2%) from the Republic of Korea SRL.

Second-line–Drug Susceptibility

Among patients from the 13 SRLs, resistance to aminoglycosides was detected in 489 (8.7%) isolates and to fluoroquinolones in 298 (5.3%) (Table 2). Among isolates from Republic of Korea patients, resistance was most commonly seen to fluoroquinolones (n = 524, 4.4%) and thioamides (n = 259, 2.2%).

From all SRLs, isolates that were resistant to at least INH and RIF (i.e., MDR TB; n = 3,520) and tested for susceptibility to ≥3 second-line drugs were combined for analysis of second-line–drug resistance patterns. Resistance to ≥1 class of second-line drug was present in 1,542 (43.8%) MDR TB patients (Table 3). The most commonly observed patterns were resistance to aminoglycosides (n = 630, 18.3%), fluoroquinolones (n = 673, 19.3%), and thioamides (n = 605, 19.3%).

MDR TB patients whose isolates had further resistance to ≥3 classes of second-line drugs were classified as XDR TB (Table 3). A total of 347 (9.9%) MDR TB patients met criteria for XDRTB. According to the revised Global XDR TB Task Force definition (www.who.int/mediacentre/news/notes/2006/np29/en/index.html), 234 (6.6%) isolates met criteria for XDR TB. Among XDR TB patients, combination drug-resistance patterns included 90 (3.4%) with resistance to aminoglycosides, capreomycin

Table 2. Second-line–drug resistance patterns for *Mycobacterium tuberculosis* isolates, 2000–2004 (N = 17,690)*†

Pattern	Other 13 SRLs‡ (n = 5,751)		Republic of Korea SRL‡ (n = 11,939)	
	No. tested	No. (%) resistant	No. tested	No. (%) resistant
Any resistance	5,751	1,237 (21.5)	11,939	849 (7.1)
Aminoglycosides§	5,620	489 (8.7)	11,939	227 (1.9)
Capreomycin	4,347	197 (4.5)	11,939	122 (1.0)
Fluoroquinolones	5,580	298 (5.3)	11,939	524 (4.4)
Thioamides	5,131	556 (10.8)	11,939	259 (2.2)
Cycloserine	2,715	70 (2.6)	11,939	80 (0.7)
Para-aminosalicylic acid	3,571	262 (7.3)	11,939	403 (3.4)

*SRLs, Supranational Reference Laboratories.
†Not all isolates were tested for each second-line–drug class (with the exception of the Republic of Korea SRL), so results are reported as a proportion of isolates tested to the specified class of drugs.
‡Cells are not mutually exclusive.
§Other than streptomycin (e.g., kanamycin, amikacin).

Table 3. Second-line–drug resistance patterns for multidrug-resistant *Mycobacterium tuberculosis* isolates, 2000–2004*†‡

Pattern	No. tested	No. (%) resistant
Any resistance (total)	3,520	1,542 (43.8)
Aminoglycosides (AG)§	3,442	630 (18.3)
Capreomycin (CM)	2,743	279 (10.2)
Fluoroquinolones (FQ)	3,492	673 (19.3)
Thioamides (TA)	3,132	605 (19.3)
Cycloserine (CS)	2,615	141 (5.4)
Para-aminosalicylic acid (PAS)	2,860	450 (15.7)
Extensively drug-resistant TB (XDR TB, total)¶	3,520	347 (9.9)
AG + CM + FQ	2,656	90 (3.4)
AG + CM + TA	2,498	77 (3.1)
CM + FQ + TA	260	50 (19.2)
AG + FQ + TA	3,040	102 (3.4)
AG + FQ + CS	139	39 (28.1)
FQ + TA + PAS	2,505	94 (3.8)

*Tested for ≥3 second-line drug classes; SRLs, Supranational Reference Laboratories.
†Not all isolates were tested for each second-line drug class (with the exception of the Republic of Korea SRL), so results are reported as a proportion of isolates tested to the specified class of drugs. For combination resistance patterns, results are reported as a proportion of isolates tested to all of the classes of drugs in the specific combination.
‡Cells are not mutually exclusive.
§Other than streptomycin (e.g., kanamycin, amikacin).
¶XDR TB, extensively drug-resistant tuberculosis, i.e., multidrug-resistant tuberculosis (resistant to at least isoniazid and rifampin) with additional resistance to ≥3 classes of second-line drugs.

and fluoroquinolones; 102 (3.4%) with resistance to aminoglycosides, fluoroquinolones, and thioamides; and 94 (3.8%) with resistance to fluoroquinolones, thioamides, and para-aminosalicyclic acid. Nearly half (n = 167, 48.1%) of all XDR TB isolates were resistant to all 4 first-line drugs, bringing the total to ≥7 drugs to which the isolate was resistant.

The proportion of XDR TB patients by region is shown in Table 4. Among the group of industrialized nations, 53 (6.5%) MDR TB patients met criteria for XDR TB. Among patients from Russia and Eastern Europe, 55 (13.6%) MDR TB patients met criteria for XDR TB. Among patients from the Republic of Korea, 200 (15.4%) MDR TB patients, who accounted for 1.7% of all *M. tuberculosis* isolates tested, met criteria for XDR TB.

In evaluating the accuracy of second-line–drug susceptibility testing, we found that 7 (0.1%) of 11,426 patients fully susceptible to all first-line drugs were resistant to 2 second-line drugs, and 109 (1%) were resistant to 1 second-line drug. Most of these patients were resistant to fluoroquinolones.

Discussion

This study represents the first assessment of the widespread occurrence of *M. tuberculosis* with such extensive drug resistance as to be nearly untreatable with currently available drugs, according to international guidelines. We provide data on second-line–drug resistance for the largest sample of patients to date, including >5,000 patients from 47 countries, apart from the Republic of Korea. The definition of XDR TB in this survey is based on WHO guidelines for the programmatic management of drug-resistant TB; the guidelines recommend treatment with ≥4 drugs known to be effective (*15*). Therefore, with ≤3 remaining classes of second-line drugs to which the infecting organism is susceptible, treatment of these patients cannot meet international standards. XDR TB has been detected in all regions of the world. XDR TB strains in this study also have high rates of resistance to pyrazinamide and ethambutol, thereby severely limiting the treatment options available.

Analysis of combination second-line–drug resistance patterns is critical for clinicians and policymakers who design treatment regimens for these patients. Although limited data exist in the literature about second-line–drug resistance patterns among MDR TB patients, data from patients undergoing retreatment for TB in Hong Kong showed that 30 (17%) MDR TB isolates were resistant to ≥3 second-line drugs (*17*), thereby meeting criteria for XDR TB. A drug-resistance survey of 447 culture-positive new patients and patients undergoing retreatment in Abkhazia, Republic of Georgia, found that of 63 MDR TB patients, 2 (3%) had additional resistance to 3 second-line drug classes, consistent with XDR TB (*18*). More recently, clusters of XDR TB have been reported in South Africa and Iran (*19*,*20*) and have been associated with HIV infection and rapid and high death rates.

The emergence of new strains of TB that are resistant to second-line drugs, especially in settings where TB control programs have become unable to adequately monitor treatment regimens for MDR TB, is cause for concern. After the resurgence of TB in industrialized countries during the 1980s and increased awareness of this global problem, implementation of strong TB control programs based on the principles of the global directly observed treatment strategy, short course (DOTS) improved treatment outcomes and reduced TB and MDR TB incidence in several countries. This framework for DOTS, promulgated by WHO, and the pilot MDR TB management projects (DOTS-Plus projects) became the basis for programmatic management of MDR TB, which has demonstrated feasibility and effectiveness in low- and middle-income countries (*5*,*15*). However, second-line drugs are available worldwide outside of well-organized TB-control programs (WHO, unpub. data).

Improper treatment of drug-resistant TB, such as using too few drugs, relying on poor quality second-line drugs, and failing to ensure adherence to treatment, will likely lead to increases in XDR TB. Strengthening basic TB programs and infection control measures is crucial for preventing the selective pressure and environments in which resistant strains are transmitted from person to per-

Table 4. Extensively drug-resistant tuberculosis among multidrug-resistant tuberculosis isolates, by region, 2000–2004*

Geographic region	Total no. isolates tested, n†	Total MDR TB patients, n (% of all isolates tested)	Total XDR TB patients, n (% of MDRTB patients)
Industrialized nations‡	2,499	821 (32.9)	53 (6.5)
Latin America§	985	543 (55.1)	32 (5.9)
Eastern Europe¶ and Russia	1,153	406 (35.2)	55 (13.6)
Africa and Middle East#	665	156 (23.5)	1 (0.6)
Asia (other than Republic of Korea)**	391	274 (70.1)	4 (1.5)
Republic of Korea	11,939	1,298 (10.9)	200 (15.4)
Total††		3,418	345

*Region from which isolate was submitted to Supranational Reference Laboratory. MDR TB, multidrug-resistant tuberculosis; XDR TB, extensively drug-resistant tuberculosis, i.e., multidrug-resistant tuberculosis (resistant to at least isoniazid and rifampin) with additional resistance to ≥3 classes of second-line drugs.
†Total no. of isolates tested for resistance to ≥3 second-line drug classes, including aminoglycosides (amikacin or kanamycin), polypeptides (capreomycin), fluoroquinolones (ofloxacin or ciprofloxacin), thioamides (ethionamide or prothionamide), cycloserine, and para-aminosalicyclic acid.
‡United States, Canada, United Kingdom, countries in Western Europe (Ireland, Portugal, Germany, France, Belgium, Spain), Japan, and Australia.
§Argentina, Bolivia, Brazil, Chile, Ecuador, Guyana, French Guiana, Peru, Mexico, Guatemala, El Salvador, Costa Rica.
¶Republic of Georgia, Czech Republic, Azerbaijan, Armenia.
#Afghanistan, Algeria, Egypt, Tunisia, Botswana, Burundi, Cameroon, Central African Republic, Côte d'Ivoire, Djibouti, Madagascar, Rwanda, South Africa, Senegal, Uganda.
**Bangladesh, Indonesia, Papua New Guinea, Thailand, East Timor.
††For 2 XDR TB patients, data were missing about geographic region.

son. Additionally, MDR TB programs that rely on quality-assured and internationally recommended treatment regimens according to WHO guidelines must be scaled up and strengthened to stem further second-line–drug resistance and spread of XDR TB. The Green Light Committee provides a global mechanism to help affected countries achieve these steps. A commentary published in 2000 predicted that "failure to institute [the] entire DOTS-Plus package is likely to destroy the last tools available to combat [TB], and may ultimately result in the victory of the tubercle bacillus over mankind" (*21*). XDR TB is an indirect indicator of program failure to adequately diagnose, prevent, and treat MDR TB.

Documenting the emergence of XDR TB requires a laboratory-based diagnosis that relies on first- and second-line–drug susceptibility testing. A limitation to accurate detection of XDR TB is that existing tests for resistance to second-line drugs are not yet standardized and are less reproducible than tests for resistance to INH and RIF. Lack of international recommendations for use, as well as lack of standardization and the historical unavailability of MDR TB treatment in the public sector, has limited use of second-line–drug susceptibility testing on a wider scale. As access to treatment with second-line drugs increases, standardized methods, improved diagnostics, and quality assurance for second-line–drug susceptibility testing are urgently needed to enable reliable testing and design of appropriate treatment regimens. Although internationally accepted methods were used by all laboratories, the precise methods and drug concentrations used varied among participating SRLs (*22*). Because these SRLs represent some of the most highly performing laboratories on 6 continents, results of drug-susceptibility testing are credible within the context of stated limitations. Initial studies that standardized different methods for second-line–drug susceptibility testing have been completed (*23–26*), but more are needed.

Our study has other limitations. The numbers reported for XDR TB probably represent an underestimate of the true number of cases because not all SRLs and not all national reference laboratories test for all 6 classes of second-line drugs. In the absence of test results for all 6 classes of second-line drugs, we speculate, on the basis of a patient's TB treatment history and known patterns of drug cross-resistance, that many other unidentified patients are likely to have had and died from XDR TB. For example, an MDR TB isolate that is also resistant to an aminoglycoside and a fluoroquinolone but that has not been tested for the other second-line drug classes is very likely to be resistant to an additional second-line drug class for the following reasons: INH and ethionamide have a 15%–20% rate of cross-resistance (*27*); kanamycin and capreomycin cross-resistance is common, ranging from 20%–60% (CDC, unpub. data) (*28*,*29*); and in this study, isolates that were resistant to all 4 first-line drugs as well as an aminoglycoside and a fluoroquinolone were 70%–80% likely to be resistant to at least 1 additional class of second-line drug.

Another limitation is that data from most SRLs were drawn from a convenience sample of isolates and reflect referral bias. Thus, these data can not be considered representative of a patient population or region, and actual denominators are difficult to determine. For this reason, although estimates of prevalence are possible, they cannot be generalized to the local or regional population. However, our study is the first to report XDR TB patients in multiple geographic regions; future systematic surveys are needed to determine the true extent of this disease. Data from the Republic of Korea reflect a more comprehensive policy for drug-susceptibility testing and provide an estimate of the population prevalence in this setting.

However, the 10.9% rate of MDR TB for the Republic of Korea is higher than rates reported from other national drug resistance surveys and may reflect other unknown referral biases (*1*).

Lastly, we had limited clinical information about each patient because information submitted to each SRL varied and was not reliably available for inclusion in the analysis. Data about TB treatment history, patient age and sex, or HIV status are not routinely collected by all laboratories. Genotyping data were not available to confirm whether XDR TB isolates are related to W variant of the Beijing strain, a highly drug-resistant strain of *M. tuberculosis* responsible for large nosocomial outbreaks in New York in the early 1990s (*30*).

Despite these limitations, our survey provides the first documentation of the emergence of XDR TB as a serious worldwide public health threat. XDR TB was identified on 6 continents and is significantly associated with worse treatment outcomes than MDR TB (*31*,*32*). The emergence of XDR TB, coupled with the increased use of second-line drugs, suggests that urgent measures are needed to improve rational use of quality-assured second-line drugs. In addition, population-based surveillance for second-line–drug susceptibility testing is needed to better describe the magnitude of XDR TB worldwide, track trends, and plan a public health response. Indeed, the convergence of XDR TB with the HIV epidemic may undermine gains in HIV prevention and treatment programs and requires urgent interventions. These interventions include ensuring adherence to recommended international standards of care aimed at promptly and reliably diagnosing TB, ensuring adherence to recommended treatment regimens with demonstrated efficacy, implementing infection control precautions where patients congregate, and improving laboratories' capacity to accurately and rapidly detect drug-resistant *M. tuberculosis* isolates so that patients can receive effective treatment (*33*). Other unmet needs include further development of international standards for second-line–drug susceptibility testing, new anti-TB drug regimens, and better diagnostic tests for TB and MDR TB. Such measures are crucial if future generations are to be protected from potentially untreatable TB.

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Tandem Repeat Analysis for Surveillance of Human *Salmonella* Typhimurium Infections

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In Denmark, as part of the national laboratory-based surveillance system of human enteric infections, all *Salmonella enterica* serotype Typhimurium isolates are currently subtyped by using phage typing, antimicrobial resistance profiles, and pulsed-field gel electrophoresis (PFGE). We evaluated the value of real-time typing that uses multiple-locus variable-number tandem-repeats analysis (MLVA) of *S*. Typhimurium to detect possible outbreaks. Because only a few subtypes identified by PFGE and phage typing account for most infections, we included MLVA typing in the routine surveillance in a 2-year period beginning December 2003. The 1,019 typed isolates were separated into 148 PFGE types and 373 MLVA types. Several possible outbreaks were detected and confirmed. MLVA was particularly valuable for discriminating within the most common phage types. MLVA was superior to PFGE for both surveillance and outbreak investigations of *S*. Typhimurium.

Members of the bacterial genus *Salmonella* are among the major pathogens that cause infections in humans and animals. Most human *Salmonella* infections are thought to be associated with foodborne transmission from contaminated animal–derived meat and dairy products (*1*). *Salmonella enterica* subspecies *enterica* serotype Typhimurium is the second most commonly isolated serotype in Denmark (*2*) and in other industrialized countries (*3*).

Typing is an important tool for surveillance and outbreak investigations of human infections. Many demands are placed on new typing methods, including high discriminatory power so that unrelated and related isolates can be identified (*4*). The method should be easy to perform and interpret, and it should be possible to standardize, so that results can be exchanged between laboratories and be effective for local, national, and international surveillance (*4*). The many molecular typing techniques target different areas of the genome in attempts to assess genetic variability; however, the stability of such a target area has to be taken into account when considering how relevant the area is for typing (*4*). Too much variability will complicate the interpretation of the typing data in relation to epidemiologic information (*5*).

Pulsed-field gel electrophoresis (PFGE) is one of the most widely used typing methods in local, national, and international *S*. Typhimurium surveillance (*2,6,7*). Linking of PFGE data and epidemiologic information has resulted in tracing the origin of common-source outbreaks (*8,9*), but the method has also shown limitations within certain phage types of *S*. Typhimurium (*10,11*). Multiple-locus variable-number tandem-repeats analysis (MLVA), based on amplification of variable number of tandem repeat (VNTR) areas, is a promising typing method that seems to have high discriminatory power within clonal species. Three MLVA schemes have been developed for Shiga toxin–producing *Escherichia coli* O157 (STEC O157) that had either equal or improved discriminatory power when compared with PFGE (*12–14*). Several schemes have also been developed for *Salmonella*, including a general scheme for *S. enterica* subspecies *enterica* (*15*). This method was not equally discriminatory for all serotypes investigated, and schemes have been developed that are based on overlapping and on serotype-specific VNTR areas. One scheme was developed for *S*. Typhi (*16*) and another for *S*. Typhimurium (*17*); the latter showed high discriminatory power within *S*. Typhimurium and within the uniform phage type DT104 (*18*).

The purpose of our study was to evaluate the usefulness of MLVA in surveillance of human *S*. Typhimurium infections and detection of possible outbreaks. In

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Denmark, surveillance for *Salmonella* in humans, animals, and food is extensively coordinated (*2*). *S.* Typhimurium isolates from all confirmed human infections are routinely typed by using PFGE, phage typing, and antimicrobial resistance profiles. The same standardized methods are used for typing food and animal isolates; however, PFGE is used only for selected food and animal isolates. In a 2-year period, MLVA typing was included in routine surveillance, and we evaluated its discriminatory ability and usefulness in cluster detection and outbreak investigations. Comparisons with phage typing, PFGE typing, and epidemiologic information were included.

Materials and Methods

Isolates

In Denmark, fecal samples from patients with diarrhea are examined for bacterial pathogens at either the regional clinical laboratories or at the diagnostic laboratory at Statens Serum Institut (SSI). All *Salmonella* isolates were serotyped according to the Kaufman-White scheme (*19*), and all *S.* Typhimurium isolates were submitted to SSI for further characterization. In a 2-year period beginning December 2003, all confirmed *S.* Typhimurium isolates were collected weekly and further subtyped by using phage typing, antimicrobial resistance profiles, PFGE, and MLVA as part of national surveillance.

Phenotypic Characterization

S. Typhimurium isolates were phage typed according to international standards (*20*) at the National Food Institute, Technical University of Denmark (FOOD, DTU). Antimicrobial resistance profiles were generated from susceptibility to antimicrobial agents and were performed as MIC determinations. Sensititer (TREK Diagnostic Systems, LTD, West Sussex, England), a commercially prepared dehydrated panel, was used for the following antimicrobial agents: amoxicillin-clavulanic acid, ampicillin, apramycin, ceftiofur, chloramphenicol, ciprofloxacin, colistin, florphenicol, gentamicin, nalidixic acid, neomycin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim.

PFGE Procedure

Isolates were grown overnight on blood plates, and PFGE was performed with *Xba*I by using the PulseNet USA protocol developed for *Salmonella* (*7*). The international standard *S.* Braenderup, H9812 (*21*) was used, and the gels were analyzed by using BioNumerics 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). All bands with sizes between 33 kb and 1,135 kb were included in the interpretation of PFGE patterns, and isolates differing at 1 band were assigned a new PFGE type.

MLVA Procedure

MLVA was performed by using the same primers and a modified version of the method previously described (*17*). Isolates were grown overnight on blood plates, and a small loopful of cells was placed directly into the PCR mix. One PCR reaction was performed with a multiplex kit from Qiagen (Hilden, Germany) in a total of 25 μL and included 2.50 pmol each of primers STTR3-F, STTR3-R, STTR6-F, and STTR6-R and 1.25 pmol each of primers STTR5-F, STTR5-R, STTR9-F, STTR9-R, STTR10pl-F, and STTR10pl-R. Amplification was performed with a GeneAmp9700 (Applied Biosystems, Foster City, CA, USA), starting with 15 min at 94°C, followed by 25 cycles of 30 s at 94°C, 1 min at 60°C, and 1.5 min at 72°C and ending with an extension step for 10 min at 72°C. The final products were separated with an ABI310 automated DNA sequencer (Applied Biosystems). Data collection and preprocessing were performed with GENESCAN (Applied Biosystems) and the internal size standard Geneflo-625 (CHIMERx, Milwaukee, WI, USA) for normalization. Fragment sizes for all loci were imported to BioNumerics 4.0, and allele numbers were assigned automatically for each strain by using arbitrary numbers. Unique allelic combinations were assigned a new MLVA, and all MLVA types are shown as fragment sizes (bp) in the following order: STTR9-STTR5-STTR6-STTR10-STTR3.

Clusters and Outbreak Investigations

A cluster was defined as 5 isolates with the same MLVA type collected over a period of 4 weeks. Investigations were started if these isolates also were identical with PFGE and phage typing and included typing of food and animal isolates and interviews with patients. For confirmed outbreaks closely related PFGE types (differing at 1 band) and MLVA types (differing at 1 locus) were included in the investigations when isolated within a narrow time frame.

Results

In total, 1,019 human *S.* Typhimurium isolates were characterized with PFGE, MLVA, and phage typing during the 2-year period. DT104, DT120, and DT12 accounted for 47.8% of all isolates; DT104 (including DT104b) was the most commonly isolated phage type. Approximately 20% of the isolates either were nontypeable (NT) or showed a phage pattern that did not correspond to a recognized phage type and was reported as phage type RDNC. Each of the remaining phage types accounted for ≤6% of the total number of isolates. Eighty-three isolates were assigned to phage types that were present for <1% of the total; these isolates are shown together as "others" in Table 1. PFGE typing resulted in discrimination within each phage type except DT40, for which all isolates were

Table 1. Phage type distribution for all isolates with a phage type abundance >1% of the total number of isolates*

Most common phage types	No. isolates (% of total)	No. PFGE types	No. isolates with most common PFGE type (%)	No.MLVA types	No. isolates with most common MLVA type (%)
104	173 (17.0)	11	125 (72)	84	34 (20)
120	161 (15.8)	24	65 (40)	36	40 (25)
12	153 (15.0)	14	130 (85)	47	37 (24)
193	60 (5.9)	26	21 (35)	28	20 (33)
U302	37 (3.6)	17	8 (22)	28	6 (16)
170	34 (3.3)	6	22 (65)	15	7 (21)
208	19 (1.9)	6	10 (53)	6	10 (53)
44	15 (1.5)	2	14 (93)	4	10 (67)
41	14 (1.4)	8	4 (29)	10	3 (21)
1	13 (1.3)	8	5 (38)	11	2 (15)
135	12 (1.2)	3	6 (50)	8	3 (25)
40	12 (1.2)	1	12 (100)	6	4 (33)
66	11 (1.1)	3	8 (73)	8	3 (27)
NT	116 (11.3)	36	33 (28)	49	31 (27)
RDNC	106 (10.4)	45	25 (24)	70	11 (10)
Others	83 (8.2)	–	–	–	–
All isolates	1,019 (100)	148	–	373	–

*PFGE, pulsed-field gel electrophoresis; MLVA, multiple-locus variable-number tandem-repeat analysis.

assigned the same PFGE type (Table 1). Within the most frequently seen phage types, many isolates were assigned to a single PFGE type, 85% of all isolates within DT12, 72% within DT104, and 40% within DT120 (Table 1). MLVA typing discriminated further, and all isolates were divided into 373 different MLVA types compared with a total of 148 PFGE types (Table 1). Discrimination within phage types was enhanced by using MLVA because ≤25% of the isolates within the 3 most frequent phage types were assigned to the same MLVA type (Table 1). Sixty-four PFGE types were represented by >1 isolate, 92% of these were divided into >1 MLVA type, and 53% were divided into >2 MLVA types. In total, 117 MLVA types were represented by >1 isolate; 44% of these were divided into >1 PFGE type; and 15% were divided into >2 PFGE types (data not shown).

Figure 1 shows the most common PFGE profiles, representing 75% of the isolates, as well as the most common MLVA and phage types within each PFGE type. Isolates within the most widespread PFGE types were separated into several MLVA types (PFGE014 and PFGE022, Figure 1), whereas isolates within more rare PFGE types often had

PFGE pattern*	PFGE type† (no. isolates)	MLVA pattern STTR9-5-6-10-3 (bp)	MLVA type (no. isolates)	Phage type‡ (no. isolates)
	PFGE031 (29)	172-271-336-000-517	MLVA014 (7)	DT170 (7)
		172-271-342-000-517	MLVA301 (5)	DT170 (5)
	PFGE084 (24)	172-271-342-000-517	MLVA301 (22)	DT193 (20)
	PFGE033 (20)	172-259-336-000-517	MLVA015 (14)	DT208 (9), U302 (4)
	PFGE143 (6)	172-246-342-000-517	MLVA018 (6)	DT208 (5)
	PFGE005 (17)	172-253-324-000-490	MLVA005 (7)	NT (3), DT120 (2)
		172-253-318-000-490	MLVA007 (6)	DT120 (6)
	PFGE157 (6)	172-253-324-000-490	MLVA005 (5)	RDNC (2), DT193 (2)
	PFGE074 (19)	172-283-324-000-490	MLVA435 (7)	NT (7)
	PFGE220 (6)	172-253-330-000-490	MLVA004 (6)	NT (6)
	PFGE134 (29)	172-265-318-000-490	MLVA201 (12)	DT120 (12)
		172-265-324-000-490	MLVA126 (9)	DT120 (9)
	PFGE004 (35)	172-259-318-000-490	MLVA008 (7)	DT120 (7)
		172-253-312-000-490	MLVA338 (6)	DT120 (6)
		172-253-318-000-490	MLVA007 (5)	DT120 (2),
		172-247-318-000-490	MLVA006 (5)	DT120 (5)
		172-253-324-000-490	MLVA005 (4)	DT120 (4)
	PFGE006 (68)	172-234-324-000-490	MLVA109 (41)	DT120 (39)
		172-253-324-000-490	MLVA005 (16)	DT120 (16)
	PFGE001 (13)	172-253-324-000-517	MLVA073 (9)	U302 (6), DT193 (2)
	PFGE205 (38)	172-246-371-418-517	MLVA253 (34)	DT104 (34)
	PFGE208 (9)	172-265-294-377-517	MLVA266 (9)	DT136 (9)
	PFGE099 (14)	172-253-324-000-490	MLVA005 (9)	NT (9)
	PFGE019 (42)	181-265-318-377-490	MLVA167 (13)	RDNC (10), DT12 (2)
		181-265-324-377-490	MLVA219 (5)	RDNC (5)
	PFGE047 (36)	181-271-318-370-490	MLVA059 (33)	NT (30), DT193 (3)
	PFGE012 (7)	163-300-342-387-471	MLVA034 (5)	DT85 (5)
	PFGE014 (145)	172-259-288-387-517	MLVA133 (19)	DT104 (18)
		172-265-330-487-517	MLVA238 (12)	DT104 (12)
		172-265-348-456-517	MLVA203 (11)	DT104 (11)
		172-271-348-412-517	MLVA150 (5)	DT104 (2), RDNC (2)
		172-265-342-443-517	MLVA089 (4)	DT104 (4)
		172-265-354-457-517	MLVA030 (4)	DT104 (4)
	PFGE036 (19)	163-247-294-377-523	MLVA273 (9)	RDNC (6), DT135 (2)
		163-300-342-381-471	MLVA033 (4)	DT46 (4)
	PFGE088 (14)	163-247-342-357-524	MLVA077 (10)	DT44 (10)
	PFGE027 (13)	163-241-284-000-524	MLVA171 (5)	DT40 (4)
	PFGE101 (7)	190-295-318-381-490	MLVA142 (4)	DT4 (4)
	PFGE022 (148)	181-265-330-364-490	MLVA216 (39)	DT12 (36), DT107 (2)
		181-203-348-357-490	MLVA056 (25)	DT12 (24)
		181-283-336-357-490	MLVA052 (24)	DT12 (23)
		181-271-306-363-464	MLVA062 (5)	DT12 (4)
		181-203-000-357-490	MLVA282 (4)	DT12 (4)
	PFGE160 (8)	172-221-286-351-524	MLVA086 (6)	RDNC (5)

Figure 1. Pulsed-field gel electrophoresis (PFGE), multiple-locus variable-number tandem-repeat analysis (MLVA), phage types, and number of isolates. *PFGE patterns were sorted using the Pearson correlation in BioNumerics 4.0. †Types are shown when present >6× and when ≥4 isolates had identical MLVA type within each PFGE type. ‡Phage types are only shown when ≥2 isolates within each MLVA type had the same phage type.

the same or 1 frequently seen MLVA type. Isolates within PFGE types and MLVA types often had the same or closely related phage types, and isolates within each phage type had closely related PFGE and MLVA types (except NT, RDNC, and DT193). MLVA types that had the same phage type were mostly conserved at MLVA loci STTR3 and STTR9, whereas the other 3 loci were more variable. The plasmidborne STTR10 was missing within DT120, DT170, DT208, and U302 and present within most other phage types. Exceptions were DT193, NT, and RDNC isolates, in which STTR10 could be either absent or present (Figure 1). Other trends were observed that correlate MLVA to both PFGE and phage type, including the more stable loci STTR3 and STTR9 (Figure 1), but MLVA cannot be used to predict either the phage type or the PFGE type.

Figure 2 shows the monthly occurrence of PFGE types (Figure 2A) and MLVA types (Figure 2B) within DT104. Most DT104 isolates were assigned to the same PFGE type (PFGE014) until a new PFGE type appeared in the summer of 2005 (Figure 2A). Most isolates that were assigned to this new PFGE type (PFGE205) also had a new and unique MLVA profile (MLVA253) (Figure 2B). Analyzing some isolates from animal and food products that had the same phage type and antibiotic resistance profile showed an isolate from imported beef with the same PFGE and MLVA type. An isolate with the same MLVA type was also found in Norway; this isolate originated from a patient who had been in Denmark. The rest of the isolates with the most common PFGE type (PFGE014) were divided into 83 different MLVA types (partly shown in Figure 1). Approximately 80% of the DT104 isolates were multidrug resistant (MR DT104), i.e., resistant to at least 5 microbial agents, including ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. During December 2003 to March 2004, routine resistance typing detected a small cluster of isolates (cluster 1, Table 2) that diverged from the common MR DT104 (Table 2). These isolates were resistant to only ampicillin and sulfamethoxazole; when typed with MLVA, they clustered with a unique profile (MLVA133), whereas the

Table 2. Clusters identified by MLVA typing that were investigated in the 2-y period, by typing of food and animal isolates and/or patient interviews*

Cluster no. and period	No. isolates, Danish (Norwegian)	Phage type/PFGE type/ MLVA type	Resistance profile	Description of confirmed outbreaks
1. Dec 03–Mar 04	16	DT104/PFGE014/ MLVA133	Ampiclilin, sulfamethoxazole	Human cases from narrow geographic area. Isolate match from local slaughterhouse.
2. Jun–Jul 04	21 (1)	DT12/PFGE022/ MLVA052	Sensitive	Human cases from narrow geographic area. Interviews indicated source from local butcher.
3. Aug–Sep 04	25 (1)	DT12/PFGE022/ MLVA056	Sensitive	Human cases from narrow geographic area.
4. Aug–Sep 04	28	NT/PFGE047/ MLVA059	Ampicillin, streptomycin, tetracyline	Isolate match from slaughterhouse.
5. Oct–Dec 04	9	NT/PFGE099/ MLVA005	Ampicillin, sulfamethoxazole, streptomycin, tetracycline	Isolate match from imported meat.
6. Jan–Nov 2005	40	DT120/PFGE006/ MLVA109	Sensitive	
7. Apr–Aug 2005	15	RDNC/PFGE019/ MLVA219, MLVA167	Sensitive	MLVA167 isolate match from pig herd and both MLVA167 and MLVA219 were isolated from meat from the same slaughterhouse.
8. May–Aug 2005	26	DT12/PFGE022/ MLVA216	Sensitive	Human cases from narrow geographic area. Isolate match from local slaughterhouse and from local pig herd.
9. Jun–Jul 2005	12	DT104/PFGE014/ MLVA238	MR	
10. Jun–Oct 2005	30 (1)	DT104/PFGE205, PFGE215/ MLVA253, MLVA350, MLVA351, MLVA352	MR	Interviews indicated restaurant outbreak. Isolate match from imported beef served as carpaccio in restaurant.
11. Jul–Aug 2005	9	DT136/PFGE208/ MLVA266	Sensitive	
12. Oct–Nov 2005	22	DT193/PFGE084/ MLVA301	Ampicillin, sulfamethoxazole, streptomycin, tetracycline	Human cases from narrow geographic area. Interviews indicated local butcher, and several samples collected from butcher were positive for outbreak profile.
13. Oct–Nov 2005	11	DT104/PFGE014/ MLVA203	Sensitive	
14. Oct–Nov 2005	7	NT/PFGE074/ MLVA435	Ampicillin, sulfamethoxazole, streptomycin, tetracycline	

*MLVA, multiple-locus variable-number tandem repeat analysis; PFGE, pulsed-field gel electrophoresis; MR, multiply resistant.

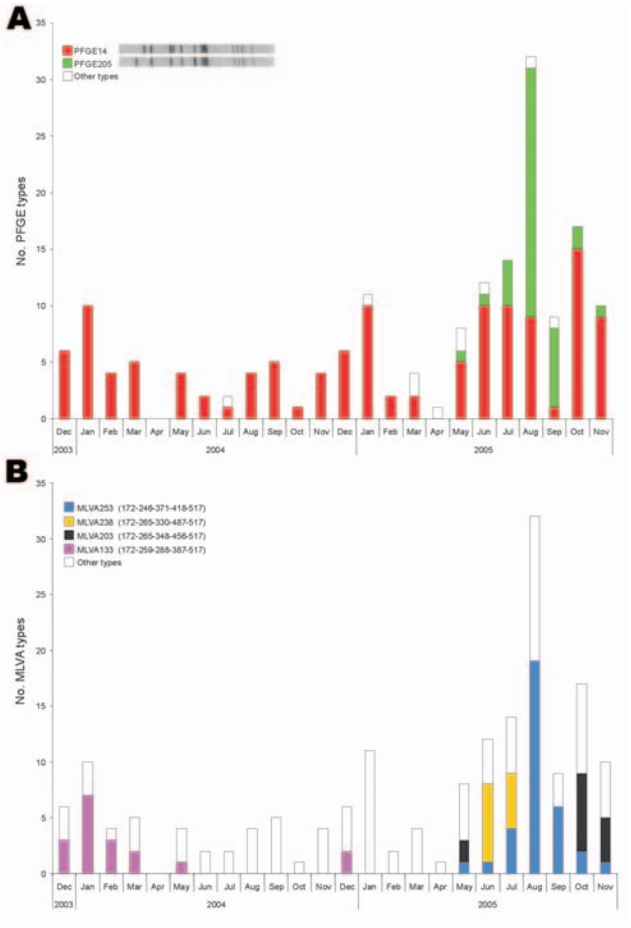

Figure 2. Monthly occurrence of pulsed-field gel electrophoresis (PFGE) types (A) and multiple-locus variable-number tandem-repeat analysis (MLVA) types (B) within *Salmonella* Typhimurium isolates with phage type DT104 over the 2-year study period. All PFGE and MLVA types that occurred ≤4× were included in other types.

isolates had the most common PFGE profile (PFGE014) (Figure 1). During October and November 2005, another small cluster (cluster 13, Table 2) that was not detected with PFGE was detected with MLVA typing (MLVA203). These isolates also diverged from the most common DT104 resistance pattern as they were sensitive to all antimicrobial agents.

Figure 3 shows the monthly occurrence of PFGE types (Figure 3A) and MLVA types (Figure 3B) within DT12. A high fluctuation was seen for DT12, but no clusters were detected from PFGE typing because 85% of all the isolates had the same PFGE type (PFGE022) (Table 1 and Figure 3A). MLVA typing showed 3 major clusters over the 2-year period, 1 in June and July 2004 (MLVA052), 1 in August and September 2004 (MLVA056), and 1 from May to October 2005 (MLVA216) (Figure 3B). The last cluster (cluster 8, Table 2) was confined to 1 region in Denmark; 1 isolate from a local slaughterhouse was positive for this type by MLVA typing of a wide selection of animal and food DT12 isolates. A national outbreak was indicated by PFGE typing and geographic distribution of the higher incidence of PFGE022 isolates in the summer of 2004 (Figure 3A). MLVA typing separated the cluster into 2 major types (Figure 3B). From a comparison of MLVA type, geographic area, and date of isolation, it was concluded that what was originally thought to be 1 outbreak was actually caused by 2 different MLVA types (MLVA052 and MLVA056), which differed at loci STTR5 and STTR6 with 13 and 2 repeat units, respectively (Figure 1). One outbreak was confined to a county in Jutland in June and July (cluster 2, Table 2), and the other was confined to the Copenhagen area in August and September (cluster 3, Table 2). The first cluster was also epidemiologically linked to a specific butcher shop, whereas no apparent source was found for the latter cluster. Two Norwegian patients who had been traveling to Denmark were identified. Characterization of these isolates showed that 1 patient was infected with MLVA52 and the other with MLVA56.

Discussion

Further investigations into clusters are started in Denmark when >5 *S.* Typhimurium isolates have the same type and are isolated within a 4-week period. In total, 14 clusters and possible outbreaks were detected and further investigated over the 2-year period (Table 2). For more than half of these clusters, a likely common source was found either by typing of veterinary and food isolates or by patient interviews (Table 2). Seven of these clusters would not have been detected when only using PFGE typing because isolates had the most common PFGE type within the assigned phage type. Two clusters would have been further divided if clusters were only assigned from MLVA types (Table 2). Cluster 7 contained 2 MLVA profiles that differed from each other with 1 repeat unit at STTR6 (MLVA167 and MLVA219 in Figure 1), but all isolates had a unique RDNC phage type that had not been identified before in Denmark, and both MLVA types were isolated from meat from the same slaughterhouse (Table 2). Cluster 10 contained 4 different MLVA profiles; most isolates had the same MLVA type, but 3 isolates were assigned to MLVA types that differed at STTR6 with 1, 2, or 8 repeat units, respectively. One isolate was also included in cluster 10 that differed at 1 band to the most common PFGE profile but had the most common MLVA type. Clusters that could be detected with MLVA were often supported by a unique PFGE profile (clusters 4, 10, 11, 12 and 14; Table 2), a characteristic antimicrobial resistance profile that differed from what was normally seen within the concerned phage types (clusters 1, 4, 12, 13, and 14; Table 2), or epi-

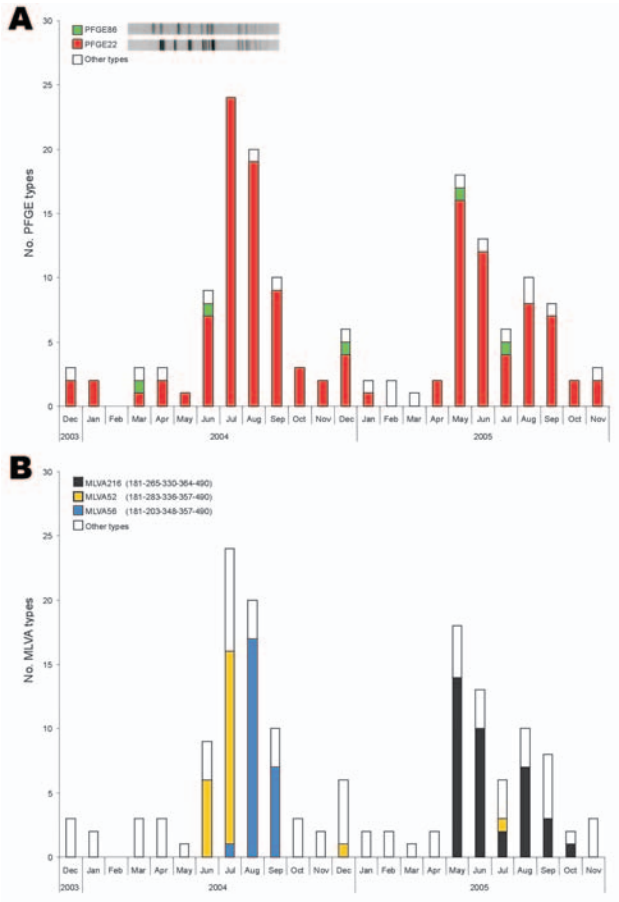

Figure 3. Monthly occurrence of pulsed-field gel electrophoresis (PFGE) types (A) and multiple-locus variable-number tandem-repeat analysis (MLVA) types (B) within *Salmonella* Typhimurium isolates with phage type DT12 over the 2-year study period. All PFGE and MLVA types that occurred ≤4× were included in other types.

demiologic information, or typing of food and animal isolates (clusters 1, 2, 3, 4, 5, 7, 8, 10, and 12; Table 2)

In Denmark, *S.* Typhimurium accounts for ≈30% of all human *Salmonella* infections (*2*), and as part of the national surveillance, all human *S.* Typhimurium isolates are subtyped by using phage typing, PFGE typing, and antimicrobial resistance profiles. These typing methods are not always discriminatory enough for surveillance and detection of common-source outbreaks. Some of the most common PFGE types account for a high percentage of isolates within each phage type and are also among the most common PFGE types in other European countries (*22*). We started routine MLVA typing (*17*) of all *S.* Typhimurium isolates over a 2-year period.

Our data supported the improved discrimination of MLVA within the uniform phage type DT104 (*18*) and furthermore showed an enhanced discrimination when compared with PFGE for almost all other phage types investigated (Table 1). The improved discrimination when we used MLVA was dependent on the phage type investigated, but with the *S.* Typhimurium level that is seen in Denmark, MLVA was especially useful for detecting clusters in the most common phage types, DT104, DT120, and DT12 (47.8% of all *S.* Typhimurium isolates), as well as isolates assigned to either NT or RDNC (21.7% of all *S.* Typhimurium isolates). No other phage types accounted for >6% of the total number of isolates, and PFGE typing would probably be sufficient for cluster detection within these less common phage types. In rare phage types, present in 1% of the total number of isolates, most of the isolates had the same antimicrobial resistance profile, PFGE type, and MLVA type; therefore, phage typing would probably be sufficient for detecting possible human outbreaks. On the other hand, phage typing would probably not be sufficient when trying to trace the source to animal or food isolates because phage types that are rare in humans can be common in animals and food (e.g., DT170 and DT193).

Most clusters that were detected with MLVA were supported by a unique phage type, PFGE profile, or antimicrobial resistance profile but none of these methods would have resulted in detecting as many clusters if used alone for surveillance. Both MLVA and PFGE were variable within clusters that were defined by other typing methods. One cluster defined by both PFGE and MLVA included 2 PFGE types that differed in 1 band and 4 MLVA types that differed at 1 locus. Another cluster contained 2 MLVA types, but all isolates had the same RDNC profile and PFGE type, which indicated that these patients were infected by a common source. For STEC O157, including isolates that differ by 1 repeat unit at 1 or 2 loci in outbreak investigation has been suggested (*13*,*14*). Results from our study suggest that including *S.* Typhimurium isolates that differ at 1 locus but with a variable number of repeat units would be useful. If including isolates that differ at 1 MLVA locus together with date of isolation for cluster detection, all reported clusters from Table 2 would have been detected and no additional cases included. Another possibility could be to include another typing method such as phage typing or PFGE together with MLVA for surveillance and outbreak investigations.

During the 2-year study period, clusters detected with MLVA were linked to a common source by MLVA typing of animal and food isolates or with interview information. Seven clusters were linked with animal or food isolates with the same MLVA and PFGE profile. One outbreak was caused by imported carpaccio, a finding further supported by interviews that showed that most patients had eaten at the same restaurant, which served carpaccio. Another outbreak was caused by meat from a local slaughterhouse from the same region as most of the patients (*23*). Another local outbreak was caused by a local butcher; samples

were found positive for the same MLVA and PFGE profile. Finally, 4 outbreaks were linked to slaughterhouses or to imported meat, samples from which were positive for the same MLVA and PFGE profile as the outbreak profile. We were unable to identify a possible source for some of the MLVA clusters, but many clusters were supported by epidemiologic information that indicated a common source. Most MLVA clusters with human cases that were linked to animal or food isolates were also supported by epidemiologic information.

For daily surveillance, MLVA has many advantages when compared with PFGE. Expensive equipment is needed to perform both processes; however, reagents for MLVA typing are cheaper and the process is less labor-intensive and faster to perform than PFGE. MLVA can be completely automated and its data are easier to analyze and interpret. The standardization of MLVA makes it possible to exchange data between laboratories. We routinely exchange data, either as fragment sizes or allelic combinations, between Denmark and Norway (*24*). Three isolates have been found in Norway that had the same MLVA profile as 3 different clusters detected in Denmark. All 3 Norwegian patients had been traveling to Denmark, and interviews revealed that 1 patient had eaten at the same restaurant as all other patients with the same MLVA type found in Denmark. MLVA has also been used to trace a common-source outbreak in Norway caused by imported meat. Two Danish patients were found with this MLVA type (*25*), and patient information showed that both patients had traveled to the same country from which the meat was imported.

In conclusion, MLVA improved surveillance of human *S.* Typhimurium infections in Denmark. MLVA was faster to perform, easier to interpret and analyze, and more discriminatory than PFGE. Several possible outbreaks were detected that otherwise would not have been detected. Some of these outbreaks were solved either by linking MLVA and epidemiologic information or by MLVA typing of animal and food isolates. We were also able to link human cases from Denmark and Norway to the same common-source outbreak. MLVA might provide an advantage to local, national, and international surveillance of *S.* Typhimurium infections.

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In Vitro Cell Culture Infectivity Assay for Human Noroviruses

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Human noroviruses cause severe, self-limiting gastroenteritis that typically lasts 24–48 hours. Because of the lack of suitable tissue culture or animal models, the true nature of norovirus pathogenesis remains unknown. We show, for the first time, that noroviruses can infect and replicate in a physiologically relevant 3-dimensional (3-D), organoid model of human small intestinal epithelium. This level of cellular differentiation was achieved by growing the cells on porous collagen-I coated microcarrier beads under conditions of physiological fluid shear in rotating wall vessel bioreactors. Microscopy, PCR, and fluorescent in situ hybridization provided evidence of norovirus infection. Cytopathic effect and norovirus RNA were detected at each of the 5 cell passages for genogroup I and II viruses. Our results demonstrate that the highly differentiated 3-D cell culture model can support the natural growth of human noroviruses, whereas previous attempts that used differentiated monolayer cultures failed.

Human noroviruses are the leading cause of nonbacterial, self-limiting gastrointestinal illness worldwide (*1–4*). Infected persons may develop symptoms of severe nausea, vomiting, and watery diarrhea within 12–24 hours of exposure and typically remain symptomatic for 1–2 days (*5*). Infections may lead to death in immunocompromised persons. The most common routes of norovirus transmission are ingestion of contaminated food and water and person-to-person contact (*5*).

Noroviruses are nonenveloped, positive-sense, single-stranded RNA viruses ≈27–35 nm in diameter (*6*,*7*). They belong to the genus *Norovirus* in the family *Caliciviridae* and consist of 3 genogroups (I, II, and IV) that infect humans (*8–11*). On the basis of sequence diversity of the capsid gene, noroviruses can be classified into 8 genetic clusters within GI, 17 in GII, and 1 in GIV (*11*).

Understanding of the pathogenesis of human noroviruses has been limited by our inability to propagate these viruses in vitro (*12*). Studies of viral attachment to cultured gastrointestinal epithelial cells (Caco-2) using recombinant virus-like particles or infectious noroviruses indicate that specific histo–blood group antigens play a key role in the attachment of the virus to the host cells (*13–17*).

Recently, the first in vitro norovirus cell culture model was reported for a norovirus that infects mice (*18,19*). Asanaka et al. (*20*) reported production of Norwalk virus particles (norovirus GI.1, the prototype strain) after transfection of cultured kidney cells. However, these models do not answer the fundamental questions of human norovirus attachment to, entry into, and replication within cells of the human gastrointestinal tract, and the resulting symptoms. In vitro differentiation of small intestinal epithelium that approaches physiologic functionality of the in vivo host may allow for the development of a pathogenesis model for norovirus.

Representative models of differentiated human intestinal epithelium can be established by growing cells in 3 dimensions (3-D) on collagen-I–coated porous microcarrier beads in rotating-wall vessel (RWV) bioreactors that model the physiologic fluid-shear environment in their respective organs (*21–24*). The design of the RWV bioreactor is based on the principle that organs and tissues function in a 3-D environment and that this spatial context is necessary for development of cultures that more realisti-

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cally resemble in vivo tissues and organs (*25*). We present the results of our first attempts to infect a physiologically relevant 3-D small intestinal epithelium model (INT-407) with genogroup I and II human noroviruses.

Materials and Methods

Generation of the Small Intestinal Epithelium Model

We summarize results from 4 different infectivity trials that used 3-D small intestinal epithelial cells (Table 1). The human embryonic intestinal epithelial cell line INT-407 was obtained from the American Type Culture Collection (Manassas, VA, USA) (CCL-6). It was initially grown as standard monolayers in GTSF-2 medium (Hyclone, Logan, UT, USA) containing penicillin/streptomycin and Fungizone (Invitrogen, Carlsbad, CA, USA) in T-75 flasks at 37°C in a 5% CO_2 environment in preparation for seeding into the RWV. GTSF-2 medium is a triple-sugar minimal essential medium α-L-15 base supplemented with 10% heat-inactivated fetal bovine serum, 2.2 g/L $NaHCO_3$, and 2.5 mg/L insulin-transferrin-sodium selenite (*26*).

Cells were trypsinized at 70% confluency, and 5×10^6 cells were added to the RWV. Cells were assayed for viability by trypan blue dye exclusion. Then they were introduced into the RWV (Synthecon, Inc., Houston, TX, USA) containing 5 mg/mL porous Cytodex-3 microcarrier beads (collagen type-I–coated porous microspheres, average size 175 μ in diameter, Sigma, St. Louis, MO, USA), which produced a final ratio of ≈10 cells/bead (*21*,*22*). Cells were cultured in the RWV bioreactors, with the rotation speed adjusted to maintain the cell aggregates in suspension during the entire culture duration (≈18–20 rotations/min initial and 24–28 rotations/min final, depending on the size of the aggregates).

Cell growth was monitored daily and fresh medium was replenished by 90% of the total vessel volume each 24–72 hours, depending on the growth and metabolic activity (as monitored by tissue culture media color change) of the cultures. 3-D cells were harvested 35 days after seeding into the RWV except for the fourth infectivity trial, for which aggregates were harvested starting on day 29 and continuing to day 35. Using a 10-mL wide-bore pipette, mature 3-D aggregates were placed into 24-well plates (≈40,000 cells/well) and infected with norovirus on the same day they were harvested.

Before each infectivity assay, immunohistochemical staining was performed on aliquots of the 3-D INT-407 cells to ensure differentiation. Aliquots of the mature tissue aggregates were fixed with paraformaldehyde (4% paraformaldehyde in 1× phosphate-buffered saline [PBS]) for 30 min at room temperature and then stained with antibodies specific for tight junction markers ZO-1, Occludin, Claudin-1, and E-cadherin (Zymed Laboratories [Invitrogen], South San Francisco, CA, USA). The aliquots were then imaged using confocal laser scanning microscopy (Zeiss Axioplan II microscope, Carl Zeiss, Thornwood, NY, USA). Correct localization of these markers at cell membranes is highly indicative of differentiated cells (*22*). Previous characterization of the 3-D INT-

Table 1. Summary of methods from the 4 norovius infectivity trials*

Infectivity trial (date)	Virus stocks/comments†	Time points assayed	Assays performed
First (Mar 2005)	Combined equal volumes of strains 149, 155, and flag2 (P0). Effective dilutions of 10^{-1} to 10^{-3} were assayed. Supernates were harvested from all infected wells, dilutions of viral stock, and time points combined (≈15 mL final volume).	1 h–72 h; media changed in all wells at 24 h postinfection.	CPE, RT-PCR (Table 3), thin section light microscopy and ultrathin section TEM (Figure 1).
Second (Jun 2005)	Supernate cocktail from first infectivity trial (P1), strains 149 and flag2 tested alone (P0 stool samples). Supernates from each time point were harvested for subsequent infectivity trials (≈3 mL/time point).	Same as first infectivity trial; media changed every 24 h.	Same as first infectivity trial, except that CPE (Figure 2) was documented photographically (Figure 3 refers to TEM).
Third (Aug 2005)	Supernate from combined stock (P2), 149, and flag2 (P1), stool sample flag2 (P0) were harvested. Controls generated by ultrafiltration (10,000 MWCO).	Same as second infectivity trial.	Same as second infectivity trial (Figure 4); first attempt with FISH
Fourth (Dec 2005)	Infectivity followed through 5 passages in cell culture using strains 155 and flag2 (P0–P5). Effective dilution of viruses at P5 = $1{:}10^6$, if replication was not occurring.	Infected aggregates were processed at 24 h postinfection, and the viruses were used for subsequent passage.	CPE, RT-PCR, molecular beacon FISH (Figure 5). PCR products from P3 of both strains were cloned and sequenced. Compared sequences with sequenced PCRs from original stools.

*CPE, cytopathic effect; RT-PCR, reverse transcription–PCR; TEM, transmission electron microscopy; MWCO, molecular weight cutoff; FISH, fluorescence in situ hybridization.
†Passage no. (P#) definitions: P0, viruses from a stool sample and used to infect a cell culture for the first time; P1, viruses harvested from P0 cell cultures and used to infect cell culture a second time; P2, viruses harvested from P1 and used to infect cell culture a third time; P3, viruses harvested from P2 and used to infect cell culture a fourth time; P4, viruses harvested from P3 and used to infect cell culture a fifth time; and P5, viruses harvested from P4 and used to infect cell culture a sixth time.

407 model also included collagen type-II, fibronectin, sialyl Lewis A antigen, villin, and periodic acid–Schiff staining to show mucin production (*22*).

Viruses, diluted 1:5 to 1:1,000 in GTSF-2 media, were applied to the 3-D cell cultures as 0.1-mL aliquots per well across a minimum of 3 wells per time point for each of the infection trials described in Table 1. Viruses were introduced to the cells by gentle mixing of the aggregates with the viral suspension. The infected aggregates were incubated for 1 h at 37°C in a 5% CO_2 incubator before being overlaid with 1 mL of fresh GTSF-2 media.

Preparation and Characterization of Virus Stocks

Stool samples were obtained from persons who became ill during acute gastroenteritis outbreaks on cruise ships (identified as samples 149 [GII] and 155 [GI]) and in a nursing home (identified as flag2 [GII]). Approximately 1 g of stool was suspended in 0.01 M PBS to obtain a 10%–20% stool suspension (≈5–10 mL). The suspension was vortexed for 60 s, centrifuged at 1,000× *g*, and processed through a 0.22-μ filter to remove bacterial contamination. Virus samples were stored at –80°C for future assays.

The presence of norovirus in the purified samples was confirmed by reverse transcription–PCR (RT-PCR) and sequencing (*10*). BLAST (www.ncbi.nlm.nih.gov/BLAST) was performed on these sequences to determine genogroup (Table 2). Stool extracts were screened for other enteric viruses by 3 passages on Buffalo Green Monkey cells and Caco-2 cells grown as conventional monolayers. Stool extracts were also tested for enterovirus by RT-PCR (*27*).

Microscopic Analysis

Cellular pathology of 3-D tissue aggregates for the second and third infection trials was documented by using an Olympus DP70 CCD camera and inverted microscope system (Nikon Eclipse TE300, Kanagawa, Japan) at each time point assayed. Subcellular pathology was assessed by using light and transmission electron microscopy (TEM). These samples (≈40,000 cells per well) were fixed in 3.5% glutaraldehyde/0.5% paraformaldehyde in PBS and processed by washing cells 3× with 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA, USA) before incubation in 1% osmium tetroxide diluted in 0.1 M sodium cacodylate buffer for 1 h at room temperature. Cells were washed with buffer and dehydrated by using a graded series of ethanol rinses (33, 50, 75, 90, and 3× 100% ethanol). Samples were then embedded in hard-grade LR WhiteTM Resin (London Resin Co., Berkshire, England) at 60°C for 24 h. Block faces were cut into the samples by using a Leica EM Trim (Wetzlar, Germany). Thin (60 nm) and ultrathin (30 nm) sections were cut by using a Diatome Ultra 45° (Biel, Switzerland) diamond knife on a Leica Ultracut UCT ultramicrotome. Thin sections were affixed to microscope slides and stained with toludine blue, then viewed on a Nikon Optiphot-2 light microscope. Ultrathin sections were affixed to copper mesh grids, stained and contrasted with uranyl acetate and lead citrate for 7 min each, and then viewed on a JEOL-2010 (Tokyo, Japan) transmission electron microscope at 106 kV.

Viral RNA Extraction and RT-PCR

RNA from tissue samples was extracted by using either an RNEasy or a ViralAmp RNA extraction kit (Qiagen, Valencia, CA, USA). RT-PCR was performed by using the OneStep RT-PCR kit (Qiagen) according to manufacturers' instructions. Primer sequences for RT-PCR and seminested PCR to amplify the RNA-dependent RNA polymerase gene are listed in Vinje et al. (*10*), with the exception of the MP290 primer for seminested PCR, which is 5′-GAYTACTCYCSITGGGAYTC-3′. Viral RNA was subjected to RT-PCR for 60 min at 42° C and 15 min at 95° C to inactivate the RT enzyme and activate Taq. A 3-step PCR was then conducted for 40 cycles (30 s at 94°C, 30 s at 50°C, and 30 s at 72° C).

Sequencing

PCR products amplified from cell cultures (P3 passage) of the fourth infection trial were sequenced after cloning into the PGEM-T Easy Vector System (Promega, Madison, WI, USA). Sequences have been deposited in GenBank under accession nos. DQ531707 (for outbreak sample 155) and DQ531708 (for outbreak sample flag2).

Fluorescence in Situ Hybridization

The molecular beacon fluorescence in situ hybridization (FISH) assay performed during the fourth infection trial used modified reverse PCR primer sequences for genogroup I and II viruses (*28*). For genogroup 1, the modified probe sequence was 5′-TAMRA-CAGGCCCTTAGACGCCATCATCATTGCCTG-DABCYL-3′, and for genogroup 2, the modified probe sequence was 5′-TAMRA-CTCGGTCGACGCCATCTTCATTCACACC-

Table 2. Genetic characterization of the RNA-dependent RNA polymerase sequence of the norovirus strains used in the study

Sample ID	Date collected (2004)	Setting	Strain in GenBank with closest sequence similarity (%)	Genogroup
149	Apr 14	Cruise ship	AJ487474 NLV/Castell/2001/Sp (97%)	II
155	Jun 21	Cruise ship	DQ157140 Hu/Offenburg1155/2004/D (100%)	I
flag2	Dec 14	Nursing home	AJ626578 NV/GII/Stockholm/IV1138/2003/SE (97%)	II

GAG-DABCYL-3′. (Underlined sequences for each probe indicate the self-complementary regions.)

Cells in the tissue aggregate were fixed in 4% paraformaldehyde for 30 min and then washed 3 times in 1× Dulbecco's PBS (DPBS, Sigma). The weight of the aggregates allowed these to settle by normal gravity to the bottom of the microfuge tube. Tissues were permeabilized with 0.1% Triton X-100 in 1× DPBS for 15 min at room temperature and then washed 3× with 1× DPBS. Molecular beacon (either genogroup 1 or genogroup 2) was suspended to a final concentration of 1 μM in 1× DPBS. The molecular beacon was incubated with the tissues in a water bath for 1 h at 37ºC. The aggregates were then washed 3× with 1× DPBS and transferred to 12-well tissue culture plates. Cell aggregates were imaged by using a Leica confocal laser scanning microscope with a 63× water immersion objective. Captured images were digitally stacked to create 3-D images (VOLOCITY, Improvision Inc., Lexington, MA, USA).

Results

First Infection Trial (March 2005)

This first attempt was performed with a cocktail of norovirus strains 149, 155, and flag2 (stool samples are defined as Passage 0 [P0]). At 24 h postinfection, infected cell aggregates exhibited vacuolization and shortening of the microvilli (Figure 1) and were detached from the cytodex beads, exhibiting cytopathic effect (CPE) (data not shown). CPE was first observed in tissue aggregates receiving the highest concentration of virus and then developed in aggregates receiving the lowest concentration of virus. TEM showed the presence of uniform 29-nm–diameter particles, consistent with the size of norovirus particles, which invaded the microvilli within 1 h postinfection (Figure 1, Panel C) and accumulated within the tissue aggregates within 24–66 h postinfection (Figure 1, Panels D and E, respectively). Concomitant with microscopic observations, viral RNA was detected as early as 1 h postinfection for the 1:10 and 1:100 dilutions and at 66 h postinfection for the 1:1,000 dilution (Table 3).

Table 3. Relative increases in viral RNA as measured by limiting dilution PCR*

Effective dilution of working viral stock applied to cells	Hours postinfection			
	1	24	66	72
1:10	+	+	+	ND
1:100	+	+	+	ND
1:1,000	-	-	+	+
Negative control	-	-	-	-

*ND, not done.

Second Infection Trial (June 2005)

Figures 2 and 3 show results for CPE and TEM, respectively, for P1 of the combined virus stocks and P0 for strains flag2 and 149 (Figure 2, Panels B, C, and D and Figure 3, Panels B, C, and D). As with the first infection trial, viral CPE was manifested by cells sloughing off the collagen beads as a mat, with the individual cells becoming highly elongated or distorted within 24–48 h postinfection. Similar to other studies that used murine norovirus (*18*), TEM exhibited uniformly sized 27–29-nm particles in infected cell aggregates and internal membrane rearrangement. By using RT-PCR, norovirus RNA was detected in all infected samples. Both the combined stock (P1) and flag2 (P0) showed an increase of viral RNA as detected by limited dilution during RT-PCR.

Third Infection Trial (August 2005)

In trial 3, we used P2 of the virus cocktail (strains 149, 155, and flag2), P1 of flag2, and the stool sample from flag2 (P0) to infect a new batch of differentiated 3-D INT-407 cells. For virus-negative controls, we filtered the virus inoculum through a 10,000–molecular weight cutoff (MWCO) filter. Both the cocktail and the flag2 isolate were able to generate CPE within 24 h postinfection (Figure 4, Panels B, D, and E), whereas the MWCO filtrates did not show CPE and tested negative by RT-PCR (Figure 4, panels A and C).

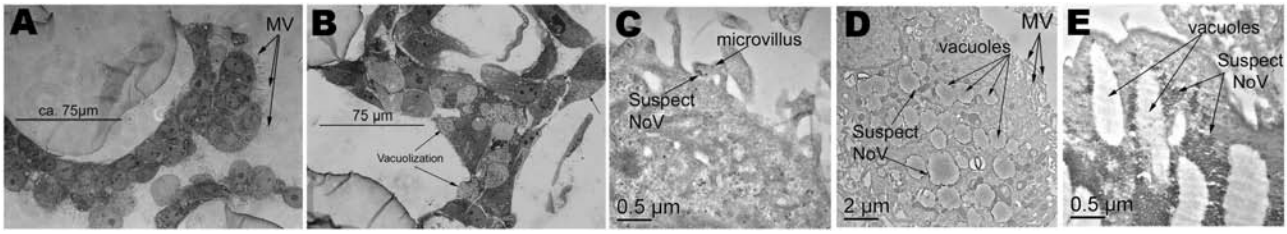

Figure 1. Light and transmission electron micrographs of uninfected and infected tissue aggregates with a combined stock of noroviruses (NoV) representing 3 strains (Passage 0 [P0]). A) Uninfected tissue aggregates displaying well-formed microvilli (MV). B) Infected tissue aggregates exhibiting vacuolization and shortening of the microvilli. C) Transmission electron microscopy (TEM) at 1 h postinfection showing possible norovirus in a microvillus. D) TEM at 24 h postinfection showing significant vacuolization, and internal membrane rearrangement. E) TEM at 66 h postinfection showing accumulation of suspected norovirus particles. For a larger reproduction of these panels, see online Figure 1 available at www.cdc.gov/EID/content/13/3/396-G1.htm

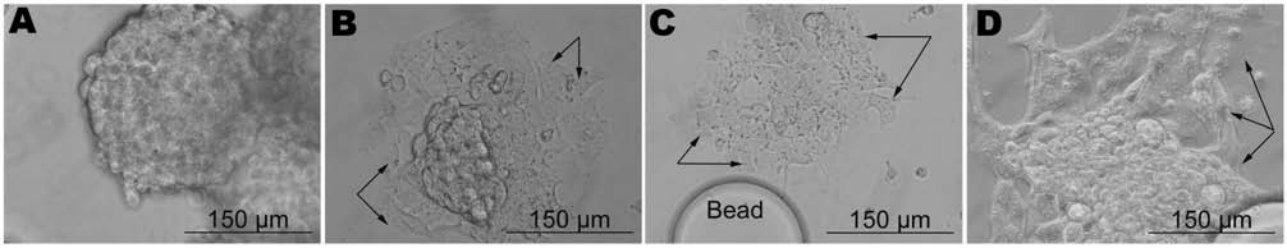

Figure 2. Demonstration of cytopathic effect in infected tissue aggregates during the second infection trial. A) Uninfected aggregate, 24 h into the experiment. B) Cells infected with lysate from the first infection trial (P1) at 24 h postinfection. C) Stool sample flag2 at 24 h postinfection (P0). D) Stool sample 149 at 48 h postinfection (P0). Arrows indicate cells exhibiting unusual pathology.

Fourth Infection Trial (December 2005)

In trial 4, we used strain 155 (genogroup I) and flag2 (genogroup II) and infected 3-D INT-407 cells. We followed viral infection for both of these strains through 5 passages in cell culture. With each viral passage, cell cultures showed CPE after 24–48 h, and norovirus RNA for both strains was detected by FISH with genogroup specific molecular beacons (Figure 5). We further sequenced RT-PCR products from the original stool sample that contained strains 155 and flag2 and both strains from passage 3 in cell culture. Only 1 nucleotide substitution for passage 3 flag2 was observed in a 261 bp product, and no nucleotide change was shown for strain 155.

Discussion

Our primary goal was to develop an in vitro cell culture assay for human noroviruses. This assay is necessary before we can even begin to understand the mechanisms of pathogenesis for this virus. Our starting point for developing an infectivity assay for human noroviruses was to use the 3-D INT-407 small intestinal epithelium model previously developed for the study of *Salmonella* pathogenesis (*22*). Multiple factors were considered for choosing this model. First, early biopsy studies that used human volunteers indicated that norovirus infection targets the human small intestine (*29*,*30*). Second, reports showing differentiation of INT-407 cells in 3-D in the RWV essentially produces a "co-culture" model of multiple intestinal cell types (enterocytes, goblet cells, and M cell–like markers) (*22*). This phenomenon of multicellularity has been hypothesized as a factor likely needed for norovirus infectivity (*12*). Finally, extensive characterization of this model 3-D system (*22*,*23*) showed apical expression of certain cell-surface antigens (e.g., Lewis antigen A), which are thought to be important in the attachment of noroviruses to cells (*4*,*13*–*17*,*31*).

However, expression of these antigens alone is not sufficient for a successful cell culture of human norovirus, because our attempts to infect 3-D aggregates established from the large intestinal (colon) cell lines Caco-2 and HT-29 cells were unsuccessful (data not shown). We are not sure whether this phenomenon is due solely or in part to 1) correct presentation of the cell surface receptors that would be necessary for viral attachment and efficient entry into cells or 2) physiologic relevance of the 3-D small intestinal model that confirms previous human biopsy studies that show human noroviruses have an affinity for cells of the small intestine (*29*,*30*).

We have developed the first successful in vitro cell culture assay for norovirus based on multiple lines of orthogonal evidence. CPE has been a measure of viral infectivity, but this measure alone can be deceiving. Duizer et al. (*12*) noted CPE in several samples but on further investigation found that it was caused by contaminating

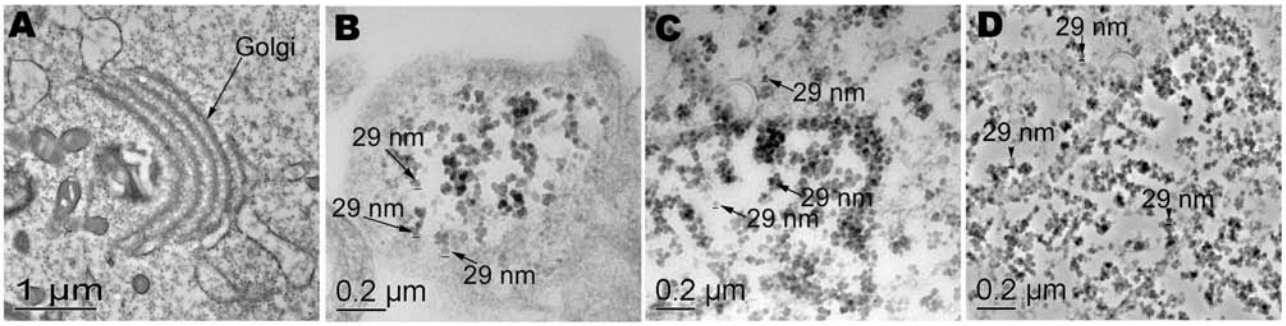

Figure 3. Transmission electron microscopy of uninfected and infected cell cultures from the second infection trial at 48 h postinfection. A) Uninfected cells showing normal internal membrane organelles. B) Suspected 29-nm particles in cells, viruses from cell culture lysate from the first infection trial (P1). C) Stool sample flag2 (P0). D) Stool sample 149 (P0) showing numerous 29-nm particles and internal rearrangement of membrane-bound organelles.

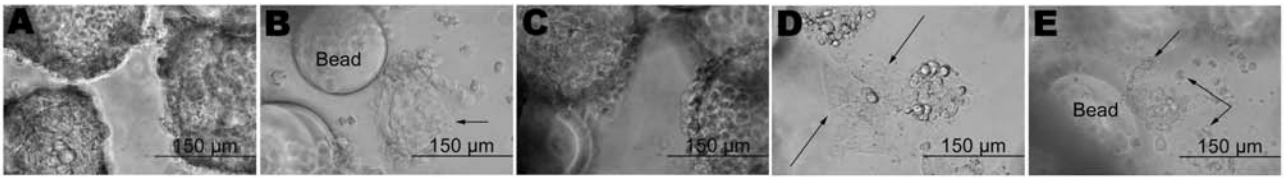

Figure 4. Cytopathic effect results from the third infectivity trial at 24 hours postinfection. A) Virus-free control of B) combined viral stock lysate from second passage experiment (second infectivity trial, P1), which was used to infect naive cells (P2). C) Virus-free control of the flag2 stool sample. D) Corresponding infection with the flag2 stool sample (P0). E) Flag2 in cell culture (P1). Cells in Panels B, D, and E were confirmed as positive for norovirus by reverse transcription-PCR (RT-PCR) and seminested PCR. Cells in uninfected controls were negative for norovirus by both RT-PCR and nested PCR. Arrows indicate cells exhibiting unusual pathology. For a larger reproduction of these panels, see online Figure 4 available at www.cdc.gov/EID/content/13/3/396-G4.htm

viruses. For the 3 virus strains we investigated, we took several measures to ensure that the viruses were indeed noroviruses. First, patients from these 3 outbreaks showed clinical symptoms typical of norovirus infection. Second, these virus isolates failed to produce CPE through 3 passages in conventional monolayers of buffalo green monkey and Caco-2 cells. Third, RT-PCR for co-infecting enteroviruses was negative. However, successful norovirus replication was demonstrated through 5 passages in the 3-D small intestinal model, as confirmed by CPE, RT-PCR, and FISH with genogroup-specific molecular beacons.

Although the Duizer et al. (*12*) study noted infrequent CPE, our study demonstrated positive CPE and norovirus-positive RT-PCR every time viruses came in contact with cell culture. This occurred whether viruses originated from stool samples or at any passage in the 3-D INT-407 cell culture. Furthermore, positive CPE from a viral sample could be abrogated by passing the sample through an ultrafilter. The filtrate, when applied to cells, did not cause CPE and was norovirus negative by both RT-PCR and seminested PCR. Additionally, for virus infected cell cultures but not uninfected cells, light microscopy and TEM demonstrated both the pathology and evidence of accumulation of viral particles that are the correct size for human norovirus. Thus, cellular pathology was due to norovirus infection and not caused by other enteric viruses or sample toxicity.

In vitro cell culture models used to study the host-pathogen interaction have benefited from the recognition that organs and tissues function in a 3-D environment and that this spatial context is necessary for development of cultures that more realistically resemble the in vivo tissues and organs from which they were derived (*21–23*). We used RWV bioreactor technology to engineer 3-D models of human small intestinal epithelium to investigate susceptibility for norovirus infection. This method to generate 3-D organoid models has been used to study *Salmonella typhimurium* and *Escherichia coli* infection by using small and large intestinal models (*21–23*,*32*), *Pseudomonas* infection by using lung epithelial models (*33*), cytomegalovirus infection by using placental tissue models (*34*), and Epstein-Barr virus by using lymphoblastoid cell models (*35*).

Our study shows that selecting the appropriate cell line, growing the cells as 3-D aggregates, and infecting them when they are fully differentiated is key for successful in vitro cell culture of human noroviruses. Future research with this model will include further testing of a broader panel of genetically diverse human noroviruses,

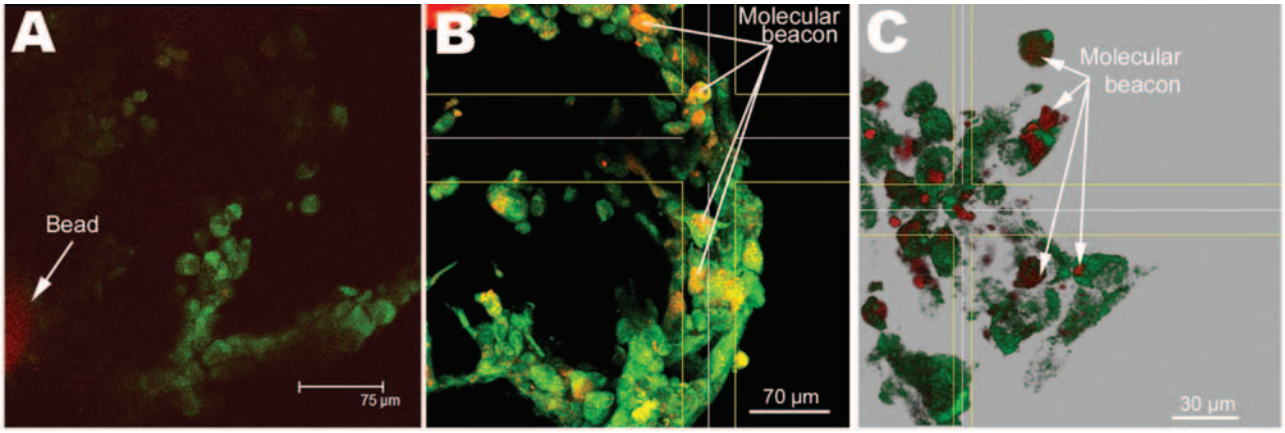

Figure 5. Deconvolved confocal laser scanning micrographs of the molecular beacon fluorescence in situ hybridization assay, demonstrating viral infectivity of a genogroup I virus (Sample 155) and genogroup II virus (flag2) at 24 hours postinfection. A) Typical response for uninfected cells, no molecular beacon observed. B) Sample 155, P5 in cell culture. C) Sample flag2, P5 in cell culture.

determining the sensitivity, identifying neutralizing epitopes and protective immune responses, and obtaining a better understanding of the molecular biology of norovirus replication and transcription to develop improved prevention protocols.

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etymologia

Norovirus

[nor′-o-vi′rəs]

Genus of viruses that cause viral gastroenteritis. Noroviruses are named after the original strain, "Norwalk virus," which caused an outbreak of acute gastroenteritis among children at an elementary school in Norwalk, Ohio, in 1968. Numerous outbreaks of disease with similar symptoms have been reported since, and the etiologic agents were called "Norwalk-like viruses" or "small round-structured viruses." Noroviruses are transmitted primarily through the fecal-oral route and are highly contagious; as few as 10 viral particles may infect a person.

Sources: Mahy BWJ. A dictionary of virology. London: Academic Press; 2001; http://www.cdc.gov/ncidod/dvrd/revb/gastro/norovirus-qa.htm; http://www.medicinenet.com/norovirus_infection/article.htm

Surveillance of Influenza A Virus in Migratory Waterfowl in Northern Europe

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We conducted large-scale, systematic sampling of influenza type A virus in migratory waterfowl (mostly mallards [*Anas platyrhynchos*]) at Ottenby Bird Observatory, southeast Sweden. As with previous studies, we found a higher prevalence in fall than spring, and among juveniles compared with adults. However, in contrast to other studies, we found that prevalence in spring was sometimes high (mean 4.0%, highest 9.5%). This finding raises the possibility that ducks are capable of perpetuating influenza A virus of different subtypes and subtype combinations throughout the year and from 1 year to the next. Isolation of the H5 and H7 subtypes was common, which suggests risk for transmission to sensitive domestic animals such as poultry. We argue that wild bird screening can function as a sentinel system, and we give an example of how it could have been used to forecast a remote and deadly outbreak of influenza A in poultry.

The influenza A virus, including all its subtypes and most of their subtype combinations, is commonly found in aquatic birds such as ducks, geese, gulls, and shorebirds, while only a limited number of subtypes have been found in nonavian hosts. Therefore, waterfowl, in particular wild dabbling ducks (genus *Anas*), are believed to constitute the main natural viral reservoir for low pathogenic influenza A virus, from which strains occasionally arise that are transmitted to other species, including humans and poultry (*1*)

Current knowledge of influenza A virus ecology in wild birds is derived mainly from North American studies (*1*,*2*), which show seasonal changes and between-year fluctuations in prevalence and subtype distribution. Highest incidences occur in juvenile and thus immunologically naïve ducks during fall migration. At other times of the year, however, the observed prevalence is very low, which raises the question as to how the multitude of subtypes are maintained and perpetuated (*1*).

Influenza A virus has diversified into 2 separate avian lineages, North American and European (*3*,*4*), so it is reasonable to ask whether the ecology of influenza A virus in Europe differs from that in North America. Unfortunately, few studies have been conducted in Europe, so more data are urgently needed.

We report results from a 4-year study of influenza A virus occurrence in migrating ducks (mainly mallards [*Anas platyrhynchos*]) in Sweden. We show that prevalence patterns remained similar over the study period but that important differences regarding seasonality and subtype distribution occurred when compared with previous studies from North America. We also compare our data to other long-term systematic surveillance studies of influenza A virus in wild ducks, review geographic patterns and prevalence of influenza A virus subtypes, and discuss their modes of perpetuation in waterfowl.

Materials and Methods

From 2002 to 2005, we collected samples from wild waterfowl at Ottenby Bird Observatory (56°12′N, 16°24′E), on Öland, a Swedish island in the Baltic Sea (Figure 1A and B). Birds were caught in a funnel live-trap

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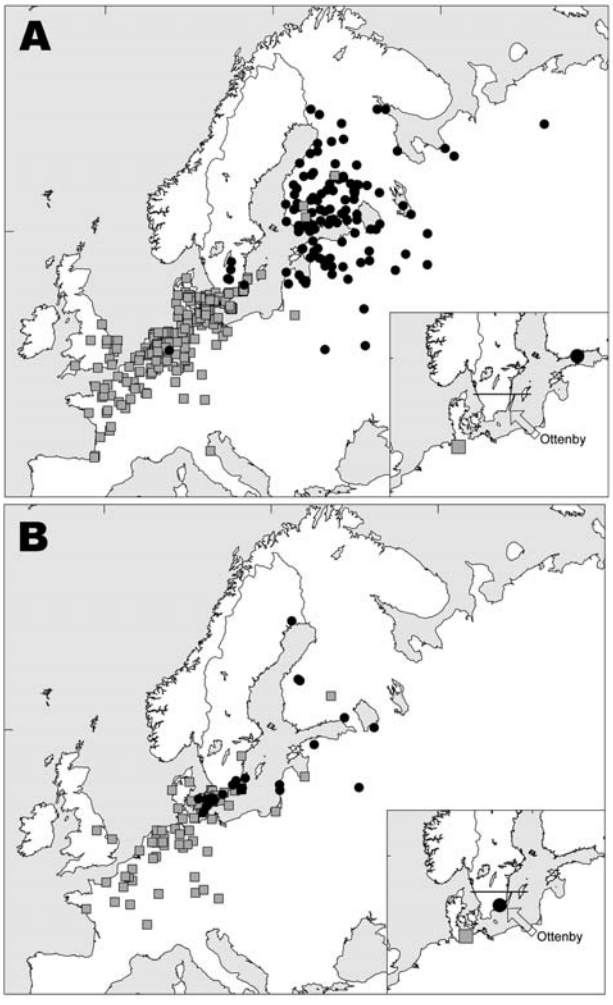

Figure 1. Female mallards banded in Sweden south of 57°30′N (indicated by a solid line in the inserted figures) in Oct–Dec (A) and May–Sep (B) and recovered in winter (Nov–Feb, n = 255 and n = 98) and summer (May–Aug, n = 135 and n = 53). Black dots represent summer recoveries; gray squares represent winter recoveries. Symbols on inset maps represent calculated mean positions and the location of Ottenby Bird Observatory.

mainly during migration (see Table 1 for precise dates). We defined the period March–June as spring (comprising the spring migration and some early summering birds), and the period July–December as fall (comprising the fall migration and perhaps some late summering birds). Captured birds were banded with steel rings and identified for species, sex, and (when possible) age. Aged individual birds were assigned to the following categories: fall (juvenile or adult), spring (first spring bird, i.e., juvenile after first winter, or adult) (*5*).

Collection and Preservation of Samples

We placed each captured duck in a box with a clean (unused) paper bottom. Using sterile cotton swabs, we then sampled each bird either by swirling the swab in its cloaca (20% of individual birds) or by swabbing its fresh droppings on the paper bottom. Cotton swabs were immediately put in vials containing virus transport media (Hanks balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/mL penicillin, 200 μg/mL streptomycin, 100 U/mL polymyxin B sulfate, 250 μg/mL gentamicin, and 50 U/mL nystatin [ICN, Zoetermeer, the Netherlands]) and frozen to –70°C within 30 min.

Virus Detection

Influenza A virus was detected by 2 different methods (Table 1). Samples collected in fall 2002 were analyzed at the Erasmus Medical Center in Rotterdam, the Netherlands, by using RNA isolation and Taqman as described by Munster et al. (*6*). To ensure efficient influenza A virus detection, the published probe sequence was changed to 5′-6-FAM-TTT-GTG-TTC-ACG-CTC-ACC-GTG-CC-TAMRA-3′, based on avian influenza A virus sequences available from public databases. Pools of 5 individual samples were prepared and processed in parallel with several negative and positive control samples in each run. Upon identification of influenza A virus–positive pools, RNA isolation and Taqman procedures were repeated for the individual samples within each positive pool, and individual Taqman-positive samples were subsequently used for virus isolation.

At the Swedish Institute for Infectious Disease Control (SMI) in Stockholm, we screened samples collected in 2003 by using a real-time PCR (RT-PCR) method directed at the conserved matrix gene with SYBR green technique as developed at SMI (M. Karlsson et al., unpub. data). Some samples from the end of 2003 and samples collected in 2004 and 2005 were screened at the Section for Zoonotic Ecology and Epidemiology, Kalmar University, by using the same method as at SMI with slight local adjustments. The following adjustments were used: RNA was isolated from 100 μL of the original sample by using an EZ1 Virus Mini Kit (QIAGEN, Germantown, MD, USA), with the extraction Biorobot EZ1 (QIAGEN) set to obtain 75 μL of elution volume. Amplification of the selected part of the influenza A matrix gene was conducted with the LC FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany), having a final reaction volume of 20 L. The thermo cycling was performed in a LightCycler 1.5 (Roche Diagnostics GmbH) under the following conditions: polymerase activation for 10 min at 95°C, and then 43 cycles of 10 s at 95°C, 10 s at 60°C, and 10 s at 72°C. After the amplification, the melting temperature of the PCR product was determined by progressively increasing the temperature from 65°C to 95°C (melting curve analysis).

Table 1. Sampling data and locations of analyses*

Season	No. mallards screened	Dates	PCR screening location	PCR method used	Subtyping location
2002 Fall	897	9/29–12/16	EMC	Taqman	EMC
2003 Spring	348	4/14–6/30	SMI	Cyber-green	EMC
2003 Fall	755	7/1–12/18	SMI	Cyber-green	EMC
2004 Spring	346	3/26–6/30	KU	Cyber-green	EMC
2004 Fall	789	7.1–12/15	KU	Cyber-green	Not performed
2005 Spring	155	4/3–6/30	KU	Cyber-green	Not performed
2005 Fall	816	7/5–12/13	KU	Cyber-green	Not performed
Total	4,106				

*EMC, Erasmus Medical Center; SMI, Swedish Institute for Disease Prevention and Control; KU, Kalmar University.

Virus Isolation and Characterization

Virus isolation and characterization of positive samples collected in 2002–2004 were performed at the Erasmus Medical Center, Rotterdam. For all influenza A virus RT-PCR–positive samples, 200 μL of the original specimen was injected into the allantoic cavity of 11-day-old embryonated chicken eggs. The allantoic fluid was harvested 2 days after injection, and influenza A virus was detected by using hemagglutination (HA) assays with turkey erythrocytes. When the HA titer was negative, the allantoic fluid was passaged once again in embryonated chicken eggs. Virus isolates were characterized by using a hemagglutination inhibition assay with turkey erythrocytes and subtype-specific hyperimmune rabbit antisera raised against all HA subtypes (*7*).

The neuraminidase (NA) subtypes of influenza A virus isolates were characterized by RT-PCR and sequencing. RT-PCR and sequencing of the NA genes were performed essentially as described by Hoffmann et al. (*8*). Nucleotide and amino acid sequences were aligned by using the ClustalW program running within the Bioedit software package, version 5.0.9 (*9*).

Mallard Populations and Their Movements

To determine breeding grounds, migration routes, and wintering areas of the mallard populations studied, we analyzed recovery data from all mallards banded (ringed) at Ottenby Bird Observatory from 1962 through 1982 (*10*), and in southern Sweden from 1962 to the present (south of 57°30′N). We obtained these data from the Bird Ringing Centre at the Swedish Museum of Natural History.

Female mallards show stronger philopatry than males, i.e., a higher proportion of the former return to natal areas to breed in consecutive years (*11*). Pair formation takes place in winter, and males that pair-bond with females follow the mate to her natal area. As a consequence, males shift breeding areas between years to a higher degree than females do. We therefore analyzed the banding recovery data according to sex; data on females banded in 1 year and recovered in consecutive breeding seasons were used to outline the general breeding range of mallard populations that pass Ottenby. Recoveries were further divided into birds trapped in summer (May–September) or fall (October–December). All recoveries with uncertainties concerning the date were excluded. The mean geographic position for the different groups was calculated according to Perdeck (*12*). The breeding season was defined as May–August and the winter season as November–February.

Results

Sampling Overview

We collected samples from 4,800 individual waterfowl of 16 species (Table 2). Only mallard, common shelduck (*Tadorna tadorna*), northern pintail (*Anas acuta*), and Eurasian teal (*A. crecca*) yielded >20 samples each (Table 2). Most (85.5%) of the samples were from mallards. Twice as many birds were sampled in fall (3,323) as in spring (1,477). In fall, 78% of birds aged were juveniles. Most birds were caught during the peak migratory periods of October–December and May–June (Figure 2).

Mallard Populations and Their Movements

Mallards banded in southern Sweden in the fall belonged to a different population than those banded there in summer. Females caught in the fall months of October–December were usually found east of the Baltic Sea the next breeding season (May–August) in Finland, Russia, and the Baltic States (Figure 1A). In contrast, females banded in summer (May–August) were usually found in nearby areas of Sweden or in Denmark the following breeding season (May–August) (Figure 1B).

Both groups of females wintered mainly in coastal areas of western Europe, from southern Sweden to France and Great Britain; the mean recovery position of females banded during late fall (Figures 1A) was located more to the southwest than that of females banded in summer (Figure 1B). Recoveries of males showed a general pattern similar to that of females but with much more geographic scatter, as predicted from the gender differences in philopatry.

Table 2. Number of processed samples and influenza A virus prevalence in waterfowl (*Anatidae*) sampled at Ottenby Bird Observatory, 2002–2005

Common name	Scientific name	Spring	Fall	Total	No. positive	Prevalence, %
Mute swan	*Cygnus olor*		9	9		
Greylag goose	*Anser anser*	2	1	3		
Barnacle goose	*Branta leucopsis*		6	6		
Brent goose	*B. bernicla*		12	12		
Common shelduck	*Tadorna tadorna*	504	7	511	14	2.7
Eurasian wigeon	*Anas penelope*		16	16		
Gadwall	*A. strepera*	1	1	2		
Eurasian teal	*A. crecca*	18	44	62	8	12.9
Mallard	*A. platyrhynchos*	950	3,156	4,106	575	14.0
Northern pintail	*A. acuta*	2	28	30	3	10.0
Common pochard	*Aythya ferina*		2	2		
Tufted duck	*A. fuligula*		18	18		
Common eider	*Somateria mollissima*		15	15		
Long-tailed duck	*Clangula hyemalis*		1	1		
Goldeneye	*Bucephala clangula*		3	3		
Red-breasted merganser	*Mergus serrator*		4	4		
Total		1,477	3,323	4,800	600	

Prevalence Overview

Total prevalence of influenza A virus in all waterfowl sampled during the 4-year period was 12.5%. However, 575 (95.8%) of the 600 influenza A virus PCR-positive samples were from mallards, and only 25 samples came from other host species (Table 2). Prevalence in mallards at different seasons varied among years but followed the same general pattern, i.e., lower values in spring and early summer compared with late summer and fall (Figures 3 and 4). The highest overall prevalence was found in October 2005 (25.7%) and the lowest in April and May 2005 (0).

Seasonal Differences

Monthly prevalence in mallards was higher in fall (3.0%–25.7%) than in spring (0–9.5%). Mean fall prevalence (15.0%, n = 2,714) was significantly higher than the corresponding spring value (4.0%, n = 817), both when data were analyzed for each year separately (proportion infected vs. noninfected 2003–2005; χ^2_1 = 19.0–41.1, n = 971–1,135, p<0.001) and for the combined dataset of 4 years (χ^2_1 = 93.1, n = 4,106, p<0.001).

Species Differences

Mallards were caught in substantial numbers in spring as well as in fall, whereas common shelducks were caught mainly in spring (Figure 2) and Eurasian teal and northern pintail mainly in fall. Regardless of this caveat, and the much smaller n values, the prevalence rates of these species were similar to those of mallards. For instance, spring prevalence in common shelducks was 2.8%, similar to the 4.0% seen in mallards. Likewise, fall prevalence rates of northern pintails and Eurasian teal, 10.7% and 18.2%, respectively, were within the range of such rates in mallards.

Age and Sex Patterns

Looking only at the species with the largest dataset (mallard), we also found differences between age groups. In fall, 11.7% of adults (n = 468) and 20.4% of juveniles (n = 1,944) were positive for influenza A virus. In spring, 1.7% of birds aged as second spring or older (n = 242) and 6.0% of the first spring birds (n = 390) were positive for influenza A virus (Figure 5). Adult birds had consistently lower prevalence than younger birds, both in fall (χ^2_1 = 11.41, n = 2,412, p = 0.001) and in spring (χ^2_1 = 5.05, n = 632, p = 0.025). We could not detect any differences in influenza A virus prevalence between male and female mallards in either of the 2 comparisons in which the sample sizes permitted statistical testing (juveniles fall: χ^2_1 = 3.16, n = 1,944, p = 0.076; adults fall χ^2_1 = 0.00, n = 468, p = not significant).

Subtype Overview

Of 213 mallard samples positive by RT-PCR during the first 2 years of sampling, 129 could be isolated by egg

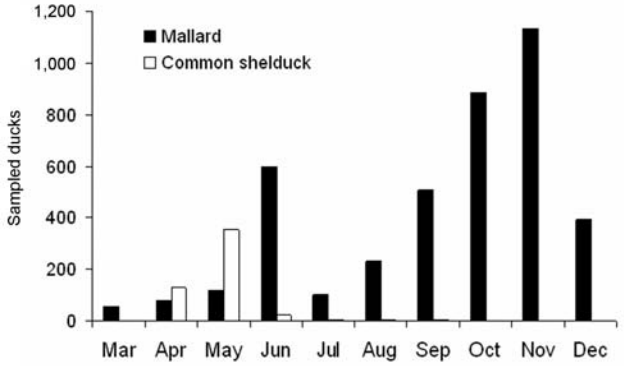

Figure 2. Seasonal variation in the number of sampled mallards and common shelducks. Data from 2002–2005 have been pooled.

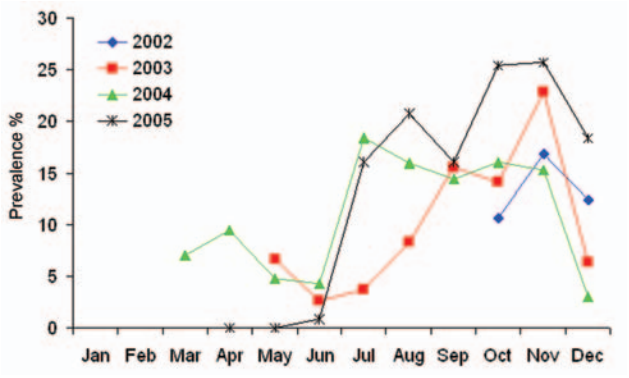

Figure 3. Seasonal influenza A virus prevalence in mallards (n = 4,106) in the 4 study years. Data from months represented by ≤5 samples are not included.

culturing. During this period, 11 different HA subtypes and all 9 NA subtypes were found in 40 different subtype combinations. Isolates for 39 of the subtype combinations came from mallards (Table 3). An additional subtype, H3N3, was found in a sample from a Eurasian teal among the 6 isolates obtained from Eurasian teal and common shelducks. All H5 and H7 virus strains were characterized as low pathogenic. The most prevalent combinations were H4N6 (14.7%), H7N7 (12.4%), and H6N2 (9.3%) (Table 3). While most subtype combinations were isolated only during short periods, H4N6 and H2N3 were isolated during longer periods (3 months) (Figure 6).

Discussion

Our large dataset came mainly from mallards, a species known to play a central role in the perpetuation of influenza A virus in nature (*13*). Because the greatest numbers of ducks were caught in fall, juvenile birds predominated in the sample. This finding reflects the age structure of the mallard population at that time, when juvenile birds making their first migration typically outnumber adults. In spring, the ratio of young ducks to older ones is smaller because juveniles experience higher mortality over winter (*14*).

Our banding analysis confirms that mallards migrate from breeding areas in northwestern Russia and Fennoscandia to wintering areas in northwestern Europe. Mallards from breeding areas close to the Baltic Sea start migration to their wintering areas in August–September (Figure 2), while the population in eastern Finland and Russia starts to appear in southern Sweden beginning in October. The wintering area of the latter mallards was, on average, more to the southwest than that of conspecifics breeding in southern Sweden, thus showing a leapfrog migration pattern (Figure 1A and 1B). The distance between breeding and wintering grounds of the northeastern populations is >3× longer than that of the more "resident" population in southern Scandinavia. The chance of receiving reports of banded birds found in Finland has probably been much higher than in Russia because of lower human population density in parts of western Siberia. Thus, a larger proportion of the mallards that pass southern Sweden during October–December might come from breeding areas in western Russia than is actually indicated by the recovery distribution.

Influenza A virus was present in a significant proportion of migrating mallards, in both fall and spring. The prevalence in all mallard samples during the entire 4-year study was 14.0%. The total prevalence of influenza A virus in our study showed consistent patterns across years and seasons (Figures 3 and 4); up to 25.7% of the ducks were infected in fall compared with up to 9.5% in spring.

We compared our results to those of multiyear studies from North America (*2*) and Germany (*15*). However, because so few multiyear studies have been made, all 3 may merely show some of the variation that can be found everywhere, rather than differences between Europe and America. Results from recent studies conducted in other parts of North America support this suggestion (*16,17*).

Overall prevalence in our study was similar to that found in the 2 studies mentioned above. However, important differences also occurred. In North America, mallards had the highest prevalence of influenza A virus (10%–60%) in August and September (*1*); this rate dropped sharply in subsequent months to <1% in winter and spring (*18*). In our study, influenza A virus was detected from August to December with peaks in October–November (3.0%–25.7%) and with comparatively high prevalence in most spring months (range 0–9.5%).

Different theories are offered to explain how the various subtypes can be perpetuated in North America, despite a low prevalence in spring. For example, influenza A virus

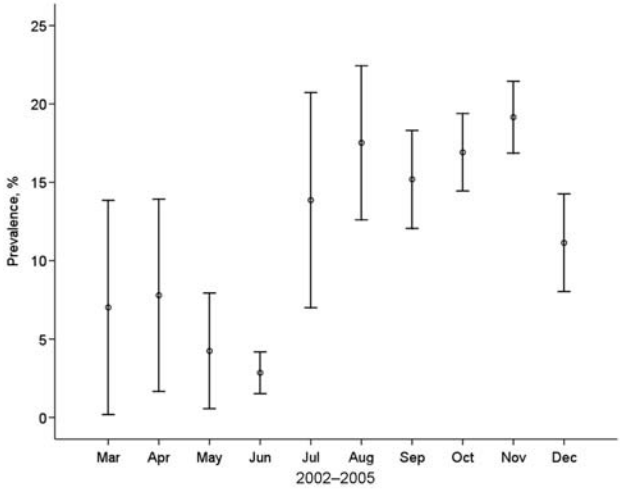

Figure 4. Monthly average influenza A virus prevalence in mallards (n = 4,106), 2002–2005, with bars indicating the standard error. Data from months represented by ≤5 samples are not included.

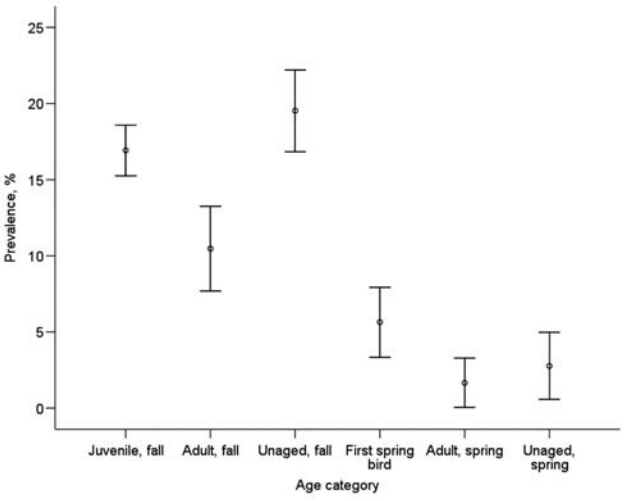

Figure 5. Mean influenza A virus prevalence in the 4 age classes. Birds that we were unable to age correctly were denoted as unaged. Bars indicate standard error.

is suggested to survive in frozen lakes and to reinfect birds when they return in spring to breed (*19*). Alternatively, influenza A virus might be carried by other bird species during the months in which the prevalence in ducks is low. In North America, shorebirds in the Delaware Bay area had a 14.2% prevalence of influenza A virus during spring migration and could thus bring influenza A virus back to the ducks' breeding areas (*2*,*20*).

Our study shows that, contrary to the findings in the North American study (*2*), influenza A virus in migrating dabbling ducks might be perpetuated by the ducks themselves. This view is based on our findings of influenza A virus prevalence of up to 9.5% in some spring months and is also supported by the 8% influenza A virus prevalence at breeding grounds in eastern Siberia (*21*) and a 4.1% influenza A virus prevalence among wintering mallards in Italy (*22*).

In our study, only mallards were caught in substantial numbers in both spring and fall. Thus, our conclusions about seasonal patterns are limited to this species. However, data from the less numerous ducks showed similar frequencies of influenza A virus prevalence.

In mallards, the higher prevalence in juvenile ducks suggests that they are more prone to be infected with influenza A virus. This may reflect their immunologic status and indicates a key role for juveniles in the perpetuation of influenza A virus, as suggested by Hinshaw et al. (*23*). This age difference remained in spring, when adult birds had significantly lower infection rates than second-year birds.

We found all 9 NA types and 11 of 16 recognized subtypes of HA. These were isolated in 40 different combinations (Table 3). Such diversity of influenza A virus subtypes is similar to that found in other large studies of wild duck populations (*2*,*15*). In our study, the HA subtypes H4, H6, and H7 were the most common, followed by H1-H3, H5, H10, and H11. The H8 and H12 subtypes were rarely isolated, and subtypes H9 and H13–16 were never isolated.

The subtype distribution in our study shows both similarities and differences to that found in studies in Germany and North America (*2*,*15*). The H4 subtype was common in all 3 studies, but while our study and the North American study share a high number of H6 isolations, the German study had higher numbers of H1 and H2 (Table 4). The fact that some subtypes such as H13 and H16 were not

Table 3. Influenza A virus subtype combinations in mallards sampled at Ottenby Bird Observatory, 2002–2004

	Neuraminidase									
Hemagglutinin	1	2	3	4	5	6	7	8	9	Total
1	5	2				1				8
2	2		7		1					10
3		1				1		5		7
4		3				19				22
5		5	3			1			3	12
6	2	12			1	1		3		19
7							16		1	17
8				4						4
9										0
10		2		1	1	2	2	2	3	13
11	1	1	2		1	1		1	7	14
12					2				1	3
13										0
14										0
15										0
16										0
Total	10	26	12	5	6	26	18	11	15	129

Table 4. Comparison of multiyear influenza A virus screening studies of ducks in North America (*2*), Germany (*16*), and Sweden (present study)*

Study region	Sweden	Germany	North America
Prevalence during fall	15.0% (in mallards)	8.7%	22.2%
Prevalence during spring	4.0% (in mallards)	No data	0.03%
Most prevalent HA subtypes	H4, H6, H7	H4, H2, H1, H6, H7	H6, H3, H4
HA subtypes not found	H9, H13–16	H5, H12–16	H13–16
Most prevalent NA subtypes	N2, N6, N7	N1, N3, N6	N8, N2, N6
NA subtypes found	N1–9	N1–9	N1–9
Most prevalent subtype combinations	H4N6, H7N7, H6N2	H2N3, H4N6, H1N1, H6N2, H7N7	H3N8, H6N2, H4N6

*HA, hemagglutinin; NA, neuraminidase.

found in our survey or in the other surveys may indicate a difference in host preference for these subtypes, as suggested by other studies (*7*,*20*). A high prevalence of influenza A virus of the H7 subtype was found in the German study as well as in ours, but ours had a higher prevalence of the H5 subtype.

In our study, NA subtypes N2, N6, and N7 dominated, while N4 and N5 were uncommon. As in the German data, N1 and N3 were less prevalent and N2 and N7 more prevalent. In North America, N2, N6, and N8 were the most frequent; N7 was rarely isolated (Table 4).

Although we only present subtype data from 2 complete sampling years, we detected just as many subtype combinations as found in the 12-year German study (*15*). The most prevalent subtype combinations we found, H4N6, H7N7, and H6N2, were also found in the German study. Similarly, both the H4N6 and the H6N2 subtype combinations were among the most common in the North American survey (*2*). The H4N6 subtype stands out as being prevalent in ducks worldwide and across years (Table 4). The H7N7 combination was common in both the German survey and in ours. However, this combination was never isolated in the North American survey, although it has been isolated in another North American study (*16*).

Most subtype combinations we found in fall were short-lived or varied in prevalence over time. Only the H2N3 and H4N6 subtype combinations in the fall of 2002 (Figure 6) showed a constant occurrence. These data, combined with those about mallard migration patterns (Figure 1A and 1B), suggest that different duck populations arriving from different breeding areas may bring different subtype combinations with them. The subtype combinations found late in the fall, for example, were probably brought in by mallards that arrived from breeding areas farther east.

In late fall 2002, we observed a sharp increase in the number of migrating mallards that carried H7N7 (Figure 6). If one assumes that these birds followed their normal migration route after leaving Ottenby (Figure 1A and 1B), they would be on their wintering grounds in western Europe a few months later. At that time, in February 2003, a large H7N7 epizootic in poultry began in the Netherlands. This epizootic also caused human illness, including 1 fatal case (*24*). Retrospectively, we found that the H7N7 samples from our Ottenby ducks were closely related phylogenetically to the H7N7 that caused the outbreak (*6*). Our data suggest that an increase in the incidence of H7 or H5 viruses among wild birds might signal an increased likelihood for transfer to poultry and that bird observatories such as those at Ottenby could play an important role as early warning systems.

Science has barely scratched the surface of the ecologic–virologic–epidemiologic interface of influenza A virus. Further research needs to focus on how the influenza A virus affects individual fitness, vital rates, and population structure in wild ducks, for both low as well as for highly pathogenic strains.

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Dr Wallensten is a physician in Kalmar County, Sweden, and conducts research at Kalmar University in affiliation with the medical faculty at Linköping University. His research interests include the epidemiology, control, and prevention of zoonotic diseases.

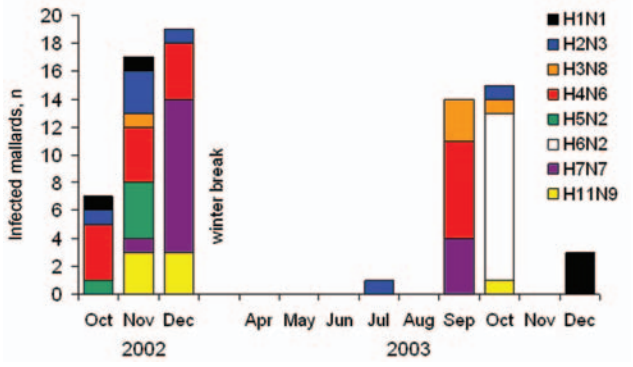

Figure 6. Occurrences of the most common influenza A virus subtype combinations (≥5 isolates) in mallards over time.

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Risk for Infection with Highly Pathogenic Influenza A Virus (H5N1) in Chickens, Hong Kong, 2002

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We used epidemiologic evaluation, molecular epidemiology, and a case-control study to identify possible risk factors for the spread of highly pathogenic avian influenza A virus (subtype H5N1) in chicken farms during the first quarter of 2002 in Hong Kong. Farm profiles, including stock sources, farm management, and biosecurity measures, were collected from 16 case and 46 control chicken farms by using a pretested questionnaire and personal interviews. The risk for influenza A (H5N1) infection was assessed by using adjusted odds ratios based on multivariate logistic regression analysis. Retail marketing of live poultry was implicated as the main source of exposure to infection on chicken farms in Hong Kong during this period. Infection control measures should be reviewed and upgraded as necessary to reduce the spread of influenza A (H5N1) related to live poultry markets, which are commonplace across Asia.

The spread of highly pathogenic avian influenza (HPAI) type A virus (subtype H5N1) infection in poultry in Asia and beyond poses threats to both human and animal health. Attempts to control outbreaks of this disease in poultry have become a regional and global priority (*1*,*2*). However, little reliable epidemiologic data exist on routes of virus transmission and perpetuation in poultry within affected countries.

Repeated outbreaks of HPAI (H5N1) outbreaks in poultry occurred in farms and live poultry markets within the Hong Kong Special Administrative Region beginning in 1997 (*3*–*6*). The first outbreak was associated with the first recorded transmission of influenza A (H5N1) to humans, with 18 cases and 6 deaths (*7*,*8*). These outbreaks were controlled by slaughtering all poultry in all markets and local farms and stopping all trading of live poultry for 7 weeks. No additional human cases were reported after these interventions, and this particular genotype of influenza A (H5N1) virus has not been detected since (*4*).

No further outbreaks of influenza A (H5N1) in poultry were recorded until 2001, when the virus was detected in live poultry retail markets in Hong Kong. Poultry farms were unaffected. This outbreak led to a slaughter of poultry in live poultry markets in Hong Kong. However, in January 2002, influenza A (H5N1) was again detected in Hong Kong wholesale and retail poultry markets (*3*,*9*). Trace-back from the wholesale poultry market led to detection of the virus on February 1, 2002, on a chicken farm in a densely populated chicken farming area of the New Territories area of Hong Kong. By late March, 17 chicken farms located within 2 km of the index farm, and 4 farms located within 2 to 5 km, were confirmed as infected (*5*,*6*). This outbreak was controlled by a combination of depopulation of infected and contact farms, quarantine and enhanced biosecurity, and vaccination (*10*).

We report the results of an epidemiologic investigation of the 2002 outbreak, including a case-control study to

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identify risk factors associated with poultry infection in farms. These findings may provide insight into the mechanisms of the spread of HPAI (H5N1) in Asia.

Materials and Methods

Study Population

At the time of the 2002 outbreak, 146 commercial chicken farms in Hong Kong were operating, with a combined holding capacity of ≈3 million birds. These farms supplied ≈20% of the poultry consumed within Hong Kong, with the remaining ≈80% imported from farms in the nearby southern provinces of China. Land-based poultry shipments from both Hong Kong and China were usually delivered to 1 wholesale poultry market and then resold to individual live poultry retail markets. Ducks and geese that were reared on poultry farms in China and imported by boat were delivered daily to a geographically separate wholesale market for slaughter and sale as chilled poultry in the live poultry markets.

A cultural preference in Asian countries for freshly killed poultry has resulted in a large volume of sales through live poultry markets, with ≈850 retail poultry market stalls in operation across Hong Kong. Several process changes were introduced in 1998 to reduce the risk of reintroducing the virus into the live market system. Plastic poultry cages were introduced for transporting land-based poultry, and cages that contained poultry from local poultry farms or from China were washed in the wholesale market and then returned to the place of origin. At the wholesale markets, poultry were sorted according to weight and transferred to other washed plastic cages and then transported to the retail poultry markets the next day. Direct sale of poultry from farms to retail markets was discouraged but continued to occur on a limited basis. Details of the live poultry marketing system and compliance requirements imposed by Hong Kong authorities are described separately (N.Y. Kung et al., unpub. data).

After 1997, the Hong Kong government set up a Farm Hygiene section under the Agriculture, Fishery, and Conservation Department for local poultry farm surveillance. This entailed monthly testing for avian influenza and Newcastle disease viruses, testing for serologic evidence of influenza A (H5), and on-farm monitoring of disease and production. Discovery of influenza A (H5N1) in retail poultry markets triggered trace-back, which identified clinically affected farms and led to intensive on-farm investigations that identified more infected farms.

During the 2002 outbreak, clinical disease and influenza A (H5N1) isolations occurred on 22 of the 146 active chicken farms in Hong Kong. For our study, case farms were defined as farms that had high death rates caused by influenza A (H5N1) infection or farms where influenza A (H5N1) was isolated from chickens during the outbreak. Each unaffected farm (n = 124) was assigned a unique identification number, and 46 were selected by using numbers generated with a random number generator in Microsoft (Redmond, WA, USA) Excel for Windows.

Data Collection

Data were obtained from the 46 unaffected farms and 16 of the 22 case farms. Representatives (farmers or farm managers) from 6 case farms and 3 of the selected control farms were either unavailable or declined to participate in the study. Subsequently, 3 additional unaffected farms were selected by using the random number method to yield the final total of 46 control farms.

Most chickens raised in Hong Kong were sold through 1 wholesale market and distributed from there to retail live poultry markets located throughout Hong Kong. However, some farms also had direct arrangements with retail market stall holders. These chicken farms were concentrated in several areas in the New Territories: Kam Tin, Pak Sha, Ha Tsuen, San Tin, Ngau Tam Mei, and Ta Kwu Ling. The affected farms were clustered in 3 areas: Kam Tin (n = 17), Hung Shui Kiu (n = 1), and Pak Sha (n = 4). Control farms were located in different areas of the New Territories; however, none were in Kam Tin because all the chickens in this region were quarantined and then slaughtered during the January–February 2002 outbreak.

Survey Methods

We pretested our questionnaire (available from the principal author on request) on 5 chicken farms, then conducted all study interviews during March 2002. The online Appendix Table (available from www.cdc.gov/EID/content/13/3/412-appT.htm) shows the list of potential risk factors surveyed by the questionnaire. Data on geographic location, farm characteristics, stock information, flock health history, farm biosecurity, farm management, and marketing practices were collected by trained interviewers during farm visits. Additional information such as farm area, number of sheds, and incoming day-old chick numbers were obtained from official records held by the Department of Agriculture, Fisheries, and Conservation and used to validate the information collected during on-farm interviews. The questionnaire contained 62 closed and 26 open-ended questions. Of the 88 questions, 77 offered single value and 11 offered multiple value answers.

Virus Isolation, Subtyping, and Genotyping

Cloacal swab samples were collected from both dead and apparently healthy chickens on farms and tested by using standard procedures for virus isolation (*3*). All influenza virus isolates were subtyped by hemagglutination and neuraminidase inhibition tests by using specific

antiserum. Results were confirmed with reverse transcription–PCR specific for influenza A (H5N1). Genetic sequencing and phylogenetic analysis were completed on selected virus isolates (*9*).

Spatial Analysis

Global positioning system coordinates for all chicken farms in Hong Kong were obtained and entered into a digitized map (Land Information Centre, Survey and Mapping Office, Land Department, Government of Hong Kong) by using a geographic information system program (ArcView 3.1, Environmental Systems Research Institute, Redlands, CA, USA). Coordinates were converted where necessary from latitude and longitude form to map grid on a Hong Kong 80-data format (Survey and Mapping Office, Land Department, Government of Hong Kong) (*11*) to allow for digital mapping and calculation of distances (Figure).

Statistical Procedures

We entered data into a customized database (Microsoft Access 2000) and then transferred it into a statistical package for analysis (SPSS for Windows version 11.0, SPSS Inc., Chicago, IL, USA). We then used descriptive statistics to calculate distributions of all variables by case and control status. We conducted univariate analyses to test for associations between disease status and each explanatory variable by using *t* tests for continuous variables and χ^2 tests for categorical variables. Where appropriate, we categorized responses before analysis, with categories selected on the basis of the distribution of responses for that variable.

Variables from the univariate analyses with a p value <0.25 were retained for consideration in a multivariate statistical model. Multivariate logistic regression analysis was then used to assess associations between independent variables and the outcome of interest (case or control status), while controlling for other possible risk factors. We constructed the final model by using both forward and backward stepwise procedures. We also used an adapted "best subsets" approach, by which variables of particular interest were forced into the initial equation and the influence of key variables was tested by using the fit of various possible equations. We then assessed model fit by using the Hosmer-Lemeshow goodness-of-fit test (*12*) and the ratio of the deviance to the degrees of freedom. Adjusted odds ratios (ORs) and their 95% confidence intervals were calculated. In all tests, a p value <0.05 was considered significant.

Results

Temporal and Spatial Pattern of Genotypes

The Figure shows the locations of the 22 infected farms (16 case-control study and 6 nonparticipant farms), 46 control farms, and 78 other unaffected farms. Three different genotypes of influenza A (H5N1) were identified: 13 case farms were infected with genotype Z, 8 with genotype Y, and 1 with genotype X (Table 1). The spatial pattern showed strong clustering of genotypes Z and Y, with some outliers. The 1 farm infected with genotype X was physically separate from the other 2 clusters. At the time of the outbreak, genotypes Y, Z, and X were isolated from poultry farms, while genotypes Z, B, X_0, X_1, X_2, and X_3 were detected in live poultry markets. Genotype Y was found only on chicken farms (*9*,*13*).

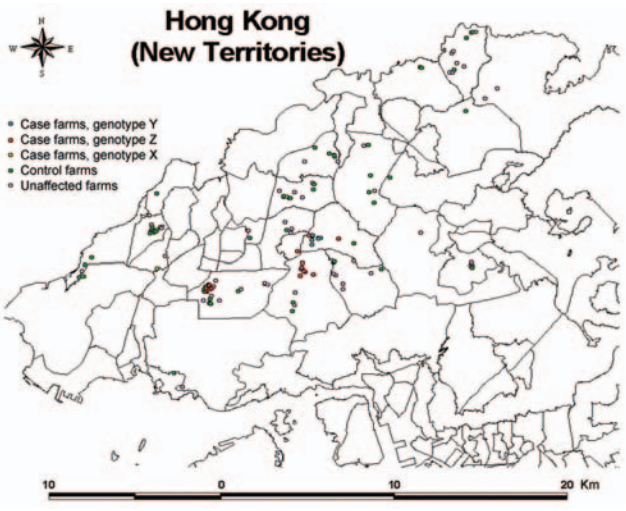

Figure. Map of Hong Kong showing the locations of the 22 infected farms (16 case-control study and 6 nonparticipant farms), 46 control farms, and 78 other unaffected farms active during the 2002 outbreak of highly pathogenic avian influenza A virus (subtype H5N1).

Risk Factors for Infection of Farms

Univariate Analysis

Statistical comparisons were not done for 9 of the variables from the questionnaire because of either uniformity of response across all farms or excessive missing data. Summary information for farm area, stock numbers, and shed numbers on each farm are presented in Table 2. We performed χ^2 tests of association on 60 variables in the univariate analysis. Table 3 shows the variables that were associated with a p value <0.25 in the univariate analysis. Affected farms were concentrated in a small number of districts compared with controls, which were more widely distributed across districts (OR 123.0, p<0.01).

Other factors positively associated with case farms: number of chickens on farm; stock density; death rate higher for birds >30 days of age than for younger birds (OR 7.40, p = 0.02); survival rate at 1–30 days of age (OR 1.54, p<0.01); medication use during January–February

Table 1. Date of identification of avian influenza type A virus (H5N1) infection, farm location, and genotypes for all infected farms, Hong Kong, 2002

Farm ID	Date	District	Genotype
1	1 Feb	Kam Tin	Z
2*	4 Feb	Kam Tin	Z
3	4 Feb	Hung Shui Kiu	X
4	4 Feb	Kam Tin	Y (*4*)
5	4 Feb	Kam Tin	Z
6	6 Feb	Kam Tin	Y
7	6 Feb	Kam Tin	Y
8*	6 Feb	Kam Tin	Y
9	8 Feb	Kam Tin	Z
10	8 Feb	Kam Tin	Z
11	8 Feb	Kam Tin	Z
12	8 Feb	Kam Tin	Z
13	8 Feb	Kam Tin	Z
14	9 Feb	Kam Tin	Y (*4*)
15*	9 Feb	Kam Tin	Y (*4*)
16	15 Feb	Kam Tin	Y
17	16 Feb	Kam Tin	Y
18*	17 Feb	Kam Tin	Z
19	20 Feb	Pak Sha	Z
20	02 Mar	Pak Sha	Z
21*	15 Mar	Pak Sha	Z
22*	18 Mar	Pak Sha	Z

*Infected farms not included in this study.

2002 (OR 4.67, p = 0.02); whether chickens were sold directly to retail markets (OR 11.15, p<0.01); whether automatic manure scrapers were installed (OR 4.55, p = 0.02); whether persons from retail markets visited during January–February 2002 (OR 10.00, p = 0.01); and whether a visitor went inside the shed during this period (OR 3.94, p = 0.04). Factors that had ORs significantly <1.0 for case farms were reports of wild birds eating in the chicken feed trough (OR 0.20, p = 0.04), farm owner living on farm (OR 0.05, p<0.01), and visitors from another chicken farm during January–February 2002 (OR 0.23, p = 0.02).

Multivariate Analysis

Three alternative final models were identified from the model-building procedures, each containing variables that had significant p values (Table 4). Three variables appeared in all models: owner lives off farm, age group with highest death rate at >30 days old, and sale of chickens direct to retail markets. Each model had 1 additional variable, which was different for the 3 models; wild birds in feed trough (protective, model A), number of chickens on farm (model B), and relative working in poultry industry (model C). On the basis of the Hosmer-Lemeshow statistic, model A provided the best fit to the data, while by the adjusted R^2 statistic, model B had the highest explanatory value. In this model, farms with a nonresident owner were 12.8× more likely to be a case farm; farms that sold chicken directly to retail markets were 30.3× more likely to be a case farm; farms with highest death rate in birds >30 days old were 20.5× more likely to be a case farm; and farms with higher chicken numbers were 1.1× more likely to be a case farm.

Residual components from all 3 models showed 1 farm (Farm ID 19) with a large standardized residual. This was a farm in Pak Sha area where influenza A (H5N1) was isolated on February 20, 2002, but the model predicted it would be a control farm. Farm 19 imported day-old chicks from China during mid-February and sold some chicks 10 days later to another nearby farm (case farm, Farm ID 20). Infection may have entered this farm directly with imported birds; therefore, it did not share risk factors with the other case farms.

Discussion

We describe the use of a combination of descriptive and analytic epidemiologic techniques to investigate possible risk factors associated with the 2002 influenza A (H5N1) outbreak in Hong Kong. The small sample size limited the number of risk factors we could combine in a multivariable model and prevented consideration of interaction terms. Models containing ≥4 variables and models containing interaction terms either did not converge or showed evidence of multicollinearity. Inspection of counts for combinations of explanatory variables indicated that these occurrences were likely to be the result of zero counts. Because the study included only 16 case farms and related directly to transmission processes that exposed these specific farms, our inferences apply only to the specific circumstances of this outbreak, and caution should be used in applying these findings to other situations.

Comparison of the clusters of case farms with the spatial distribution of randomly selected control farms indicated strongly that locally operating contagious risk factors had a strong influence on which farms become infected. That is, either the virus was spread between farms

Table 2. Descriptive analysis of farm area, standing population of chickens, and number of sheds of chicken farms included in survey, Hong Kong, 2002*

	Case (n = 16)					Control (n = 46)					
	Mean	Median	Max	Min	SD	Mean	Median	Max	Min	SD	p value
Farm area (m^2)	4,008	1,700	18,600	900	4,684	3,275	1,975	28,350	207	4,331	0.86
Chicken count (×1,000)	41.3	27.5	101.4	5.6	33.3	16.2	16.0	51	3.5	10.2	<0.001
Shed no.	7.4	5.5	20	2	5.9	8.0	7.5	19	1	4.4	0.65

*Max, maximum; Min, minimum; SD, standard deviation.

Table 3. Results of univariate analysis of risk factors for avian influenza type A virus (H5N1) infection among chicken farms, Hong Kong, 2002*†

Variable	Category	Case	Control	OR	95% CI	p value
Stock	No. chickens on the farm	NA	NA	NA	NA	<0.001
	Stock density (chicken no./farm area)	NA	NA	NA	NA	0.007
Age group with highest death rate	Most deaths in birds ≥30 d old	5	3	7.40	1.49–36.82	0.017
	Most deaths in birds <30 d old	9	40	1		
Survival rate	≥90% at 1–30 d	15	28	1.54	1.23–1.91	0.003
	<90% at 1–30 d	0	18	1		
	≥90% at 30–60 d	11	42	0.26	0.06–1.22	0.093
	<90% at 30–60 d	4	4	1		
	≥90% at >60 d	13	44	0.20	0.03–1.31	0.103
	<90% at >60 d	3	2	1		
Does the farm sell chickens directly to a retail market?	Yes	7	3	11.15	2.41–51.56	0.002
	No	9	43	1		
Farm owner lives on farm	Yes	7	43	0.05	0.01–0.25	<0.001
	No	9	3	1		
Visitors from another chicken farm?	Yes	2	0	0.23	0.15–0.37	0.015
	No	14	46	1		
Visitors from retail markets?	Yes	5	2	10.00	1.71–58.59	0.010
	No	11	44	1		

*OR, odds ratio; CI, confidence interval; NA, not applicable.
†A longer version of this table is available from **http://www.cdc.gov/EID/content/13/3/412-T3.htm**

or nearby farms were exposed to a common source of infection.

Many of the commercial chicken farms in Hong Kong operate as small, family-run businesses and are located in close proximity to each other. Some larger scale commercial chicken farms (concentrated in the Kam Tin area) operate with modern facilities such as automatic manure scrapers, drink dispensers, and feeders. On all of these farms except 1, chickens are raised in 3- to 4-tier cages located in open-sided sheds with fan-assisted ventilation. These farms also use a "continuous flow of stock" operation, which means they contain different age groups of chickens at any 1 time.

Only 1 chicken farm in Hong Kong at the time of the 2002 outbreak operated on an "all-in, all-out" basis, with ≈9,000 chickens at same age group raised in 2 levels of open (not individual), net wire–fenced area. Consequently, this farm had less contact with markets and enhanced biosecurity compared with other case farms. A notable point is that this farm was the only case farm located outside the main affected district and the only farm where the X genotype was isolated during the 2002 outbreak. Joint interpretation of the epidemiologic investigation findings and gene sequence results shows that the disease apparently entered a small number of chicken farms as a single transmission event and then either was controlled on that farm (the geographically isolated farm affected by genotype X) or spread laterally to farms that shared local exposure factors (farms clustered in certain areas affected by genotypes Y and Z).

Visits to a farm by ≥1 persons from retail markets was a strong risk factor for infection. This supports the hypothesis that infection began in the retail markets, where locally produced and imported poultry were mixed and kept for several days (*14*). A US study showed that avian influenza virus (subtype H5N2) amplified in the retail poultry mar-

Table 4. Comparison of different multivariate models of risk factors for avian influenza type A virus (H5N1) infection among chicken farms, Hong Kong, 2002*

		OR (95% CI)		
Variable	Category	Model A	Model B	Model C
1	Owner lives off farm	37.04 (3.18–431.63)	12.64 (1.18–135.35)	45.84 (3.65–575.69)
2	Sell to retail markets	20.11 (1.47–274.98)	30.26 (2.26–405.09)	28.39 (2.30–350.40)
3	Highest death rate >30 d	17.37 (1.03–292.01)	20.51 (1.51–277.96)	24.28 (1.62–364.87)
4	Wild birds in feed trough	0.07 (0.01–0.85)		
5	Chicken count		1.07 (1.01–1.12)	
6	Relative in poultry industry			19.41 (1.46–257.74)
Cox and Snell R^2		0.42	0.46	0.43
Nagelkerke R^2		0.63	0.68	0.65
Significance of Hosmer and Lemeshow test		0.91	0.67	0.82
Degrees of freedom		3	8	3

*OR, odds ratio; CI, confidence interval.

ket setting (*15*). Research in Hong Kong has shown that that "rest days," when markets are emptied of all poultry and cleaned, can interrupt virus perpetuation (*16,17*). Therefore, influenza A (H5N1) introduced into poultry markets in 2002 likely was amplified within them and transmitted back to a few index farms, initiating each genotype-specific outbreak. Each genotype then spread to other farms (Y, Z) or remained limited to the index farm (X), depending on the proximity and operation design of the farm. The virus may also have been carried among farms by retail poultry market personnel who visited multiple farms.

Factors that require particular attention in risk management include movement of humans (e.g., buyers, bird catchers) and inanimate objects (e.g., cages, trucks) between retail markets and farms, or among multiple farms, because these movements may carry virus in ways that expose birds to an infectious dose. Airborne spread from affected birds, either while infection was spreading within a flock or during slaughter of a flock, may explain a small number of cases (especially those associated with the Y genotype), but most secondary cases appeared to be due to transfer of virus between farms in ways that could be prevented with enhanced biosecurity.

In addition, influenza A (H5N1) has been isolated from terrestrial birds (*13,18,19*), which raised the concern that local resident wild birds could introduce virus into a flock. However, although the presence of wild birds in the vicinity of the chicken farms was considered a possible risk factor for introducing avian influenza, it was not significant in this analysis. In fact, wild birds being observed in feed troughs was a protective factor for infection cases in both univariate and multivariate analyses. This information should be interpreted with caution, however, because the operators of case farms underwent questioning by government field officers after the farm was identified as infected and thus may have been more aware of the possibility of transmission of avian influenza from wild birds. This may have decreased the frequency with which case farms reported of the presence of wild birds in feed troughs in case farms compared with control farms.

The death rate in chickens >30 days old was higher on case farms than on control farms, which is to be expected because avian influenza kills chickens of all ages and will increase the death rate in older age groups. In addition, chickens in this age group were more likely to be visited by the stock agents, catchers, or farmers before being sent to the markets. Notably, all 3 models showed that the owner living off the farm was a significant risk factor. These farms may have outside visitors, or the owners may be more likely to employ nonfamily workers, and this increased activity increases the likelihood that the virus will be brought onto the farm. Owners who live on the farm may also be more attentive to implementation of protective measures.

The evidence from this study points toward influenza A (H5N1) moving from retail markets to farms for each of the genotype-specific outbreaks. Genotype Y was not isolated from retail poultry market samples at the time of the outbreaks, likely because of the relatively small numbers of live poultry markets that were under routine virologic surveillance at that time. However, we cannot rule out the possibility of an alternative route of introduction of this genotype into the farms, e.g., through wild birds or smuggled poultry.

Enhancement of farm biosecurity would be a useful measure to reduce entry of virus onto farms and interfarm spread. Good farm management and strict biosecurity measures are beneficial actions available to prevent entry of infection to farms and transmission between sheds within farms (e.g., only allowing authorized persons to enter the farm, providing a change of clothes and footwear for all visitors, requiring a stand-down period for anyone who had been in retail poultry markets, ensuring strict control of equipment and transport vehicles entering farms). The role of live poultry markets in the amplification and dissemination of influenza viruses is likely to be related to the maintenance of HPAI (H5N1) across Asia, where such live poultry markets serve the demand for the consumption of freshly killed poultry. One way of reducing the risks associated with live poultry marketing is to reduce the levels of virus circulating in these markets, which has been achieved in Hong Kong through a combination of compulsory vaccination and strict biosecurity measures on poultry farms.

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Behavioral Risks for West Nile Virus Disease, Northern Colorado, 2003

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In 2003, residents in 2 adjacent cities in northern Colorado (Loveland and Fort Collins) had severe outbreaks of human West Nile virus (WNV) disease. Unexpectedly, age-adjusted neuroinvasive disease rates were higher in Loveland (38.6 vs. 15.9 per 100,000), which had a more extensive mosquito control program and fewer mosquitoes. A survey was conducted to assess differences in personal protection and risk practices by each city's residents. During May and June 2004, a random-digit dial telephone survey was conducted among adults to assess personal protection behavioral practices used to prevent WNV infection during the 2003 outbreak. After we adjusted for identified risk factors, Loveland residents were 39% more likely to report seldom or never using N,N-diethyl-*m*-toluamide (DEET), and ≈30% were more likely to report being outdoors during prime mosquito-biting hours than Fort Collins residents. Personal protective practices may directly influence rates of WNV infection and remain important even when comprehensive community mosquito control measures are implemented.

In the United States, the mantra familiar to public health workers and residents living in West Nile virus (WNV)–affected areas is to practice the 4 Ds of prevention: 1) DEET (N,N-diethyl-*m*-toluamide): wear an insect repellent containing DEET; 2) dress: wear long sleeves and long pants; 3) drain: drain standing water around the home; and 4) dusk to dawn: limit time outdoors during this time. Although the 4 Ds have been used by many state and local health departments to promote personal prevention, the question remains, how well do these tactics work to prevent infection?

In 2003, ≈30% (2,947) of human WNV cases in the United States were reported in Colorado. Among infected residents, 63 died (*1*). WNV transmission was especially intense in northern Colorado, including Larimer County. Among county residents, 546 laboratory-confirmed cases of WNV disease, including 63 neuroinvasive disease cases and 9 deaths (*2*), occurred. Officials at the Larimer County Department of Health and Environment noted differences in age-adjusted rates of WNV neuroinvasive disease between the 2 largest cities in the county. WNV neuroinvasive disease rates were used because neuroinvasive cases are more likely to be captured in surveillance systems because of illness severity, which often requires hospitalization and prompt laboratory diagnosis, unlike the generally milder West Nile fever. Furthermore, WNV neuroinvasive disease cases are typically used to draw comparisons between geographic areas and assess rates over time (*3*,*4*). The city of Loveland had a much higher age-adjusted rate of neuroinvasive disease (38.6/100,000) than the city of Fort Collins (15.9/100,000); standardized risk ratio 2.43 (95% confidence interval [CI] 1.21–4.87, $p<0.01$). These findings were unexpected given the ecologic and demographic similarities of the 2 cities and a long-term comprehensive mosquito control program in Loveland.

Among the 265,489 Larimer County residents in July 2003, most lived in the cities of Fort Collins (125,461) and Loveland (55,905) (*5*). Although some limited ecologic differences exist (i.e., Loveland water surface area is ≈2.5× greater), Fort Collins and Loveland are largely similar. Demographically, both cities are ≈90% white with 9% reported Hispanic ethnicity, 8%–13% of the residents are ≥65 years of age, and the annual household estimated median income is ≈$45,000 per year (*5*).

Loveland and Fort Collins are both situated in a high plains ecologic zone. The dominant WNV vector mosquito species are *Culex tarsalis* and *Cx. pipiens* (*6*). During the 2003 outbreak, ≈20–40 mosquito traps were collected

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per city per week by using CO_2 baited Centers for Disease Control and Prevention (CDC) miniature light traps. Mosquitoes were collected by the Colorado Mosquito Control (Brighton, CO, USA) and the Division of Vector-Borne Infectious Diseases at CDC (Fort Collins, CO, USA). During the height of the outbreak, from July 26, 2003, to September 5, 2003, the mean ± standard deviation number of *Cx. tarsalis* and *Cx. pipiens* mosquitoes collected per trap night was higher in Fort Collins (*Cx tarsalis* 76 ± 62, *Cx. pipiens* 31.5 ± 13.2,) than in Loveland (*Cx. tarsalis* 43 ± SD 34, *Cx. pipiens* 7 ± 1) (Figure). On the basis of 7,037 mosquitoes tested (4,999 *Cx. tarsalis* and 2,038 *Cx. pipiens*), the WNV infection rates (estimated number of mosquitoes infected/1,000 tested) were approximately equivalent in the 2 cities during that period (*Cx tarsalis* 14.7 in Fort Collins, 12.8 in Loveland; *Cx. pipiens* 25.9 in Fort Collins, 21.2 in Loveland). A vector index was calculated to estimate the average number of WNV-infected mosquitoes collected per trap night (i.e., summation of the product of the average number *Culex* mosquitoes collected per trap night and the proportion infected for each species). More WNV-infected mosquitoes were present in Fort Collins than in Loveland (Figure) (CDC, unpub. data). This finding was consistent with mosquito control efforts occurring during that period; Loveland had an integrated mosquito control program in place since 1986, and Fort Collins reacted to the outbreak by implementing an emergency mosquito control program later in the outbreak (mid-August through early September).

Larimer County health officials encouraged residents to "Fight the Bite," specifically, to practice the 4 Ds of prevention. The difference in age-adjusted rates of WNV neuroinvasive disease between the 2 cities was unexpected because Fort Collins residents were exposed to a larger number of WNV-infected mosquitoes. To understand the differences in rates of neuroinvasive disease in the 2 cities, Larimer County health officials commissioned a survey to assess city residents' knowledge, attitudes, and beliefs about WNV and to measure reported personal protective practices during the 2003 WNV season. The purpose of this study was to increase our understanding of the role of individual preventive measures by assessing post hoc the behavioral practices among residents of Fort Collins and Loveland.

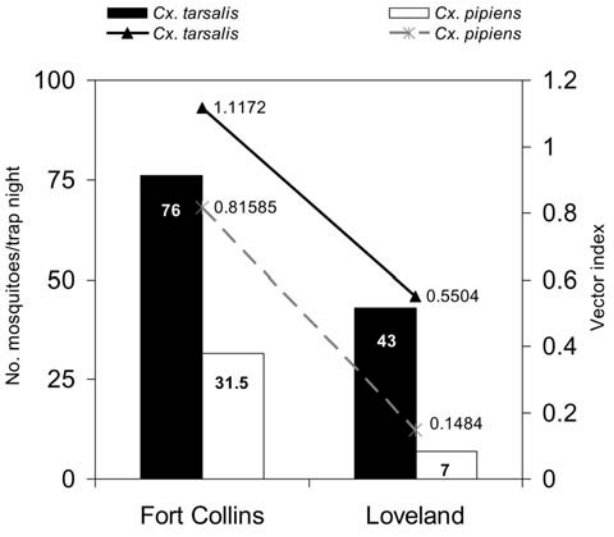

Figure. *Culex tarsalis* and *Cx. pipiens* density (average number collected per trap night) and vector index (reflecting the average number of infected mosquitoes collected per trap night), Fort Collins and Loveland, Colorado, July 26, 2003 through September 5, 2003.

Methods

From May 4 to June 7, 2004, the Survey Research Unit of the Colorado Department of Public Health and Environment in Denver performed a random-digit-dial telephone survey among residents of Fort Collins and Loveland. The survey was developed by the Larimer County Department of Health and Environment, CDC, and the Colorado Department of Public Health and Environment. The survey consisted of 42 questions; 27 (64.0%) questions were specifically related to WNV, and 15 (36.0%) questions concerned demographic information. Questions were derived from the 2003 Colorado Behavioral Risk Factor Surveillance System, local agency staff, and a survey conducted by the Mississippi Department of Health (*7*,*8*).

All households with telephones in Fort Collins and Loveland were eligible for inclusion. One adult ≥18 years of age who lived within the city limits of Fort Collins or Loveland from July through August 2003 was randomly selected from each household to participate. Each phone number in the sample was called ≤15 times, with at least 3 attempts in the evening, 3 during the day, and 3 on the weekend until the total number of desired completed interviews was obtained. Interviews were conducted in either English or Spanish. All interviews were completed by using Computer-Assisted Telephone Interviewing software (Sawtooth Technologies, Northbrook, IL, USA).

The exposure factor of interest was residence (Fort Collins vs. Loveland). Outcomes were based on self-reported WNV preventive practices during the 2003 outbreak. Five outcomes were used: DEET repellent use (DEET was the only insect repellent active ingredient recommended in 2003); draining standing water; dressing in long clothing (pants and long-sleeved shirts); minimizing hours outside from dusk to dawn on weekends; and minimizing hours outside from dusk to dawn on weekdays. Outcome variables were dichotomized as follows: DEET and dress (sometimes, nearly always, or always vs. seldom

or never); drain (yes vs. no); dusk to dawn on weekends (≤2 h vs. 3–40 h outside); and dusk to dawn on weekdays (≤5 h vs. 6–60 h outside).

Explanatory variables investigated included sex, age, ethnicity, income, education, owning an air-conditioner or swamp cooler, WNV risk perception, and awareness of a local mosquito control program. Variables were classified as follows: sex (male and female); age in years (18–44 and ≥45); ethnicity (non-Hispanic and Hispanic); income in US dollars (<$25,000, $25,000–50,000, and >$50,000); education (a high school diploma or less and college or higher); an air-conditioner or swamp cooler in home (yes and no); risk perception (very worried, somewhat worried, and not worried about getting sick from WNV); and awareness of a mosquito control program in city of residence (yes and no).

Analysis was conducted by using anonymized data. A Z test was used to identify statistically significant ($p<0.05$) differences in the proportion of residents reporting selected characteristics. Five unconditional logistic regression models were used to estimate the odds of each outcome among Fort Collins and Loveland residents while adjusting for identified risk factors. Models were built by using purposeful selection, a 5-step method for selecting variables based on both biologic importance and statistical significance. As outlined by Hosmer and Lemeshow (*9*), the 5 steps of purposeful selection are 1) test for univariate significance ($p<0.25$); 2) build the multivariate model ($p<0.05$); 3) test for confounding; 4) assess continuous variables for linearity; and, 5) test for effect modification and include interaction terms that are both significant ($p<0.05$) and biologically plausible. Model fit was determined by using the Hosmer–Lemeshow goodness-of-fit test (*9*). Because 5-point Likert scales can place persons in the middle category (*10*), final unconditional logistic regression models were retested to determine if similar results would be found between models in which the response "sometimes" was included in the opposite category (seldom and never). Statistical analyses were performed by using SAS version 9.1 software (SAS, Cary, NC, USA).

Results

Among the 3,739 county households identified, 1,230 were sampled. Of these, 256 (20%) refused sampling 11 (1.1%) terminated the interview before it was completed, 6 (0.9%) had a language barrier, and 957 (78%) were surveyed. Fort Collins residents included 424 (44.3%) of those interviewed. Survey demographics reflect the general populations of Loveland and Fort Collins (*5*).

Among those surveyed, significant ($p<0.05$) differences were found between Fort Collins and Loveland residents by sex, age group, income, education, and ownership of an air-conditioner or swamp cooler. Compared with Loveland survey participants, more residents of Fort Collins were female, younger (18–44 years of age), had a higher income (>$50,000), educated (more than a high school diploma), and reported no air-conditioner or swamp cooler in the home (Table 1).

Significant differences ($p<0.05$) between Fort Collins and Loveland residents were observed for those reporting DEET use and those spending time outdoors from dusk to dawn on both weekends and weekdays (Table 2). The proportion of persons who reported seldom or never using DEET was higher among Loveland residents than among Fort Collins residents. Likewise, a higher proportion of Loveland residents reported spending >2 h outdoors from dusk to dawn on weekends and spending >5 h outdoors from dusk to dawn on weekdays.

Five unconditional multivariate logistic regression models were built to test for an association between city of residence and reported WNV preventive behavior. The drain model was omitted after careful review of the survey question deemed it too vague for a meaningful interpretation. This was unfortunate because draining water from around a residence may reduce exposure to mosquito-breeding sites.

When we adjusted for sex, age, and risk perception, Loveland residents were 39% (95% confidence interval [CI] 1.04–1.76) more likely to report that they seldom or never used DEET than Fort Collins residents (Table 3). Among residents surveyed about DEET use, persons >45 years of age were 62% (95% CI 1.21–2.18) more likely to report seldom or never using DEET than younger respondents. Persons who were not worried about WNV were 4× (95% CI 2.90–7.51) more likely to report that they seldom or never used DEET than persons who sometimes, nearly always, or always worried about WNV. Similar results were obtained when the model was tested with the sometimes response included in the seldom or never group.

After we adjusted for identified risk factors, no statistically significant difference was observed between Fort Collins and Loveland residents who reported seldom or never wearing long clothes to protect against mosquitoes. However, persons who reported that they were not worried about getting sick from WNV were 2.5× (95% CI 1.25–5.28) more likely to report not wearing protective clothing. Similar results were obtained when the sometimes response was included in the seldom or never response group.

Participants were surveyed regarding amount of time spent outdoors from dusk to dawn during the week and on weekends. Compared with the Fort Collins residents, Loveland survey participants were 35% (95% CI 1.01–1.82) more likely to report spending >5 h outdoors during the week from dusk to dawn when adjustments

Table 1. Distribution of Fort Collins and Loveland residents by selected characteristic (potential risk factor), Larimer County West Nile Virus survey, Colorado, 2003

Characteristic	Fort Collins (n = 424) 44.3%, no. (%)	Loveland (n = 533) 55.7%, no. (%)	Total (N = 957) 100.0%, no. (%)
Sex			
Male	166 (39.1)	249 (46.7)	415 (43.4)*
Female	258 (60.9)	284 (53.3)	542 (56.6)*
Age group, y			
18–44	212 (50.0)	204 (38.3)	416 (43.5)*
≥45	212 (50.0)	325 (60.9)	537 (56.1)*
Unknown	0	4 (0.8)	4 (0.4)
Ethnicity			
Non-Hispanic	392 (92.5)	498 (93.4)	890 (93.0)
Hispanic	32 (7.5)	30 (5.7)	62 (6.4)
Unknown	0	5 (0.9)	5 (0.6)
Income, US $			
<25,000	72 (16.9)	106 (19.8)	178 (18.7)
25,000–50,000	121 (28.8)	159 (29.8)	280 (29.2)
>50,000	195 (45.9)	207 (38.8)	402 (42.0)*
Unknown	36 (8.4)	61 (11.6)	97 (10.1)
Education			
High school diploma or less	89 (21.0)	174 (32.8)	263 (27.5)*
College†	332 (78.3)	356 (66.7)	688 (71.9)*
Unknown	3 (0.7)	3 (0.5)	6 (0.6)
Air-conditioner, swamp cooler, or both, in home			
Yes	296 (69.8)	420 (78.8)	716 (74.8)*
No	128 (30.2)	113 (21.2)	241 (25.2)*
Risk perception			
Very worried	60 (14.1)	68 (12.8)	128 (13.4)
Somewhat worried	229 (54.0)	287 (53.8)	516 (53.9)
Not worried	134 (31.6)	177 (33.2)	311 (33.0)
Unknown	1 (0.2)	1 (0.2)	2 (0.2)
Knowledge of mosquito control program			
Yes	332 (78.3)	408 (76.5)	740 (77.3)
No and did not know	92 (21.7)	125 (23.5)	217 (22.7)

*Statistically significant differences by Z test ($p<0.05$)
†College: some college, college degree, some graduate school, or graduate degree.

were made for sex, age, education, and income. The model also held a statistically significant and plausible interaction term; males earning <$25,000 per year were more likely to report spending >5 h outside from dusk to dawn during the week.

Compared with Fort Collins residents, Loveland residents were 30% (95% CI 1.00–1.74) more likely to report spending >2 h outdoors from dusk to dawn on weekends when adjustments were made for sex, age, and risk perception. Similarly, persons who reported they were somewhat worried or not worried about getting sick from WNV were 68% and >2× as likely (95% CI 1.07–2.65 and 1.31–3.51), respectively, to report spending >2 h outdoors from dusk to dawn on weekends than persons very worried about getting sick from WNV.

Discussion

During the 1999 WNV outbreak on Staten Island, New York, a serosurvey conducted by Mostashari et al. found the highest seroprevalence of WNV among persons who spent >2 h outdoors from dusk to dawn; persons were even more likely to be seropositive if they reported never using a repellent containing DEET (*11*). In a recent national study, 40% of survey participants reported using a repellent containing DEET and draining standing water, 29% avoided perceived areas with mosquitoes, 28% avoided being outdoors from dusk to dawn, and 27% wore long sleeved-shirts and long pants to avoid bites (*12*).

The results of this study suggest that differences in WNV neuroinvasive disease rates may be due, in part, to lower use of repellents containing DEET and greater dusk-to-dawn outdoor exposure among Loveland residents. These findings support the benefit of promoting personal prevention approaches, particularly by using effective insect repellents and reducing exposure to mosquitoes during prime-biting hours.

An alternative explanation for the differences in neuroinvasive disease rates among Loveland and Fort Collins residents may be unexplained ecologic differences that influence the risk for infection. Loveland has a greater pro-

Table 2. Distribution of Fort Collins and Loveland residents by outcome, Larimer County West Nile Virus survey, Colorado, 2003

Outcome*	Fort Collins (n = 424) 44.3%, no (%)	Loveland (n = 533) 55.7%, no. (%)	Total (N = 957) 100.0%, no. (%)
DEET use			
Sometimes/nearly always/always	271 (63.9)	298 (55.9)	569 (59.5)†
Seldom/never	147 (34.7)	223 (41.8)	370 (38.6)†
Unknown	1 (1.4)	12 (2.3)	18 (1.9)
Drain water			
Yes	192 (45.3)	213 (39.9)	405 (42.3)
No	67 (15.8)	92 (17.2)	159 (16.6)
Not applicable	165 (38.9)	227 (42.8)	393 (41.0)
Unknown	0 (0.0)	1 (0.1)	1 (0.1)
Dress (long sleeves and pants)			
Sometimes/nearly always/always	100 (23.6)	141 (26.5)	241 (25.2)
Seldom/never	134 (31.6)	186 (34.9)	320 (33.4)
Unknown	190 (44.8)	206 (38.6)	396 (41.4)
Dusk to dawn, weekends			
≤2 h outside	191 (45.0)	200 (37.5)	391 (40.8)†
3–40 h outside	174 (41.0)	234 (44.0)	408 (42.6)
Unknown	59 (14.0)	99 (18.5)	158 (16.6)
Dusk to dawn, weekdays			
≤5 h outside	261 (61.6)	279 (52.4)	540 (56.4)†
6–60 h outside	136 (32.0)	202 (38.0)	338 (35.3)
Unknown	27 (6.4)	52 (9.6)	79 (8.3)

*DEET, N,N-diethyl-*m*-toluamide.
†Statistically significant differences by Z test ($p<0.05$).

portion of water surface area than Fort Collins, a difference that has been hypothesized to influence mosquito populations, local bird populations, and human behavior. However, on the basis of vector indices for 6 weeks of entomologic data collected during the height of the 2003 outbreak, more WNV-infected mosquitoes were present in Fort Collins than in Loveland. This finding was predictable, given that Fort Collins implemented an emergency mosquito control program late in the WNV season.

Many results of this study are consistent with those of previous reports. Older persons and those not worried about WNV infection were more likely to report seldom or never using a repellent containing DEET (*12*,*13*). Similarly, persons with lower incomes reported practicing fewer preventive behavioral measures. This finding was evident in the dusk-to-dawn weekday model in which an interaction term appeared; men with the lowest income levels were more likely to be outdoors during the week from dusk to dawn. This result seems plausible given that weekday workers have less control over outdoor exposure than nonworking hours during the weekend and may specifically capture those engaged in agricultural or landscaping work. No differences between Fort Collins and Loveland residents were observed for those reporting seldom or never wearing long clothes, which is not surprising, given that few people use this strategy (*12*).

Although explanatory variables help identify the proportion of surveyed persons not following the 4 Ds of prevention, they do not explain why Loveland residents were less likely to practice personal prevention behavioral measures. Loveland residents may have had less knowledge of these prevention strategies. However, this was unlikely, given widespread WNV educational efforts in both cities and local and state media coverage of the outbreak. Furthermore, bivariate analysis of reported risk perception indicates that both Fort Collins and Loveland residents perceived very similar risks for WNV infection.

Perceived risk for disease was a consistent factor in the multivariate models. Persons who were not worried about WNV were more likely to report seldom or never using a repellent with DEET, not wearing long clothes, and spending more time outdoors from dusk to dawn on the weekend. As noted by other authors (*14–16*), risk perception is only one of many factors that directly contribute to practicing preventive behavioral measures. For example, environmental triggers may play a role. In a model proposed by Zielinski-Gutierrez and Hayden, a person's experience with their environment (i.e., seeing mosquitoes, getting bitten, or both) is one of the most immediate triggers for taking protective action (*17*). This was true for residents in Mississippi who in 2003 reported feeling a mosquito bite as the most important reason for taking precautions against mosquito bites (*8*). During the outbreak in Larimer County, biting pressure from the nuisance mosquito *Aedes vexans* (Fort Collins, 39.6/trap night), and Loveland (22.6/trap night) along with *Culex* sp. may have prompted residents to use repellent and practice other avoidance strategies. Environmental triggers, such as biting pressure, may

Table 3. Adjusted odds ratios and 95% confidence intervals for logistic regression models* assessing self-reported West Nile virus preventative outcomes by identified risk factors, Larimer County West Nile Virus survey, Colorado, 2003

Risk factors	DEET (n = 842), seldom/never use DEET	Dress (n = 507), seldom/never wear long clothes	Dusk to dawn, weekdays (n = 797), >5 h outside from dusk to dawn	Dusk to dawn, weekends (n = 795), >2 h outside from dusk to dawn
City				
Fort Collins	Referent	Referent	Referent	Referent
Loveland	1.39 (1.04–1.76)	1.02 (0.70–1.48)	1.35 (1.01–1.82)	1.30 (1.00–1.74)
Sex				
Female	Referent	Referent	Referent	Referent
Male	0.92 (0.69–1.25)	1.36 (0.94–1.98)	0.97 (0.63–1.48)	1.20 (0.90–1.61)
Age, y				
18–44	Referent	Referent	Referent	Referent
≥45	1.62 (1.21–2.18)	0.70 (0.48–1.01)	0.91 (0.67–1.22)	0.67 (0.51–0.89)
Education				
College†		Referent	Referent	
High school diploma or less		0.89 (0.58–1.38)	1.41 (0.99–1.98)	
Income, US $				
>50,000		Referent	Referent	
<25,000		0.79 (0.47–1.31)	0.65 (0.38–1.11)	
25,000–50,000		0.72 (0.47–1.08)	1.09 (0.69–1.72)	
Risk perception				
Very worried	Referent	Referent		Referent
Somewhat worried	1.47 (0.92–2.36)	1.79 (0.88–3.61)		1.68 (1.07–2.65)
Not worried	4.68 (2.90–7.51)	2.58 (1.25–5.28)		2.14 (1.31–3.51)
Interaction			Referent	
Male <$25,000			4.36 (1.88–10.0)	
Male $25,000–50,000			1.39 (0.72–2.69)	

*Hosmer-Lemeshow Goodness-of-fit is based on deciles of risk. p values >0.05 indicate good fit (N,N-diethyl-*m*-toluamide [DEET], p = 0.67; dress, p = 0.68; dusk/dawn, weekdays, p = 0.97; dusk/dawn, weekends, p = 0.91).
†College: some college, college degree, some graduate school, or graduate degree.

explain why Fort Collins and Loveland residents responded similarly to a general question on risk perception regarding WNV but reported differences in preventive behaviors.

This possibility raises a related question, "Did Loveland residents choose to rely on the city's control program instead of practicing individual preventive measures?" Loveland residents may have been less likely to have applied personal preventive measures (the 4 Ds of prevention) given their reliance on the long-standing community mosquito control program. Although difficult to establish with any certainty, this prospect suggests the need to promote integrated prevention with both community and individual actions complementing each another. Future research should assess the multiple factors that contribute to risk perception and address the human-environmental interactions that influence protective behaviors.

Although this study is limited by recall and reporting bias because the survey was conducted 8 months after the outbreak, these information biases are most likely nondifferential since recall and reporting would likely be similar among both Loveland and Fort Collins residents. The results of this study reinforce use of personal protection efforts even in areas with strong community mosquito control measures and suggest that these personal measures may influence disease rates. Furthermore, study results suggest that persons residing in a city with greater mosquito-biting pressure, as measured by a vector index, were more likely to take preventive measures than persons in a community with less biting pressure. Future studies are warranted to understand the effects of human-environment interactions to derive the greatest benefit from community and personal efforts to reduce disease and death from WNV.

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Matrix Protein 2 Vaccination and Protection against Influenza Viruses, Including Subtype H5N1

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Changes in influenza viruses require regular reformulation of strain-specific influenza vaccines. Vaccines based on conserved antigens provide broader protection. Influenza matrix protein 2 (M2) is highly conserved across influenza A subtypes. To evaluate its efficacy as a vaccine candidate, we vaccinated mice with M2 peptide of a widely shared consensus sequence. This vaccination induced antibodies that cross-reacted with divergent M2 peptide from an H5N1 subtype. A DNA vaccine expressing full-length consensus-sequence M2 (M2-DNA) induced M2-specific antibody responses and protected against challenge with lethal influenza. Mice primed with M2-DNA and then boosted with recombinant adenovirus expressing M2 (M2-Ad) had enhanced antibody responses that cross-reacted with human and avian M2 sequences and produced T-cell responses. This M2 prime-boost vaccination conferred broad protection against challenge with lethal influenza A, including an H5N1 strain. Vaccination with M2, with key sequences represented, may provide broad protection against influenza A.

Yearly development of influenza vaccines that are antigenically matched to circulating strains poses extraordinary challenges. A rapidly developing pandemic would shorten the time for strain identification and vaccine preparation; meanwhile, antigenic changes would continue. Moreover, the need to immunize an entirely naive population would exacerbate problems with vaccine production and supply.

Vaccines based on conserved antigens would not require prediction of which strains would circulate during an approaching season and could avoid hurried manufacturing in response to outbreaks. Test vaccination with DNA constructs that express conserved influenza A nucleoprotein (NP) or NP plus matrix (M) induced antibody and T-cell responses and protected against heterosubtypic viruses (*1*,*2*). Despite the virulence and rapid kinetics of challenge infection, DNA vaccination with NP and M achieved limited protection against an H5N1 virus strain isolated from the 1997 human outbreak in Hong Kong (*3*).

The M gene of influenza A encodes 2 proteins, both highly conserved: M1, the capsid protein, and M2, an ion channel protein. M2 contains a small ectodomain (*4*), M2e, which makes it a target for antibody-based immunity. The ability of anti-M2 monoclonal antibody (MAb) to reduce viral replication (*5*) implicates M2, in particular M2e, as a vaccine target. M2 vaccine candidates that have been explored include peptide-carrier conjugates (*6*), baculovirus-expressed M2 (*7*), fusion proteins (*8*,*9*), multiple antigenic peptides (*10*), and M DNA constructs that potentially express M2 (*11*,*12*). In those studies, mice were protected against challenge with homologous or heterosubtypic viruses, but even the heterosubtypic viruses had an M2e sequence identical to the vaccine constructs or differed by only 1 amino acid.

Although most human influenza viruses of H1, H2, or H3 subtypes share identity with the M2e consensus sequence (M2e-con) (*9*,*13*), some influenza A viruses do not. In a study of M2e-carrier conjugate vaccines, serum antibodies specific for M2e-con or M2e-A/PR/8/34 (H1N1) did not cross-react with M2e peptides from H5 and H7 subtype avian viruses that have 3 or 4 mismatches (*6*). In another study, monoclonal and polyclonal antibodies reacted with a subset of avian sequences (*14*). Although a recent study used M2e peptide-liposome vaccines of subtypes

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including H5N1 with matched challenge viruses (*15*), no prior work has documented protection against challenge with influenza viruses in which M2e sequences differed substantially from those of the immunizing antigen.

Priority is being given to developing vaccines that offer broad protection against multiple influenza subtypes, including H5N1. Indeed, development of conserved-antigen vaccines, and specifically M2-based vaccines, is part of the US Department of Health and Human Services Pandemic Influenza Plan (www.hhs.gov/pandemicflu/plan/). We therefore evaluated M2-based vaccine efficacy against divergent challenge viruses.

Methods

Mice

Female BALB/cAnNCR mice were purchased from Division of Cancer Treatment, National Cancer Institute, Frederick, Maryland, USA. The institutions' Animal Care and Use Committees approved all protocols for animal experiments.

Viruses

Influenza viruses used were A/PR/8/34 (H1N1) (*3*), A/FM/1/47-MA (H1N1) (*16*), and A/Thailand/SP-83/2004 (H5N1) (*17*). Some virus stocks were propagated in the allantoic cavity of embryonated hen eggs at 34°C for 48–72 h (A/PR/8) or 37°C for 24 h (SP-83). A/FM was prepared as a pooled homogenate of lungs from BALB/c mice infected 4 days previously. All experiments with H5N1 subtypes were conducted under biosafety level 3, enhanced containment.

Peptides and Peptide Conjugates

M2e 2–24 peptides (no NH2-terminal methionine) were synthesized with COOH-terminal cystine residues and conjugated to maleimide-activated keyhole limpet hemocyanin (KLH) for vaccines. The same peptides were also synthesized without COOH-terminal cystine and used for antibody and T-cell assays. Influenza A NP147–155 and M2e peptides were synthesized in the core facility of the Center for Biologics Evaluation and Research, US Food and Drug Administration. Severe acute respiratory syndrome (SARS) matrix peptide (209–221) was provided by the National Institutes of Health.

Vectors

Plasmid and recombinant adenoviral (rAd) vectors that express B/NP and A/NP have been described (*18*), as has the plasmid containing the entire M gene of A/PR/8 (*2*). The plasmid VR1012-M2 (termed M2-DNA above) was generated as follows. The plasmid pCR3-M2 was derived by PCR from the vector pCR3-M previously generated from A/PR/8 virus by reverse transcription–PCR (*19*). To modify the sequence to the widely shared M2e sequence, full-length consensus M2 cDNA with Kozak sequence at its 5′ end was generated from 2 overlapping M2 DNA fragments and subcloned into VR-1012, obtained under material transfer agreement from Vical, Inc., San Diego, CA, USA. The sequence of the M2 insert was confirmed by restriction digestion and sequence analysis. The replication-incompetent adenovirus that expresses the M2 protein with the consensus sequence (M2-Ad) was constructed by using Gateway cloning and the ViraPower Adenoviral Expression System (both from Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Briefly, the M2 cDNA from VR1012-M2 was cloned by PCR into the pENTR/D-TOPO Gateway vector and then transferred into the pAd/CMV/V5-DEST adenoviral Gateway vector by LR Clonase (Invitrogen) reaction to give pAd/CMV-M2. Integrity and proper insertion of the cloned M2 cDNA were confirmed by sequencing. M2-Ad was generated by transfection of 293A cells with pAd/CMV-M2. M2 expression was confirmed by immunohistochemical staining of M2-Ad-infected Madin-Darby canine kidney cells with M2-specific polyclonal sera (data not shown). High-titered stocks of rAd were prepared by ViraQuest, Inc. (North Liberty, IA, USA). Adenovirus stocks were stored in 3% sucrose/phosphate-buffered saline (PBS) at $1–2 \times 10^{12}$ particles/mL and confirmed as negative for replication-competent adenovirus by passage on nonpermissive cells.

Immunization

Mice were given an intraperitoneal injection of 40 μg peptide-KLH or unconjugated KLH in complete Freund's adjuvant (emulsified 1:1 with antigen in PBS). Three weeks later, the mice were given an intraperitoneal booster injection with peptide-KLH in incomplete Freund's adjuvant; 13 days later blood was collected. Injections were started at 8–10 weeks of age for peptide and 6 weeks of age for DNA. DNA vaccination at doses of 50 μg/mouse (unless noted otherwise in a figure legend) in low-endotoxin PBS (AccuGENE, Cambrex, East Rutherford, NJ, USA) was given intramuscularly in the quadriceps, half to each leg, in 3 doses 2 weeks apart. In some experiments, mice were given a booster injection of rAd intramuscularly at a dose of 10^{10} particles/mouse, 2–3 weeks after the last dose of DNA.

Challenge

Challenge virus in 50 μL of PBS was administered intranasally to anesthetized mice. Isoflurane or ketamine/xylazine was used for mice challenged with H1N1 subtype. Reported 50% lethal dose (LD_{50}) for H1N1 subtype was determined for 8-week-old naive BALB/c

mice for each anesthetic (and may vary from the actual LD_{50} for the older vaccinated mice that were challenged). Subtype H5N1 was administered intranasally to mice anesthetized with 2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Aldrich Chemical Co., Milwaukee, WI, USA). Some mice were killed so their lungs could be harvested; others were monitored for body weight and death. Monitoring continued until all animals died or were recovering, as indicated by body weight.

In Vivo T-Cell Depletion

Acute depletion of lymphocyte populations by MAb treatment on days –3, +2, +8 relative to day of challenge was performed as described previously (*18*) and used MAbs GK1.5, specific for mouse CD4; 2.43, specific for mouse CD8; and SFR3-DR5, specific for a human leukocyte antigen as a negative control. Splenocytes were analyzed 2 days after challenge (before the next injection) by flow cytometry to confirm completeness of in vivo T-cell depletion, as described (*2*).

ELISA

ELISA for M2-specific antibodies was performed on plates coated with 15 μg/mL of synthetic peptides in 0.007 mol/L borate buffer and 0.025 mol/L saline; the rest of the procedure was as described (*20*).

Passive Serum Transfer

Naive mice were given intraperitoneal injections of pooled serum, 1 mL per mouse, from mice immunized with M2-DNA plus matched Ad booster or from control mice (B/NP-DNA, M2-H5[HK] peptide-KLH conjugate, or A/PR/8 virus). Mice were challenged with a moderate dose of A/PR/8 virus the day after serum transfer; antibody levels in recipients were not measured.

Spleen Cell Fractionation

T cells were enriched by negative selection that used magnetic beads. Briefly, splenocytes were depleted of erythrocytes and labeled with biotinylated antimouse B220, CD11b, and PanNK antibodies (BD Pharmingen, San Diego, CA, USA). After labeling, cells were incubated with Streptavidin MicroBeads (Miltenyi Biotec, Auburn, CA, USA). Unlabeled T cells and labeled non–T cells were separated through the Miltenyi AutoMACS system according to the manufacturer's instructions.

Enzyme-linked Immunosorbent Spot (ELISPOT) Assay

This assay detected T-cell responses to M2 peptides. ELISPOT IP plates (Millipore; Billerica, MA, USA) were coated with 50 μL of Hank's balanced salt solution (Hyclone, Logan, UT, USA) containing 5 μg/mL of anti–interferon-γ (IFN-γ) MAb AN18 (BD Pharmingen) and incubated overnight at 4°C. The membrane was washed and then blocked with medium containing 10% fetal bovine serum for 60–90 min at room temperature. Splenocytes depleted of erythrocytes were added to wells in 2-fold dilutions, starting at 250,000 cells/well in 50 μL. Peptides (SARS-M-209–221, NP147–155 of A/PR/8, or M2–2-24 of A/PR/8) were added at a final concentration of 1 μg/mL. After incubation for 36–48 h at 37°C, bound IFN-γ was detected with 50 μL of biotinylated MAb R4–6A2 (BD Pharmingen) at 1 μg/mL. Spots were developed by using alkaline phosphatase–labeled streptavidin and 5-bromo, 4-chloro, 3-indolylphosphate/nitroblue tetrazolium substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) and counted with an ELISPOT reader (Zeiss; Thornwood, NY, USA).

Virus Quantitation

Lungs were homogenized in 1 mL of sterile PBS, clarified by centrifugation, and titrated for virus infectivity by 50% egg infectious dose (EID_{50}) assay as described (*3*). The limit of virus detection is 1.2 $\log_{10}$ EID_{50}/mL. Challenge virus stocks were titrated on Madin-Darby canine kidney cells as described previously (*18*).

Statistical Analysis

The serologic assays and detection of M2-specific T cells were performed multiple times with comparable results. Some vaccinations were repeated with independent groups (as noted in the figure legends); those performed once used numbers of animals per group adequate for statistical significance. Lung virus titers were compared by using 1-way analysis of variance on log-transformed data, followed by pairwise multiple comparison (Holm-Sidak method). Weight loss after challenge was compared for survivors on each day by also using 1-way analysis of variance followed by pairwise multiple comparison (Holm-Sidak). This method overestimates body weight in groups with deaths because the animals that died would have had very low body weights, affecting the average, especially in the negative control groups. Nonetheless, differences between vaccinated groups were significant in the instances stated. Comparison of cumulative survival rates used the log-rank test, followed by pairwise multiple comparison, again by using the Holm-Sidak method. Overall significance level for Holm-Sidak tests was $p = 0.05$. All statistical analyses were performed with SigmaStat Software v3.11 (Systat Software, Point Richmond, CA, USA).

Results

M2e-KLH Vaccination

For proof-of-concept studies, peptides representing M2e-con (9) and additional viral M2 ectodomains (Table)

Table. Sequences of matrix protein 2 (M2) ectodomains

Strain	Abbreviation	Subtype	M2e Sequence*†
Consensus	M2e-con	‡	MSLLTEVETPIRNEWGCRCNDSSD
A/PR/8	M2e-PR8	H1N1	MSLLTEVETPIRNEWGCRCN**G**SSD
A/FM/1/47-MA	M2e-FM	H1N1	MSLLTEVETP**TK**NEW**E**CRCNDSSD
A/Hong Kong/156/97	M2e-H5(HK)	H5N1	MSLLTEVET**LT**RN**G**WGCRC**S**DSSD
A/Thailand/SP-83/04	M2e-H5(SP-83)	H5N1	MSLLTEVETP**T**RNEW**E**CRC**S**DSSD

*Variations from the M2e consensus sequence are in **boldface** and underlined.
†Virus sequences available at www.ncbi.nlm.nih.gov/genomes/FLU and www.flu.lanl.gov.
‡Consensus sequence derived from human influenza viruses of H1, H2, and H3 subtypes (*9*,*13*).

were conjugated to KLH and used to immunize BALB/c mice. Immune serum samples were analyzed for M2-specific antibodies by ELISA on plates coated with synthetic M2e peptides with sequences from 2 viruses of H1N1 and 2 of H5N1 subtype. Serum from KLH-immune mice did not react with any of the peptides, but each M2e-immune serum sample reacted with M2e-PR8, M2e-FM, and M2e-H5(SP-83) peptides (Figure 1A–C). Cross-reactions were lower on the M2e-H5(HK) peptide (Figure 1D).

M2e-vaccinated mice were then challenged with either A/PR/8 or A/FM and monitored for weight loss (as a measure of illness) and death. Weight losses were consistent with a hierarchy of protection based on sequence similarity (data not shown), and differences from control mice were statistically significant. In the same groups, 100% of mice vaccinated with M2e-con or M2e-FM survived challenge with A/PR/8 and A/FM, but M2e-H5(HK)/KLH vaccination provided incomplete protection (Figure 2A and B).

M2-DNA Vaccination

To investigate whether, like M2e peptide, DNA vaccination could protect against viruses with quite divergent M2e sequence, we tested M- and M2-DNA for efficacy. M-DNA with the A/PR/8 sequence protected against challenge with A/PR/8 (Figure 3A). M2-DNA with the consensus sequence also protected against this A/PR8 challenge; however, M1-DNA did not (data not shown). For challenge with A/FM, M2-DNA was more efficacious than M-DNA (Figure 3B).

M2-DNA Vaccination Followed by M2-Ad Boost

Previously, we have shown for NP vaccination that boosting with rAd induces more potent antibody and T-cell (especially $CD8^+$) responses than does DNA vaccination alone and can protect against challenge with highly pathogenic H5N1 subtype (*18*). We investigated whether boosting with rAd would also enhance immunity to M2. The A/M2 gene with the consensus sequence was cloned into a replication-deficient Ad construct (M2-Ad). Mice were primed with DNA as before and boosted with M2-Ad or control B/NP-Ad. Two weeks later, serum samples were collected and assayed for M2-specific immunoglobulin (Ig) G by ELISA on peptides as above (Figure 4A–D). Mice given the booster of M2-DNA+M2-Ad had dramatically greater M2e-PR8–specific IgG antibody responses than mice given either component alone (M2-DNA+B/NP-Ad or B/NP-DNA+M2-Ad; Figure 4A). Moreover, IgG cross-reactivity with M2e-FM (3 amino acid differences; Figure 4B) and M2e-H5(SP-83) (3 amino acid differences; Figure 4C) was found with serum from mice given M2-Ad; cross-reactivity was even greater with serum from mice given M2-DNA+M2-Ad. These serum samples, however, did not cross-react with M2e-H5(HK) (4 amino acid differences; Figure 4D), although serum from mice immunized with M2e-H5(HK)-KLH as positive controls reacted strongly (data not shown).

Protective immunity due to vaccination with M2-DNA+M2-Ad was tested by challenge with A/PR/8 (high-dose) or A/FM (moderate dose) virus. Of mice vaccinated with M2-DNA+M2-Ad, 100% survived challenge with A/PR/8 and A/FM virus; of mice vaccinated with B/NP-DNA+B/NP-Ad, 20% survived challenge with A/PR/8 and none survived challenge with A/FM (Figure 5A,B). Thus, the prime-boost vaccination protected against challenge viruses in which M2e sequences were similar to or divergent from those of the vaccine.

T-cell Response

T-cell responses to M2 have been observed (*7*). Immunization with cDNA expressing full-length M2 protein might induce, in addition to antibody, M2-specific T-cell responses not induced by peptides. To address the contribution of T-cell responses, we immunized mice to M2 by prime-boost and acutely depleted them of T cells just before and during the challenge period. Lymphocyte depletion was confirmed to be complete; residual splenic $CD4^+$ or $CD8^+$ cells were <1% (data not shown). Of the B/NP control mice, 100% died of A/PR/8 infection by day 8 after challenge, while 100% of M2-immune mice treated with a control MAb (SFR) survived (Figure 6A). Individual depletion of $CD4^+$ or $CD8^+$ T cells did not abrogate protection. Depletion of $CD4^+$ and $CD8^+$ T cells together partially, but statistically significantly, abrogated M2-induced protection (Figure 6A) but left some protection significantly different from that in the B/NP control mice. Thus, under these challenge conditions, T cells are important. M2e-specific immunity has been reported to be natural killer (NK)-cell dependent (*21*). We found that

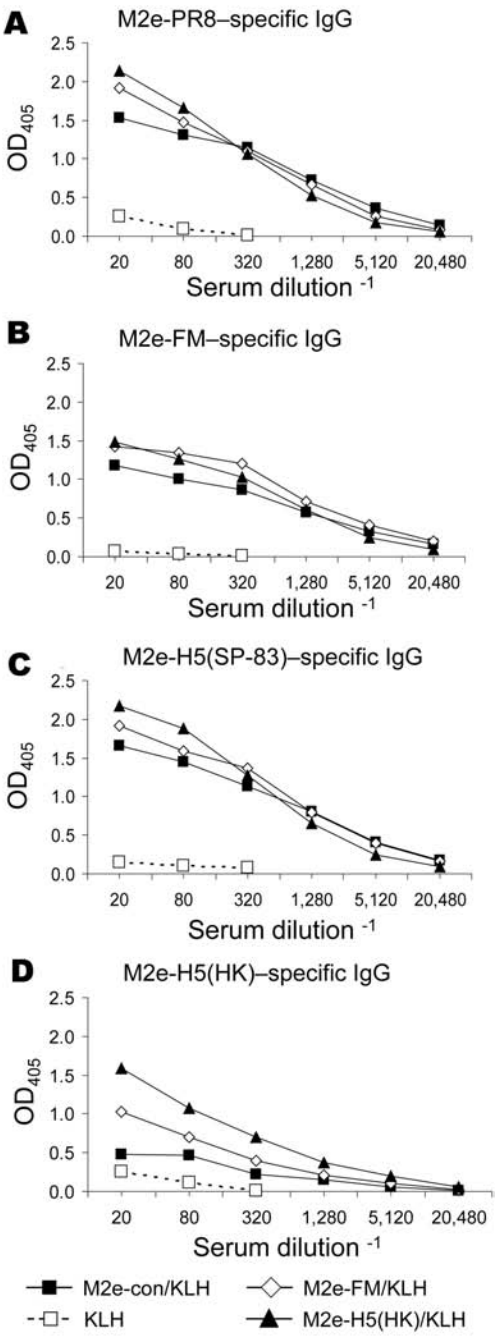

Figure 1. Results of matrix protein 2 (M2)e–keyhole limpet hemocyanin (KLH) vaccination, showing induction of cross-reactive antibody responses. Mice (7–9 per group) were immunized intraperitoneally with KLH or M2e peptides conjugated to KLH (M2e-con/KLH, M2e-FM/KLH, or M2e-H5(HK)/KLH) in complete Freund's adjuvant. After 21 days, the mice were given an intraperitoneal booster with KLH or M2e-peptide/KLH in incomplete Freund's adjuvant. Immune serum was collected 13 days after booster and assayed for immunoglobulin (Ig) G reactive to various M2e peptides by ELISA. Plates were coated with M2e-PR8 (panel A), M2e-FM (panel B), M2e-H5(SP-83) (panel C), or M2e-H5(HK) (panel D). Data are representative of multiple experiments. OD, optical density; e, ectodomain.

mice depleted of NK cells with anti–asialo-GM1 antibody were protected similarly to controls (data not shown). Thus, while NK cells may play a role, they were not required under the conditions we studied.

Given the effect of T-cell depletion, we tested for in vitro M2-specific IFN-γ–producing T cells. The IFN-γ ELISPOT assay showed positive responses to an amino-terminal M2 peptide in spleen cells from mice immune to M2-DNA+M2-Ad (Figure 6B) and to A/PR/8 (data not shown). After spleen cells were fractionated by magnetic bead separation (see Methods), the non–T-cell fraction did not respond, while the unfractionated cells and T-cell fraction maintained a strong M2-specifc IFN-γ response (Figure 6B).

Role of M2-specific Antibody

On the basis of information from previous studies (see Discussion), we tested the ability of antibodies induced by DNA prime–Ad boost to passively transfer protection. All

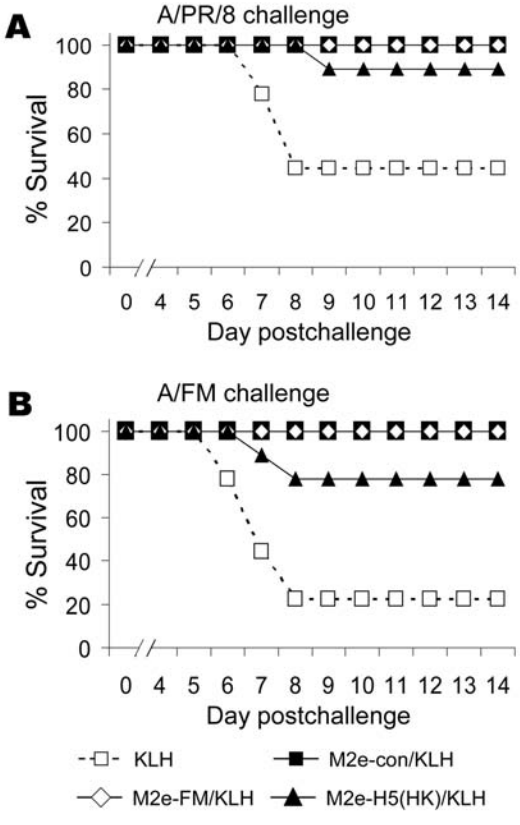

Figure 2. Results of matrix protein 2 (M2)e–keyhole limpet hemocyanin (KLH) vaccination, showing cross-protection. Mice (7–9 per group) were vaccinated as in Figure 1. Six weeks after the booster, they were anesthetized with isoflurane and challenged with 10x the 50% lethal dose (LD_{50}) of A/PR/8 (A) or A/FM (B) viruses and then monitored for survival. Cumulative survival rates after challenge with A/PR/8 or A/FM virus differed significantly from those of KLH controls for all M2e-conjugates ($p = 0.001$ and $p<0.001$, respectively, log rank). e, ectodomain.

(100%) mice given serum from M2-immune or A/PR/8-infected mice survived, while only 50% given control B/NP-immune serum survived (Figure 6C; $p<0.001$, log rank). Passive serum antibody from M2 prime–boost immune mice also conferred significant protection against weight loss (Figure 6D).

Heterologous Challenge, Including SP-83 (H5N1)

Because protection against challenge with A/FM virus that has an M2e sequence quite divergent from that of the immunizing sequence was encouraging, we tested whether M2-DNA+M2-Ad vaccination could protect against challenge with H5N1 subtype. Mice were immunized 3× with B/NP-DNA (negative control), A/NP-DNA (positive control), or consensus M2-DNA, and boosted with matched rAd. Mice were challenged with a lethal dose of A/Thailand/SP-83/2004 (H5N1) virus in which M2e differed from the consensus by 3 amino acids. On day 5 after infection, a random subset of animals was killed and their

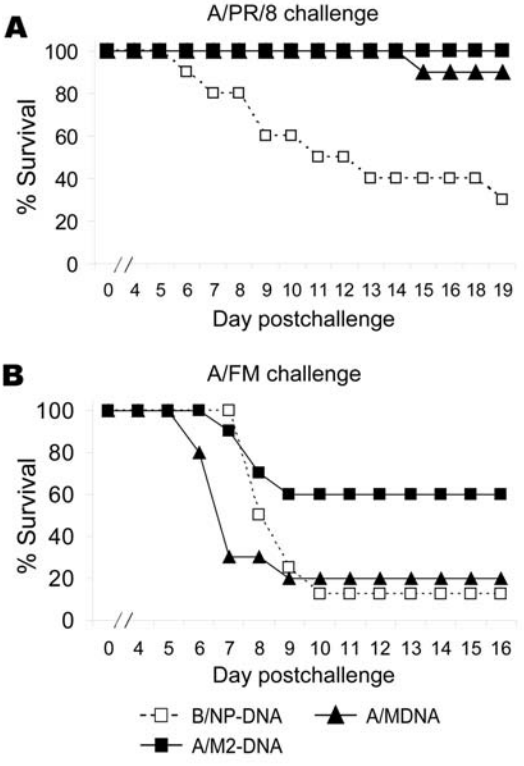

Figure 3. Results of matrix protein 2 (M2)–DNA vaccination, showing protection against divergent influenza viruses. Mice (8–10 per group) were vaccinated with DNA as described in Methods except at a dose of 100 μg/mouse. Approximately 2 weeks after the last dose of DNA, mice were challenged with 7× the 50% lethal dose (LD_{50}) of virus and monitored for survival. A) A/PR/8 challenge: Cumulative survival rate of mice vaccinated with M-DNA or M2-DNA was significantly higher than that of mice vaccinated with B/NP-DNA ($p<0.001$, log rank). B) A/FM challenge: Cumulative survival rate differed significantly among groups ($p = 0.041$, log-rank), although in post hoc Holm-Sidak tests, pairs did not differ significantly ($p\geq0.05$).

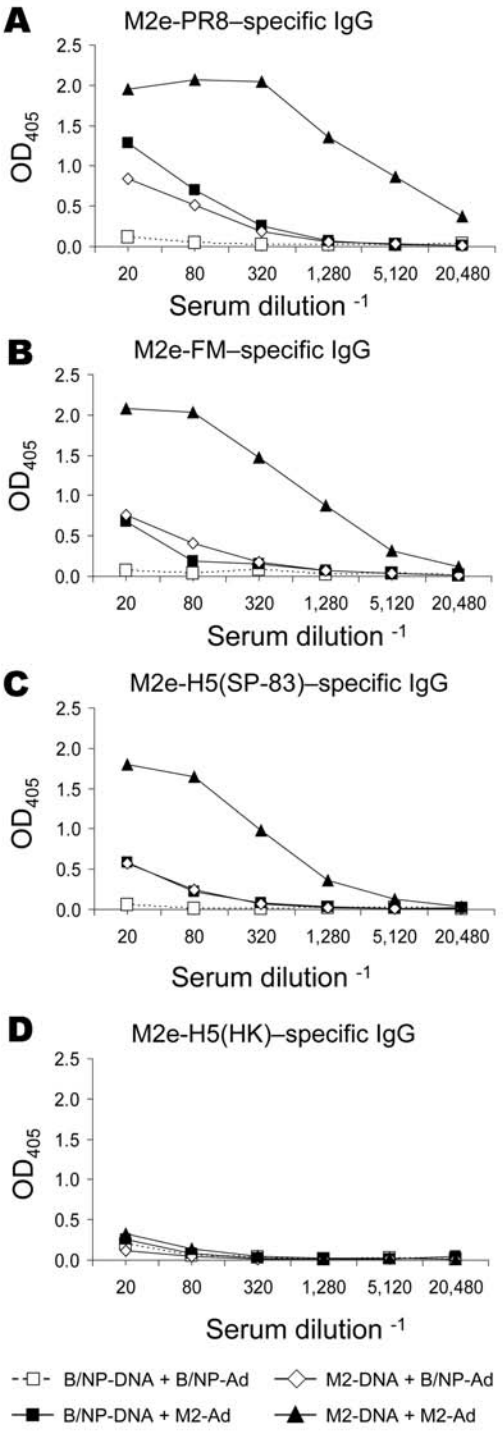

Figure 4. Results of matrix protein 2 (M2) vaccination and booster with DNA prime–adenovirus (Ad), showing cross-reactive antibodies. Mice (8–10 per group) were vaccinated with DNA and given an Ad booster as described in Methods. Immune serum collected 3 weeks after the booster was assayed for immunoglobulin (Ig) G reactive to various M2e peptides by ELISA, as described in Methods. Plates were coated with M2e-PR8 (panel A), M2e-FM (panel B), M2e-H5(SP-83) (panel C), or M2e-H5(HK) (panel D). OD, optical density. e, ectodomain.

lung virus titers were measured. Virus titers of mice vaccinated with A/NP or with M2 were significantly reduced compared with those of control mice (Figure 7A). The remaining mice were monitored for weight loss and survival. Weight loss was less in mice vaccinated with A/NP and M2 than in control mice (Figure 7B). All the B/NP-immune mice died of SP-83 infection by day 11 postchallenge. All (100%) A/NP-immune mice and all but 1 M2-immune mouse survived (Figure 7C).

Discussion

Our results indicate that M2 vaccination can induce cross-reactive antibody responses, virus-specific T-cell responses, and protection against challenge with lethal heterologous virus. As has been found in previous studies of M2-based vaccines, we found strong antibody responses to the conserved M2e region. Anti-M2 antibodies in the serum of mice immunized with M DNA suggest expression of M2 from this plasmid (not shown). However, reactivity could be due to the 9 amino acid portion shared with the M1 sequence, and we did not explore this possibility.

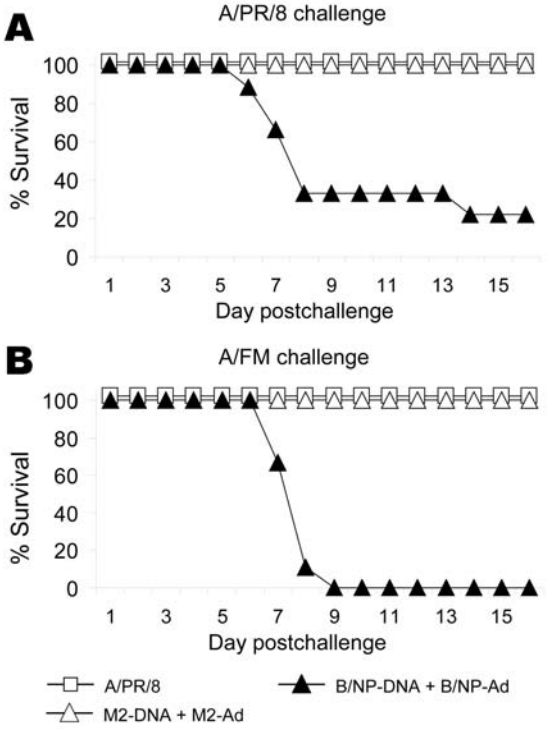

Figure 5. Results of vaccination and booster with DNA prime–adenovirus (Ad), showing cross-protection. Mice (8–10 per group) were immunized as in Figure 4 or intranasally given a sublethal priming infection with A/PR/8. Three weeks later they were challenged with a high dose of A/PR/8 (1.5×10^4 50% lethal dose [LD_{50}]) (A) or moderate dose of A/FM (10 LD_{50}) (B) and monitored for survival. The cumulative survival rate for mice immunized with A/PR/8 and M2-DNA+M2-Ad was significantly higher than that for mice immunized with B/NP-DNA+B/NP-Ad ($p<0.001$, log rank). Data are representative of multiple experiments.

Fan et al. used A/PR/8 and Aichi M2e-carrier conjugates for immunization, and the resulting antibodies did not cross-react with the avian M2e sequences tested (*6*). We found cross-reactivity with M2e peptides that had considerable sequence divergence from the human influenza M2 consensus. Immunization with M2e-con/KLH induced antibodies that were reactive with M2e-H5(SP-83) peptide but less reactive with M2e-H5(HK). However, immunization with M2e-H5(HK)/KLH induced antibodies that were reactive with all the M2e peptides. This pattern parallels the results of Liu et al. (*14*). However, neither Fan et al. nor Liu et al. investigated protection against challenge with H5N1 subtype.

In our lethal challenge studies, M2e peptide conjugates protected against not only a 1934 subtype H1N1 virus (A/PR/8) but also a 1947 subtype H1N1 virus (A/FM). The latter virus, which is virulent in mice, has an M2e sequence with 3 amino acid differences from the consensus and thus is as divergent from the consensus sequence as some M2-H5 sequences. Encouraged by this broad cross-reactivity and cross-protection, we expanded the study to DNA vaccination and DNA prime–Ad boost regimens. These approaches have the advantage of providing more epitopes than peptide immunization and relevant T-cell immunity.

Using M2 consensus DNA vaccination with or without Ad boost, we again saw cross-reactivity on avian peptides M2e-H5(SP-83) and M2e-H5(HK), although cross-reactivity was low on the HK peptide. T-cell responses to M2 peptides were detected by ELISPOT.

Several studies have shown that M2e-specific antibodies can mediate protection against influenza infection in vivo (*9*,*10*,*13*). In agreement with those studies, we found that serum antibodies induced by peptide conjugates or by prime-boost vaccination could transfer protection to naive recipients. We found that T cells were also important because depletion of $CD4^+$ and $CD8^+$ T cells during the challenge period reduced protection against a higher challenge dose. This could reflect M2e-specific memory T cells, which we have demonstrated in spleen and peripheral blood by ELISPOT, or a concurrent T-cell response to challenge virus supplementing the protective effects of antibodies.

In lethal challenge studies, the M2 consensus DNA and rAd constructs could protect against not only A/PR/8 but also against A/FM, a virus quite divergent in the M2e sequence. Furthermore, they could protect mice against challenge with SP-83 (H5N1) isolated from a fatal human case, at a dose lethal to control mice. Virus replication in lungs and illness reflected by loss of body weight were also reduced by M2 immunization. Protection against challenge with other H5N1 subtypes remains to be explored, and serologic results on M2e-H5(HK) peptide suggest

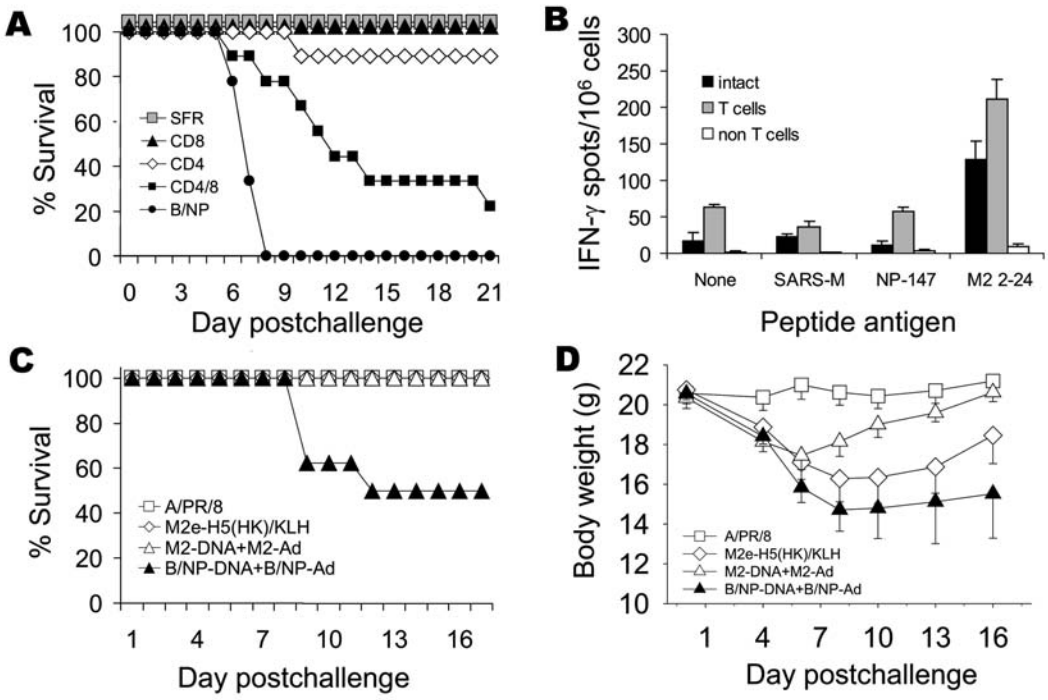

Figure 6. Role of T- and B-cell immunity in matrix protein 2 (M2)–specific protective immunity. A) Mice (9 per group) were immunized with M2-DNA or B/NP-DNA and boosted with matched adenovirus (Ad) as described in Methods. Three weeks after Ad boost, M2-DNA groups were acutely depleted of T cells with monoclonal antibodies (MAbs) to $CD4^+$ or $CD8^+$ or both, or given control MAb SFR3-DR5, as described in Methods. Mice were challenged with 1.5×10^4 50% lethal doses (LD_{50}) of A/PR/8. Compared with the cumulative survival rate for the SFR control, survival rates differed significantly for mice depleted of both T-cell subsets ($p<0.001$, log-rank), although some protection remained, which differed significantly from that of the B/NP control ($p<0.001$, log-rank). B) Mice were immunized with M2-DNA+M2-Ad as described under A. Five months after mice received the Ad boost, spleen cells were isolated and pooled from immune mice (n = 10), fractionated into T-cell and non–T-cell populations, and assayed for interferon-γ (IFN- γ)–producing cells by enzyme-linked immunosorbent spot assay, as described in Methods. C and D) Serum collected from immune mice was passively transferred intraperitoneally into naive BALB/c mice (8 per group). The recipients were challenged with 10 LD_{50} of A/PR/8 and monitored for survival (C) and weight loss (D). The cumulative survival rate for mice given A/PR/8 immune serum, M2-DNA+M2-Ad-immune serum, or M2e-H5(HK)/keyhole limpet hemocyanin–immune serum was significantly higher than that for mice given B/NP-DNA+B/NP-Ad–immune serum ($p<0.001$, log rank). For weight loss, M2 prime-boost differed from B/NP prime-boost at days 8, 10, and 13 ($p \leq 0.003$, analysis of variance; $p<0.05$, Holm-Sidak pairwise multiple comparison). SARS, severe acute respiratory syndrome; IFN- γ, interferon- γ.

results of such studies might differ on the basis of sequence variations.

M2 expression constructs with various M2e sequences could be used as vaccines. Our observation of protection across substantial sequence divergence means that H5-derived vaccines might also protect against circulating H1N1 and H3N2 subtypes. An additional advantage of protection across substantial divergence is potential protection by an M2 vaccine against an unexpected subtype that could cause a pandemic.

One concern about M2 vaccines is the possibility of escape mutants. A study of forced escape mutants found limited diversity (*13*), which indicates that structural constraints, perhaps due to requirements of the M1 structure encoded by the same segment, may limit drift.

The cross-reactivity and protective efficacy of M2-specific antibodies suggest that M2-specific MAbs could be useful for antiviral therapy. These features, combined with constraints on M2 structure, highlight the potential of M2-specific MAbs to inhibit replication of influenza viruses, including some H5N1 strains. Although traditional M2-directed drugs (e.g., amantadine) have led to drug resistance, the mutations that confer resistance are within the transmembrane region (*22*), which may have fewer structural constraints than the ectodomain.

An M2 prime-boost regimen is intended to be combined with vaccination against additional antigens rather than acting as a standalone vaccine. For example, prime-boost vaccination against conserved NP is highly protective ([*18*]; Figure 7). The use of multiple antigens has several advantages: reduced likelihood of escape mutants, better coverage of human leukocyte antigen haplotypes in the genetically diverse human population, and a broader spectrum of immune response mechanisms (with antibodies perhaps dominating for M2 and cytotoxic T lymphocytes for NP).

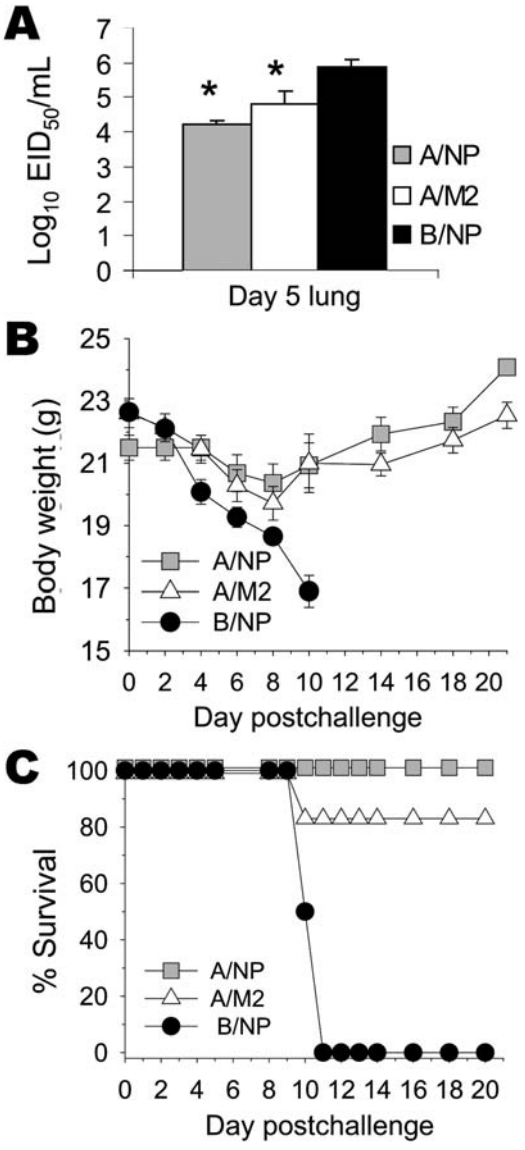

Figure 7. Results of vaccination with matrix protein 2 (M2)–DNA plus M2-adenovirus (Ad) and challenge with heterologous H5N1 subtype. Mice (10 per group) were vaccinated with A/NP-DNA, M2-DNA, or B/NP-DNA and boosted with matched Ad, as described in Methods. Seventeen days after Ad boost, mice were challenged with 10× 50% lethal dose (LD_{50}) of SP-83 (H5N1). A random subset of mice (4/group) was killed on day 5, and their lungs were assayed for virus titer, as described in the Methods (A). Remaining mice were monitored for weight loss (B) and survival (C). The cumulative survival rates for A/NP and M2 immune mice were significantly higher than those for B/NP-immune mice but did not differ from each other significantly ($p<0.001$, log-rank, Holm-Sidak pairwise comparison: $p<0.05$ comparing B/NP with A/NP or M2 groups, $p\geq0.05$ comparing A/NP and M2 groups. *Lung virus titers in A/NP- and M2-immune mice were significantly lower than in B/NP-immune mice but did not differ from each other significantly ($p = 0.004$, analysis of variance. Holm-Sidak pairwise comparison: $p<0.05$ comparing B/NP with A/NP or M2 groups, $p\geq0.05$ comparing A/NP and M2 groups).

Vaccines based on conserved antigens are not intended to replace strain-matched vaccines that induce neutralizing antibodies and thus prevent infection. However, strain-matched vaccines may be difficult to produce in adequate quantities in short time periods, and continued antigenic drift may render them ineffective. Vaccinations as described here, based on M2, might reduce deaths and severity of disease while strain-matched vaccines were being prepared and could enhance protection afforded by inactivated vaccines. Immunogenicity and safety studies in people are needed to evaluate this approach.

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Diversity and Distribution of *Borrelia hermsii*

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Borrelia hermsii is the most common cause of tickborne relapsing fever in North America. DNA sequences of the 16S–23S rDNA noncoding intergenic spacer (IGS) region were determined for 37 isolates of this spirochete. These sequences distinguished the 2 genomic groups of *B. hermsii* identified previously with other loci. Multiple IGS genotypes were identified among isolates from an island, which suggested that birds might play a role in dispersing these spirochetes in nature. In support of this theory, all stages of the tick vector *Ornithodoros hermsi* fed successfully on birds in the laboratory and advanced in their life cycle. *B. hermsii* produced a detectable spirochetemia in 1 chicken inoculated subcutaneously. Additional work is warranted to explore the role of birds as enzootic hosts for this relapsing fever spirochete.

Tickborne relapsing fever in humans in North America is most often caused by the spirochete *Borrelia hermsii*, which is transmitted by its argasid tick vector, *Ornithodoros hermsi* (*1*). The spirochete is endemic to the western United States and southern British Columbia in Canada (Figure 1) but restricted to higher elevations with coniferous forests where both the ticks and appropriate vertebrate hosts coexist (*1*). The most common exposure for humans occurs while they are sleeping in tick-infested cabins, where the nocturnal ticks seek their hosts and feed quickly within 15 to 90 minutes and then return to their refuge in the walls, floor, or attic.

The specific association of this spirochete with *O. hermsi* led to the name *B. hermsii* for the bacterium, distinguishing it from other species of relapsing fever spirochetes transmitted by other species of ticks in the western United States (*2*)**.** The ability to propagate *B. hermsii* in pure culture (*3*) and the development of molecular techniques and databases to identify, type, and compare spirochetes were critical advances for the study of these bacteria. We are now able to characterize and better define these species and to elucidate the geographic distribution and role that *O. hermsi* and various vertebrate hosts play in maintaining *B. hermsii* in nature. These advances are countered, however, by the difficulty in finding infected *O. hermsi* ticks or rodents in the wild and the infrequent access to infected blood samples from patients when they are acutely ill and spirochetemic. Additionally, although *B. hermsii* is cultivatable, establishing these spirochetes in vitro from infected samples is not always successful.

Recently, we identified 2 genomic groups in *B. hermsii* by multilocus sequence typing of 31 isolates (*4*)**.** Four

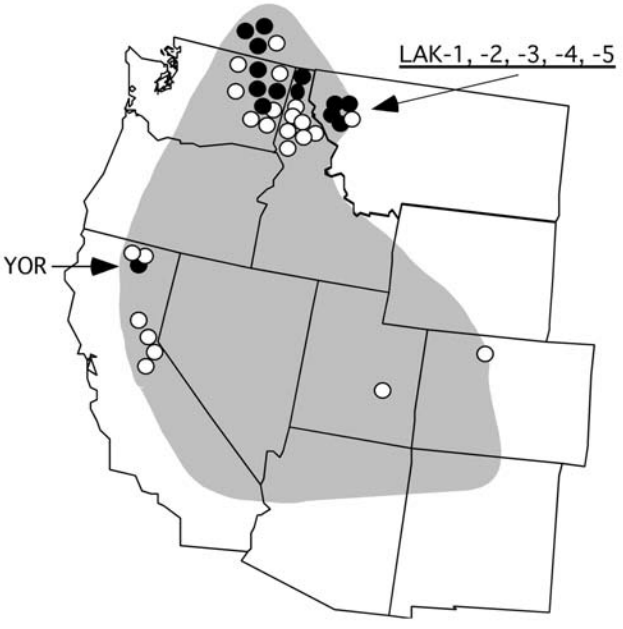

Figure 1. Western United States showing the approximate endemic range of tickborne relapsing fever associated with *Ornithodoros hermsi* and the localities of origin for the 37 *Borrelia hermsii* isolates included in this study. Genomic group I isolates are shown by open circle; genomic group II isolates are shown by filled circle. Localities of 6 isolates discussed in detail are indicated with arrows.

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loci were examined (*16S rRNA*, *flaB*, *gyrB*, and *glpQ*), which cumulatively totaled 5,197–5,203 bp per isolate. The 2 genomic groups of *B. hermsii* were also distinct from isolates of *B. turicatae* and *B. parkeri*, for which we undertook a similar analysis (*5*)**.** Bunikis and co-workers recently typed relapsing fever spirochetes based on the intergenic spacer (IGS) region of noncoding DNA located between the *16S rRNA* and *ileT* tRNA genes (*6*)**.** In their report, 4 IGS types were identified among 9 isolates or DNA extracted from tissues infected with *B. hermsii*. Given that less effort is needed to type *B. hermsii* with only the IGS sequence compared to several larger loci, we undertook an analysis of the IGS region in our isolates to determine its utility to define the 2 genomic groups in these spirochetes. Here we compare results obtained with the IGS locus to results obtained by multilocus sequence typing, which included recently acquired spirochetes from an outbreak of relapsing fever that were not examined previously. We use these data to discuss the geographic distribution of *B. hermsii* and explain how birds may help maintain and disperse these spirochetes in nature.

Materials and Methods

The *B. hermsii* examined in this study originated from infected humans (n = 32), *O. hermsi* ticks (n = 4), and 1 chipmunk (Table 1). Isolates were established by first inoculating laboratory mice (*Mus musculus*) and then passing infected mouse blood into BSK-H medium with 12% rabbit serum (Sigma-Aldrich, Saint Louis, MO, USA) (*4*)**.** Genomic DNA samples were prepared from pure cultures (*7*), and PCR amplification and DNA sequencing of *16S rRNA*, *flaB*, *gyrB*, and *glpQ* were completed as described (*4*)**.** The IGS sequences were determined by PCR amplification with primers IGS-F and IGS-R (*8*) and an initial heating at 96°C for 3 min, followed by 35 cycles with denaturation at 96°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. After the 35th cycle, an additional extension was done at 72°C for 7 min. DNA sequences of the amplicons were determined with primers IGS-F, IGS-R, Fn, and Rn (*8*).

Nucleotide sequences were analyzed with Sequencher 4.2 (Gene Codes Corp., Ann Arbor, MI, USA). DNA sequences were first aligned with the CLUSTAL V program in the Lasergene software package (DNASTAR Inc., Madison, WI, USA). Alignments were transferred into the MacClade program (*9*) and corrected manually. MacClade output files were opened in PAUP (*10*), and maximum-likelihood neighbor-joining trees were created. Alignments were also created with the DNasp package of algorithms (www.ub.es/dnasp) to calculate mean nucleotide diversity (π) per aligned base. A full heuristic search with 1,000 bootstrap replicates was performed to test the robustness of clade designations.

All stages of colony-reared *O. hermsi* were fed on hand-held 14-day-old chickens (*Gallus domesticus*) or 10-day-old northern bobwhite quail (*Colinus virginianus*) acquired from commercial hatcheries. Ticks were also fed on 5- to 10-day-old mice that were unrestrained in plastic jars with plaster-of-paris bases and screened lids. Nonfeeding ticks were kept at 85% relative humidity, 20°–22°C, natural photoperiod (Hamilton, MT, USA), and observed for development in their life cycle.

B. hermsii DAH and REN were tested first for infectivity in mice as described (*4*)**.** Next, 0.1 mL of blood with $\approx 5 \times 10^6$ spirochetes from each mouse was injected intraperitoneally into four 4-day-old chickens. The inoculum of DAH-infected blood was split between intraperitoneal and subcutaneous sites in 1 bird. The 8 birds were monitored for spirochetemia for 7 days postinoculation by intravenous collection of blood from the wing's brachial vein and darkfield microscopic examination (×400) of the wet, unstained blood. The Rocky Mountain Laboratories Animal Care and Use Committee approved the tick feeding and experimental inoculations (Protocol nos. 03–31 and 05–17).

Results

The phylogram based on the IGS sequences separated the 37 isolates of *B. hermsii* into genomic group I (GGI) and genomic group II (GGII) as defined previously (Figure 2) (*4*). Alignment of the sequences identified 3 indels (gaps resulting from insertions or deletions) of 1, 13, and 13 bp between the 2 groups. All GGI isolates contained an IGS sequence of 663 bp compared with 690 bp in all GGII isolates. Aligned sequences for all isolates, excluding the indels, demonstrated that the IGS region was more polymorphic and had greater nucleotide diversity between the 2 genomic groups than did the other loci (Table 2). IGS sequences varied little within each genomic group, with 5 polymorphic sites in GGI and only 2 polymorphic sites in GGII. GGI and GGII contained 5 and 3 sequence types, respectively (Table 1), with only a 1- or 2-bp difference within either group. IGS sequences were determined for 6 isolates of *B. parkeri* and 8 isolates of *B. turicatae* described previously (*5*) (GenBank accession nos. DQ855545–DQ855558) but are not discussed further in this study.

IGS sequences varied in their ability to identify unique genotypes associated with geographic clusters of isolates within each genomic group when compared to other loci. The phylogram based on *flaB* sequences was nearly identical to the IGS phylogram (data not shown). However, neither the IGS nor *flaB* sequences separated SIS and RAL from the other GGI isolates. SIS and RAL came from a tick and patient, respectively, from the same cabin in northern California (*11*). IGS and *flaB* sequences

Table 1. *Borrelia hermsii* isolates examined, history, origin, and IGS sequence types, western North America*

Isolate*	Year	Source	Locality	IGS type†
GGI				
HS1	1968	Tick	Spokane Co., WA	1
CON	1960s	Human	Sierra Nevada Mtns, CA	1
FRO	1987	Human	Eastern WA	1
DAH	1991	Human	Spokane Co., WA	1
FRE	1996	Human	Pend Oreille Co., WA	1
MIL	1996	Human	Kootenai Co., ID	1
BRO	1996	Human	Kootenai Co., ID	1
CAR	1996	Human	Benewah Co.,ID	1
BAK	1997	Human	Okanogan Co., WA	1
BYM	1997	Human	Kootenai Co., ID	1
RAL	1997	Human	Siskiyou Co., CA	1
SIS	1998	Ticks	Siskiyou Co., CA	1
HAL	1998	Human	Kootenai Co., ID	1
GAR	2001	Human	Okanagan Valley, BC	1
LAK-4	2004	Human	Lake Co., MT	1
MAT	2004	Human	Bitterroot–Selway Mtns, ID	1
DOU	2005	Ticks‡	Douglas Co., WA	1
EST-7	1996	Chipmunk	Larimer Co., CO	2
ALL	1997	Human	Duchesne Co., UT	2
WAD	1998	Human	Placer Co., CA	2
SWA	1996	Human	Kootenai Co., ID	5
ELD	2005	Ticks‡	Eldorado Co., CA	6
MAN	1960s	Human	Sierra Nevada Mtns, CA	7
GGII				
YOR	1964	Human	Siskiyou Co., CA	8
REN	1992	Human	Okanogan Co., WA	8
OKA-1	1995	Human	Okanagan Valley, BC	8
OKA-2	1996	Human	Okanagan Valley, BC	8
OKA-3	1996	Human	Okanagan Valley, BC	8
GMC	1997	Human	Stevens Co., WA	8
CMC	1997	Human	Stevens Co., WA	8
LAK-3	2004	Human	Lake Co., MT	8
LAK-5	2004	Human	Lake Co., MT	8
LAK-1	2002	Human	Lake Co., MT	9
LAK-2	2002	Human	Lake Co., MT	9
SIL	2002	Human	Boundary Co., ID	9
HAN	1990	Human	Boundary Co., ID	9
RUM	1997	Human	Stevens Co., WA	10

*GGI, genomic group I; GGII, genomic group II; WA, Washington; Mtns, mountains; Co., county; ID, Idaho; CA, California, BC, British Columbia; MT, Montana; CO, Colorado; UT, Utah.
†Each IGS sequence type is unique from the others and represented by the following isolate and GenBank accession nos.: type 1, DAH, DQ845746; type 2, ALL, DQ845747; type 5, SWA, DQ845746; type 6, ELD, DQ845745; type 7, MAN, DQ845748; type 8, YOR, DQ845744; type 9, LAK-1, DQ845742; type 10, RUM, DQ845743. Types 1 and 2 are identical to types of the same number in Bunikis et al. (AY515265 and AY515266, respectively) (*6*). No sequences matched types 3 and 4 identified by Bunikis and co-workers and are excluded here to avoid confusion. Types 5–10 are newly identified here.
‡DOU detected by PCR in a pool of 3 dead *Ornithodoros hermsi*. ELD based on spirochetes transmitted by *O. hermsi* to a laboratory mouse, followed by PCR of infected blood.

for both isolates were identical to most other GGI isolates. Yet, *gyrB* and *glpQ* sequences were identical in these 2 isolates and separated them from all or all but 1 of the other GGI isolates, respectively, from other localities (*4*).

Isolates LAK-1, LAK-2, SIL, and HAN in GGII had identical IGS sequences that were different from all other isolates in their genomic group by 1 base. These isolates originated from patients infected in western Montana (LAK-1 and LAK-2) and northern Idaho (HAN and SIL). The locations of exposure are only ≈110 miles (183 km) apart, which suggests a unique IGS genotype for this geographic cluster of isolates. These 4 isolates also contained a *flaB* allele unique from all other isolates, and LAK-1, LAK-2 and SIL contained a *gyrB* allele unique from the other isolates (*4*).

The *B. hermsii* isolates from 5 patients infected in 2 cabins on Wild Horse Island, Flathead Lake, Montana, USA, in 2002 and 2004 were especially intriguing. Both IGS and multilocus sequence analysis typed LAK-4 in GGI and LAK-1, –2, –3, and –5 in GGII. The 3 patients

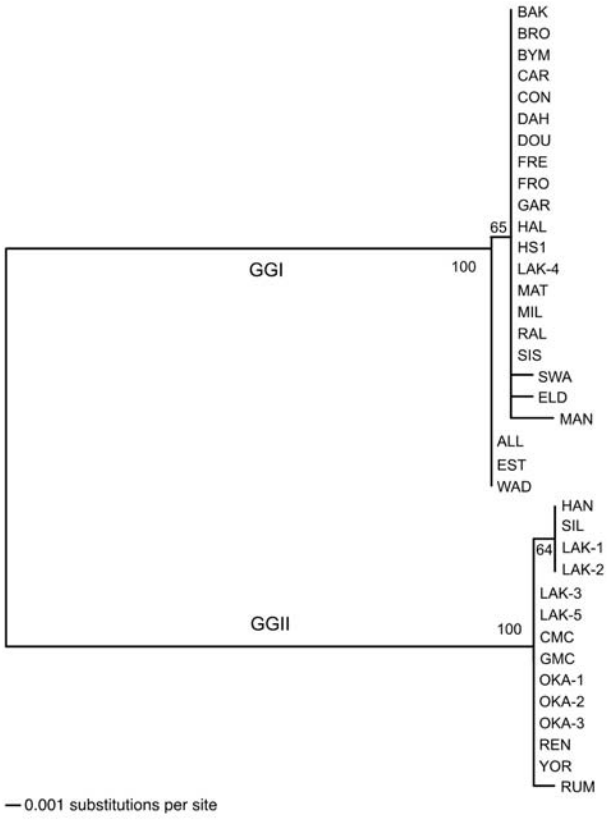

Figure 2. Phylogram of the intergenic spacer sequences of 37 *Borrelia hermsii* isolates. The tree was constructed with ClustalV and the neighbor-joining method with 1,000 bootstrap replicates. Numbers at the nodes are the percentages of bootstraps that supported this pattern. The scale bar for the branch lengths represents the number of substitutions per site. An unrooted tree is shown because a gap in the alignment with *B. turicatae* resulted in the removal of a polymorphic site in some GGII isolates of *B. hermsii*.

infected in 2004 slept in the same bed; GGI spirochetes were isolated from 1 patient, and GGII spirochetes were isolated from the others. Among the 4 GGII isolates, LAK-1 and LAK-2 originated from 1 cabin (2002) and had identical IGS, *16S rRNA*, *flaB*, *gyrB*, and *glpQ* sequences. LAK-3 and LAK-5 came from the other cabin (2004), and these isolates also had identical sequences for the 5 loci examined. However, LAK-1 and LAK-2 contained IGS, *flaB*, and *glpQ* sequences that each varied by 1 base compared to LAK-3 and LAK-5 sequences. The presence of 3 IGS, *flaB*, and *glpQ* sequences among the 5 isolates suggests multiple past introductions of *B. hermsii* to the island.

Another *B. hermsii* isolate of interest was YOR from California (*12*), which had an identical IGS sequence to most other GGII isolates (Figure 2). Multilocus sequencing also showed YOR was identical to isolates from southern British Columbia and northern Idaho (*4*). Therefore, this isolate originated far from where all other GGII isolates came (Figure 1).

The presence of multiple genotypes of *B. hermsii* on Wild Horse Island (nearest shoreline is 2 km from the mainland), and the finding of identical genotypes separated over large geographic distances, suggested that birds might play a role in dispersing these spirochetes in nature. For this to occur, birds must be suitable hosts for *O. hermsi*. Therefore, we attempted to feed various stages of *O. hermsi* on chickens and quail. Larvae, nymphs, and adults all fed within 10 to 30 minutes on the birds, and the ticks survived with a low proportion of deaths (Table 3). Female ticks laid viable eggs that produced larvae, which survived 7 months until they fed on mice. Larvae that fed on chickens molted to first nymphs, and these ticks fed on quail or mice up to 9 months later. These results demonstrated that these experimental birds were suitable hosts for all stages of *O. hermsi*.

Eight 4-day-old chickens were inoculated with *B. hermsii* and examined for spirochetemia levels for the next 7 days. Only the 1 bird injected subcutaneously had a detectable spirochetemia on day 3 postinoculation, with 2 spirochetes seen in 25 fields, which indicated that birds may be more susceptible to infection by this route of inoculation and also possibly by tick bite.

Discussion

The IGS region separated all isolates of *B. hermsii* into GGI or GGII defined by the other 4 loci. The IGS, *16S rRNA*, *flaB*, *gyrB*, and *glpQ* sequences each contained unique positions (signature bases) that were conserved among all isolates of each genomic group that are suitable for typing *B. hermsii* in 1 of the 2 groups (available in GenBank). However, the IGS sequences were more polymorphic between the 2 genomic groups than were the other loci, and the indels in this region created unique sequences in GGII isolates that were absent in GGI isolates. Thus, the IGS region is an efficient target for typing *B. hermsii* into 1 of the 2 genomic groups. Hovis and co-workers recently sequenced the factor H–binding locus, *fhbA*, in 24 of our 37 isolates of *B. hermsii* (*13*). However, possible horizontal transfer of this plasmid-encoded gene between spirochetes makes this gene unsuitable for genomic group typing.

Based on our results, the 9 isolates of *B. hermsii* that Bunikis et al. typed previously with the IGS sequence all belong to GGI, including the spirochete identified in the northern spotted owl (*Strix occidentalis caurina*) (*6,14*). Their type 2 isolates (AY515266) from New Mexico and Colorado had identical IGS sequences to isolates from Utah (ALL), Colorado (EST-7), and eastern California (WAD). Bunikis et al. also deposited an unpublished IGS sequence for a human isolate of *B. hermsii* from Idaho (LPO; AY515270), which they did not type but which is

Table 2. Descriptive statistics for 5 loci in *Borrelia hermsii* GGI and GGII, western North America*

Group	Locus†	Samples‡	Alleles	Bp	Indels§	Polymorphisms (%)	π¶
All isolates	IGS	37	8	663/690	3	54 (8.1)	0.03648
GGI	IGS	23	5	663	0	5 (0.75)	0.00088
GGII	IGS	14	3	690	0	2 (0.29)	0.00084
All isolates	*16S rRNA*	35	2	1,273	0	5 (0.39)	0.00194
GGI	*16S rRNA*	21	1	1,273	0	0	0
GGII	*16S rRNA*	14	1	1,273	0	0	0
All isolates	*flaB*	36	5	1,002	0	16 (1.6)	0.00634
GGI	*flaB*	22	3	1,002	0	5 (0.5)	0.00150
GGII	*flaB*	14	2	1,002	0	1 (0.1)	0.00044
All isolates	*gyrB*	35	5	1,902	0	40 (2.1)	0.00997
GGI	*gyrB*	21	4	1,902	0	3 (0.16)	0.00046
GGII	*gyrB*	14	1	1,902	0	0	0
All isolates	*glpQ*	36	9	1,020/1,026	1	37 (3.6)	0.01721
GGI	*glpQ*	22	6	1,020	0	4 (0.39)	0.00113
GGII	*glpQ*	14	3	1,026	0	2 (0.19)	0.00087

*GGI, genomic group I; GGII, genomic group II.
†Nucleotide sequences for *16S rRNA*, *flaB*, *gyrB*, and *glpQ* for isolates not included previously (*4*) have been deposited in the GenBank database with accession nos. DQ855527 to DQ855544.
‡The number of samples varies because only the IGS sequence was determined for DOU and only IGS, *flaB* and *glpQ* sequences were determined for isolate ELD.
§IGS with 3 indels of 1, 13, and 13 bp; *glpQ* with 1 indel of 6 bp.
¶π, mean nucleotide diversity at each aligned position.

identical to the IGS sequence in 9 of our GGII isolates (type 8). Our data, combined with those of Bunikis et al. (*6*), include 46 IGS sequences from *B. hermsii* isolates or *B. hermsii*–infected material. *B. hermsii* YOR is the only GGII isolate known from outside the inland Northwest where all other isolates of this group originated. Dispersal of spirochetes by birds could explain the occurrence of identical genotypes found in distant locations.

IGS sequences varied little within either genomic group, but as with the other loci the sequences were more polymorphic in GGI than in GGII. The 3 indels in this noncoding region could have arisen either by deletions in GGI or insertions in GGII, although the bias may be for deletions, as has been proposed for other bacterial genomes, including *B. burgdorferi* (*15*). The fewer polymorphisms in all loci examined, the larger IGS sequence, and the restricted geographic distribution of isolates all suggest that GGII is a more recent derivative of GGI.

Spirochetes in both genomic groups of *B. hermsii* are transmitted by the same species of tick (*4*), sympatric in the northern parts of their range, and pathogenic in humans. Evidence for horizontal transfer of the variable tick protein (*vtp*) gene between spirochetes in the 2 genomic groups suggests that dual infections have occurred in the same host, most likely ticks (*4*). Therefore, what might have driven the selection for 1 clone (GGII) of *B. hermsii* to diverge from another (GGI) is intriguing to consider. Might there have been a significant period of time when the populations were isolated from each other? Or might there be different primary enzootic vertebrate hosts that maintain these different spirochetes in nature? Only 1 isolate we examined (EST-7 in GGI) came from an enzootic host, a chipmunk in Colorado (*16*). The other isolates came from ticks or human patients, who are only accidental hosts for the spirochetes, so this tells us nothing about their vertebrate hosts in the wild.

The possible role of birds in the ecology and epidemiology of tickborne relapsing fever caused by *B. hermsii* in western North America is worthy of further investigation. The dogma for 70 years has been that pine squirrels (*Tamiasciurus* spp.) and chipmunks (*Tamias* spp.) are the primary vertebrate hosts of *B. hermsii* and its tick vector *O. hermsi* (*1*,*17*–*19*). During our investigation of the relapsing fever outbreak in Montana, we found *O. hermsi* and dead American robin chicks (*Turdus migratorius*) in nest material from the cabin's attic (*20*). An outbreak associated with another cabin on the same island in 2004 (source of isolates LAK-3, -4, and -5 in this report) led the infected family to suspect that barn swallows (*Hirundo rustica*) were the hosts of the infected ticks, although no investigation was done.

Few *O. hermsi* have been found in association with birds. In 1949, Gregson collected 26 *O. hermsi* in a bluebird (*Sialia* sp.) nest in southern British Columbia. Five fledgling bluebirds were in the nest, and most ticks had recently fed (*21*). Furman and Loomis reported 9 *O. hermsi* in a house sparrow (*Passer domesticus*) nest and 2 *O. hermsi* in a California gull (*Larus californicus*) nest at Mono Lake, California (*22*). However, the later record is probably accidental as extensive investigation of ticks in the gull colonies on the islands in Mono Lake has never found *O. hermsi*, except where humans slept (*23*,*24*).

B. hermsii was identified once in a wild bird, a northern spotted owl found dead in Kittitas County, Washington

Table 3. Number and stage of *Ornithodoros hermsi* fed on chickens, quail, or mice*

Cohort A	Cohort B	Cohort C
2 M, 2 F on chicken	310 L on chicken†	184 first N on chicken
127 L on mouse	261 first N on quail	179 second N on mouse
	42 first N on mice	
124 first N on mouse	84 second N on mice	169 third N on mouse

*M, males; F, females; L, larvae; N, nymphs.
†After larvae were fed on chickens, the first nymphs were fed on quail or mice. Some of the resultant second nymphs were used in other experiments, hence the smaller number of second nymphs fed on mice.

(*14*). Spirochetes were not isolated, but DNA extracted from the bird's liver contained 16S rRNA DNA that was 99.6% identical to *B. hermsii* sequences. The spirochete was subsequently called *B. hermsii* on the basis of its IGS sequence, which was compared to those of 8 isolates of this species (*6*). The authors stated that while a nest-associated transmission cycle was possible, the infection more likely resulted from direct transmission from an infected prey animal to the owl.

Little work has explored the susceptibility of birds to infection with relapsing fever spirochetes. *B. duttonii*, a cause of tickborne relapsing fever in Africa, produced a detectable spirochetemia level in chickens (*25*) and grew in chick embryos for 1 month (*26*). The inoculation experiments with *B. hermsii* in chickens suggest that some birds may be suitable hosts for this spirochete in the wild. Further experiments are needed to determine if birds become spirochetemic after being fed upon by infected ticks, and if spirochete densities become high enough in bird blood to facilitate their acquisition back to feeding ticks. Cavity-nesting birds and their young might be ideal hosts for both ticks and spirochetes and should be investigated as possible sources of human infection when established nests are present in tick-infested cabins.

Migratory birds might disseminate *B. hermsii* in nature, as has been found for *B. burgdorferi* and *B. burgdorferi*–infected ticks (*27–29*). Immature stages of *Ixodes scapularis* ticks feed for 3 to 6 days and thus can be transmitted long distances by birds. In contrast, all stages of *O. hermsi* are rapid feeders and are unlikely to be carried very far by birds. Nocturnal forest birds such as owls could possibly spread infected ticks or prey short distances while foraging at night. However, we believe that infected birds are more likely to disseminate spirochetes directly than by transporting infected ticks. The information gained from *B. hermsii* isolated from patients has been extremely informative but these spirochetes represent only a small segment of the true populations of these bacteria. More work is needed to acquire additional isolates of *B. hermsii* to determine more fully the true distribution of these spirochetes throughout their entire range and identify the species of enzootic hosts involved in maintaining the spirochetes in nature.

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Population-based Laboratory Surveillance for AmpC β-Lactamase–producing *Escherichia coli*, Calgary

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In the Calgary Health Region during 2000–2003, prospective, active, population-based laboratory surveillance for all cefoxitin-resistant *Escherichia coli* isolates was performed. Isolates were screened with an inhibitor-based disk test, and plasmid-mediated types were identified by multiplex PCR with sequencing. A total of 369 AmpC β-lactamase–producing *E. coli* isolates were identified; annual incidence rates were 1.7, 4.3, 11.2, and 15 per 100,000 residents for each year, respectively. AmpC β-lactamase–producing *E. coli* was 5× more likely to be isolated from female than male patients across all age groups except <1 year. Of these isolates, 83% were community onset, and urine was the principal site of isolation (90% of patients). PCR showed that 125 (34%) were positive for bla_{cmy} genes; sequencing identified these enzymes to be CMY-2. In this large Canadian region, AmpC β-lactamase–producing *E. coli* is an emerging community pathogen that commonly causes urinary tract infections in older women.

Organisms that produce plasmid-mediated AmpC β-lactamases were first reported in the 1980s (*1*). These β-lactamases are derivatives of the chromosomally encoded clavulanate-resistant AmpC cephalosporinases of bacteria such as *Enterobacter* spp., *Citrobacter freundii*, *Morganella morganii, Aeromonas* spp., and *Hafnia alvei* (*2*). These enzymes have been reported in *Escherichia coli*, *Klebsiella pneumoniae*, *K. oxytoca*, *Salmonella* spp., *Enterobacter aerogenes*, and *Proteus mirabilis.* Because the genes are typically encoded on large plasmids that contain additional antimicrobial resistance genes, therapeutic options are limited (*3*).

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E. coli possess a chromosomal gene that encodes for an AmpC β-lactamase. Usually, low amounts of β-lactamases are produced because the AmpC gene is regulated by a weak promoter and a strong attenuator. These *E. coli* isolates are sensitive to the cephamycins (*4*). However, surveys of resistance mechanisms in cephamycin-resistant isolates have identified promoter or attenuator mutations that result in the upregulation of AmpC β-lactamase production; these isolates are referred to as AmpC hyperproducers (*5*). Occasionally, cephamycin-resistant strains produce plasmid-mediated β-lactamases such as CMY-2, which are derived from bacteria with chromosomally encoded AmpC cephalosporinases (*3*). In addition, altered expression of outer membrane proteins constituting porins can also contribute to cephamycin resistance (*6*).

Methods for detecting *E. coli* AmpC hyperproducers or isolates that produce plasmid-mediated cephalosporinases are technically demanding for clinical laboratories. Although nonsusceptibility to the cephamycins suggests increased production of AmpC β-lactamases, organisms that produce these types of enzymes often go undetected and have been responsible for several nosocomial outbreaks (*2*,*7*).

Surveillance studies of organisms that produce plasmid-mediated AmpC β-lactamases, especially among community isolates, are needed (*8*). We noticed an increase in cephamycin-resistant *E. coli* isolates in the Calgary Health Region (CHR) during 2002 and 2003 (from 0.1% of all *E. coli* isolated in 2000 to 1.3% in 2003). To our knowledge, no surveillance studies have investigated the population-based epidemiology of AmpC β-lactamase–producing *E. coli* (i.e., hyperproducers or plasmid-mediated enzymes), although studies have shown the widespread distribution of

these isolates in Europe and North America (*2*,*9*–*11*). Our objectives were to define the population-based incidence of infections caused by *E. coli* that produce increased levels of AmpC β-lactamases in a large well-defined Canadian region and investigate whether plasmid-mediated types were present in this population.

Methods

Patient Population

CHR provides all publicly funded healthcare services to the >1 million persons residing in the cities of Calgary and Airdrie and numerous adjacent surrounding communities covering an area of 37,000 km² (www.health.gov.ab.ca/regions/RHA_comm3.html). Acute care is provided mainly through 1 pediatric hospital and 3 large hospitals for adults. A centralized laboratory (Calgary Laboratory Services; CLS) performs the routine clinical microbiology services for the community, e.g., nursing homes, physicians' offices, community collection sites (where outpatients submit specimens for investigation purposes), and hospital sites within the CHR. Our base study population consisted of all patients from whom cefoxitin-resistant *E. coli* was first identified by CLS from January 1, 2000, through December 31, 2003.

Population-based Surveillance.

Prospective, active, population-based, laboratory surveillance for all cefoxitin-resistant *E. coli* isolates was performed by CLS; all cefoxitin-resistant *E. coli* isolates were included in this study. We used the laboratory information system at CLS (PathNet Classic version 306, Cerner, Kansas City, MO, USA) to determine basic demographic information (age, sex, specimen submission site, date of hospital admission) and microbiologic data (location of isolate on patient and antimicrobial-susceptibility testing results) for all patients. Isolates were frozen and stored in batches for further analyses. Community-onset isolates were the first cultures obtained from outpatients or inpatients within 2 days of hospital admission. Hospital-onset isolates were the first cultures taken ≥2 days after admission to a hospital.

Clinical Microbiology and Molecular Laboratory Testing

We included consecutive nonduplicate isolates of *E. coli* that were intermediately resistant or resistant to cefoxitin (MIC>8 μg/mL) and that were collected at CLS during January 2000 through December 2003 and isolated from clinical specimens by standard microbiology techniques. During June 2001, we cultured urine samples that had positive screening results from an ATPase-luciferase assay and those specifically requested by a physician (*12*). Strains were identified to the species level by using Vitek (Vitek AMS; bioMérieux Vitek Systems Inc., Hazelwood, MO, USA.). MICs to the following drugs were determined by Vitek: imipenem, gentamicin, tobramycin, trimethoprim-sulfamethoxazole, and ciprofloxacin. Results were interpreted according to the Clinical and Laboratory Standards Institute criteria for broth dilution (*13*).

Clinical isolates of cefoxitin-resistant *E. coli* were tested for AmpC β-lactamases by using the combination of the AmpC β-lactamase inhibitor Syn 2190 and cefotetan disks as described (*14*). All isolates with an AmpC β-lactamase were further investigated for plasmid-mediated AmpC β-lactamase genes by using multiplex PCR conditions and primers as described (*15*). These included enzymes that originated from the chromosomally encoded AmpC cephalosporinases of bacteria. Genes of the CMY-positive isolates were identified by cycle sequencing the full-length amplified products with conditions and different primers as described (*16*).

Analysis

All analyses were performed by using Stata version 9.0 (Stata Corp., College Station, TX, USA). Variables were assessed before analysis by using histograms to identify underlying distribution. Means with standard deviations were used to describe normally or near normally distributed variables and were compared by using the Student *t* test. Medians with interquartile ranges (IQRs) were used to describe nonnormally distributed variables and were compared by using the Mann-Whitney U test. Differences in proportions were compared by using the Fisher exact test. Incidence rates (per 100,000 population per year) were calculated by using the annual number of new cases among CHR residents as the numerator and regional population estimates for each year from 2000 through 2003 (December 2003 boundaries) of the CHR as the denominator. Patients with Alberta healthcare numbers were considered CHR residents and were included; those with out-of-province healthcare numbers were excluded. Age- and sex-specific incidence rates were calculated by dividing the number of new cases within a subgroup by the population at risk. Risk ratios (RR) for incidence rates among demographic subgroups were calculated by dividing the incidence rate with the factor (as opposed to without) and were reported with 95% confidence intervals (CIs) as described (*17*).

Results

During the 4-year study period, 78,275 *E. coli* isolates were obtained from 51,735 patients; 72,756 (93%) isolates were classified as community onset and 5,519 (7%) as hospital onset. For 408 (0.7%) patients, a cefoxitin-resistant *E. coli* isolate was identified. The number of isolates (per first

isolate per patient per year) increased significantly each year: 23 (0.1%) of 17,989 patients in 2000, 53 (0.3%) of 15,907 in 2001, 141 (1%) of 14,583 in 2002, and 191 (1.3%) of 14,319 in 2003 ($p<0.0001$ for 2000 compared with 2003). Of the 408 cefoxitin-resistant isolates, 384 (94%) were available for further analysis. Of these, 369 (96%) were positive for AmpC β-lactamases according to the Syn 2190 inhibitor disk screen test, and of the 369, 359 (97%) were from CHR residents. The number of isolates identified in CHR residents during 2000, 2001, 2002, and 2003 were 18, 46, 123, and 172, with annualized incidence rates of 1.7, 4.3, 11.2, and 15 per 100,000, respectively.

Seasonal variability in the occurrence of AmpC β-lactamase–producing *E. coli* isolates was moderate; the lowest rates of isolation were in early winter and spring, and the highest rates were in late summer and fall (Figure 1). Of the 369 isolates, 61 (17%) were classified as hospital onset. Of the 308 (83%) community-onset isolates, 54 were submitted from hospital emergency departments, 24 from inpatients within the first 2 days of admission, 20 from nursing home residents, and the rest from outpatients. While the number of hospital-onset AmpC β-lactamase–producing *E. coli* isolates increased gradually during 2000–2003 (5, 12, 20, 24 each year, respectively), the number of community-onset isolates increased dramatically (13, 35, 108, 152 each year, respectively). The increase among the acute care centers was not the result of clustering of patients in a specific acute care center. However, in relation to all first *E. coli* isolates per year per patient tested by CLS, AmpC β-lactamase–producing *E. coli* were proportionally more likely to be obtained as hospital-onset isolates (Figure 2).

The median age of the cohort was 51.1 (IQR 27.3–74.3) years; most (310; 84%) patients were female. Incidence of AmpC β-lactamase–producing *E. coli* significantly increased in association with increasing age (Figure 3). Risk for isolation of AmpC β-lactamase–producing *E. coli* was 5× higher for female than male residents (14.0 vs. 2.6 per 100,000 per year; RR 5.4; 95% CI 4.04–7.31; $p<0.0001$); this increased risk was observed across all age groups with the exception of the very young (< 1 year) (Figure 3).

Among the 369 AmpC β-lactamase–producing isolates, the principal site of isolation was the urinary tract for 333 (90%) patients, bloodstream for 20 (5%), respiratory tract for 8 (2%), soft tissue for 5 (1%), and abdomen for 3 (1%). Of these same 369 isolates, 73 (20%) were not susceptible to trimethoprim-sulfamethoxazole, 32 (9%) to tobramycin, 54 (14%) to gentamicin, and 33 (9%) to ciprofloxacin. No resistance to imipenem was detected.

Multiplex PCR amplified a 462-bp amplicon among 125 (34%) of the 369 AmpC β-lactamase–producing isolates that was consistent with the plasmid-encoded

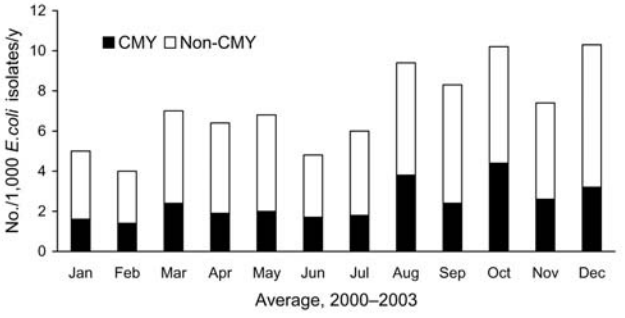

Figure 1. AmpC β-lactamase–producing *Escherichia coli* isolates per 1,000 *E. coli* isolates, Calgary Health Region, 2000–2003. Data are averaged over the 4-year period. The presence of plasmid-mediated AmpC β-lactamase genes was determined by using multiplex PCR conditions and primers as described (*8*). CMY; isolates positive for chromosomal gene of *Citrobacter freundii;* non-CMY; isolates negative by multiplex PCR.

types of AmpC β-lactamases originating from the chromosomal gene of *Citrobacter freundii* (CMY types) (*15*). No other types of plasmid-mediated AmpC enzymes were present. Sequence analysis of full-length PCR products on 15 randomly selected isolates showed 100% identity to bla_{CMY-2} (*18*). The Table shows features of CMY-type and non–CMY-type AmpC β-lactamases. With the exception of a higher rate of gentamicin resistance among CMY-2–positive strains (Table), *E. coli* isolates that produced CMY-types and those that produced non-CMY types of AmpC β-lactamases did not differ according to year of study, principal site of isolation, or demographics.

Conclusion

Limited data are available regarding hyperproduction of AmpC β-lactamases among *E. coli* in the United States

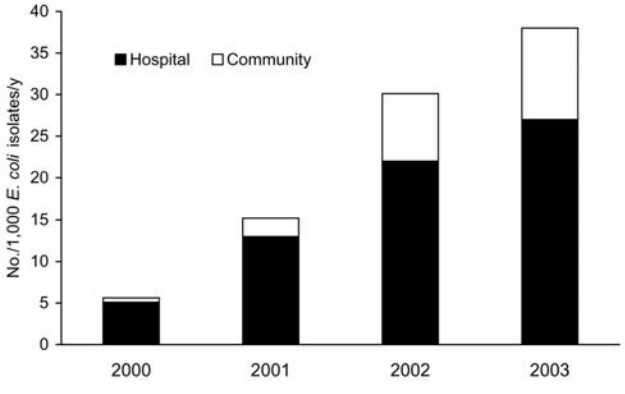

Figure 2. First AmpC β-lactamase–producing *Escherichia coli* isolates per 1,000 *E. coli* isolates per year. Calgary Health Region, 2000–2003. Community isolates were those obtained from outpatients or admitted patients who had their first cultures obtained within 2 days of hospital admission. First cultures from other hospitalized patients obtained after 2 days of admission were deemed to represent hospital-onset isolates.

Table. Features of *Escherichia coli* β-lactamases isolated from Calgary Health Region, 2000–2003

Feature*	CMY types (n = 125), no. (%)	Non-CMY types (n = 244), no. (%)	p value
Female	105 (84)	205 (84)	1.0
Community-onset	104 (83)	204 (84)	1.0
Resistant to			
Ciprofloxacin	10 (8)	23 (9)	0.6
Gentamicin	28 (22)	26 (11)	0.004
Tobramycin	13 (10)	19 (8)	0.4
Trimethoprim-sulfamethoxazole	29 (23)	44 (18)	0.3

*Median age for those with CMY types was 54.3 (interquartile range 27.7–76.1); median age for those with non-CMY types was 50.5 (interquartile range 26.6–73.3).

and Canada. A study from Canada showed that most cephamycin-resistant *E. coli* from the Toronto area in 2001 had different promoter and attenuator mutations in the chromosomal AmpC cephalosporinases (*5*). Jacoby and colleagues found plasmid-mediated AmpC-type resistance in 7 of 75 of ceftazidime-resistant *E. coli* from 25 US states; 2 of these isolates produced CMY-2 (*19*). Mulvey and colleagues studied 232 cefoxitin-resistant *E. coli* from 12 hospitals in Canada and found that 25 (11%) strains contained CMY-2 and 51 (22%) had different promoter and attenuator mutations (*20*). *E. coli* that produce CMY-2 have also had been isolated from food-producing animals in Canada and the United States (*21*,*22*).

Laboratory tests that use inhibitors of AmpC β-lactamases in *E. coli* successfully distinguish between isolates that have altered expression of outer membrane proteins and isolates that produce increased levels of AmpC β-lactamases (*23*,*24*). Multiplex PCR that detects the different types of plasmid-mediated AmpC β-lactamases is the most practical way to differentiate between isolates with promoter or attenuator mutations and those with plasmid-mediated cephalosporinases (*15*). Our study screened all cefoxitin-resistant *E. coli* for AmpC β-lactamases and used multiplex PCR to identify plasmid-mediated types. However, the cephalosporinases that originated from *H. alvei* (e.g., ACC types) are not detected by our phenotypic method. In our study, >90% of cefoxitin-resistant strains produced increased levels of AmpC cephalosporinases; 125 (34%) of these 369 were positive for CMY-2, much higher than the 11% reported by Mulvey et al. (*20*). None of the other plasmid-mediated AmpC types were present in *E. coli* isolated from patients in the CHR.

Some studies have recognized a role of AmpC β-lactamase–producing *E. coli* in nosocomial infections (*2*,*3*,*7*); however, these studies were based at institutions and did not survey community-based laboratories. Because our surveillance included all clinical specimens from hospital and community sites, we are highly unlikely to have missed many isolates. We observed that in the CHR, AmpC β-lactamase–producing *E. coli* is predominantly a community-onset pathogen. The designs of other studies in the literature (*19*,*20*) make it unclear whether AmpC β-lactamase–producing *E. coli* is an important cause of community-onset infections elsewhere. A community outbreak in CHR during 2002 resulted from CMY-2–producing *Salmonella enterica* serotype Newport associated with the handling of pet treats (*25*). A previous study from our center has shown that these salmonella isolates share similar-size plasmids with CMY-2–producing *E. coli* of multiple pulsed-field gel electrophoresis types identified in our study (D.B. Gregson, unpub. data). Thus, *E. coli* and *Salmonella* spp. may share similar plasmids.

Several investigations have shown that animals may represent a source for dissemination of AmpC-encoding genes from *E. coli* to humans. Evidence of CMY-2–producing isolates in cattle (*26*), pork (*27*), poultry (*21*,*28*), and dogs and cats (*29*) is of concern because food-producing animals and domestic pets may act as reservoirs for resistant organisms. Therefore, factors that lead to the high rate of isolation of AmpC β-lactamase–producing *E. coli* in patients from the community require further exploration.

Ours is the first report of the population epidemiology of AmpC β-lactamase–producing *E. coli*. We restricted our study to *E. coli* because other plasmid-mediated AmpC-producing organisms are rare in our region (only 17 patients infected with AmpC-producing *K. pneumoniae* and 12 with AmpC-producing *Salmonella* spp. were identified at CLS during this study period). We determined demographic risk factors associated with the isolation of AmpC β-lactamase–producing *E. coli* by comparing patient demographic characteristics with our well-defined base population. In these analyses, female and older patients were at much higher risk than male and younger patients (Figure 3). These results are similar to those we obtained in a previous study from CHR that investigated the population epidemiology of infections caused by extended-spectrum β-lactamase (ESBL)–producing *E. coli* during 2000–2002 (*30*). Incidence of ESBL-producing isolates was stable for the 3 years; incidence rates in 2000, 2001, and 2002 were 5.0, 5.6, and 5.7 per 100,000, respectively. These rates differ from those for AmpC β-lactamase–producing *E. coli* in this study, which had rates of 1.7, 4.3, and 11.2 per 100,000 for these 3 years, respectively. Our previous study also showed that most ESBL-producing *E. coli* from our region isolated during the same period were resistant to gentamicin and ciprofloxacin (*30*);

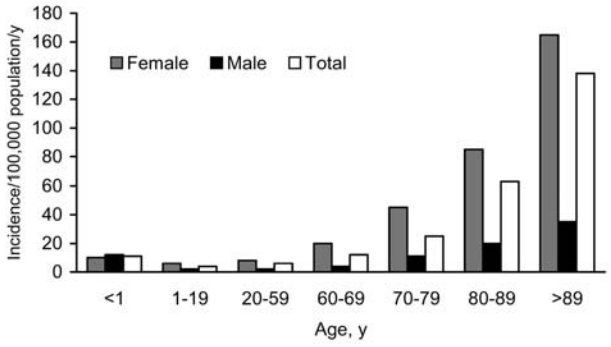

Figure 3. Age- and sex-specific incidence of AmpC β-lactamase–producing *Escherichia coli* isolates per 100,000 population, Calgary Health Region, 2000–2003.

in our current study, only 14% and 9% of AmpC β-lactamase–producing isolates were resistant to gentamicin and ciprofloxacin, respectively. Thus, susceptibility patterns differ between ESBL- and AmpC β-lactamase–producing *E. coli* from the CHR isolates during the same period.

The population-based design has some methodologic limitations. First, because this was a laboratory-based study, detailed clinical information (e.g., prior receipt of antimicrobial drugs, travel, exposure to food and water, underlying concurrent conditions) was not available. We were therefore unable to determine whether the isolates in this study truly caused infection. The lack of detailed clinical information is an inherent limitation to all laboratory-based studies. Second, isolates were defined as either community- or hospital-onset on the basis of their location of submission. Although this may in part reflect where these organisms were acquired, some isolates classified as community onset may have been associated with healthcare (*31*). Third, incidence rates were based on the assumption that all persons with Alberta healthcare numbers were CHR residents. We estimate that 10%–15% of patients in this study may have resided within other health regions in Alberta rather than within CHR. As a result, our incidence rates may be slightly higher than the true values.

In conclusion, this study demonstrates that AmpC β-lactamase–hyperproducing *E. coli* is an emerging community pathogen in the CHR with public health implications. Our results warrant increased efforts at surveillance for and the study of risk factors associated with the acquisition of these isolates in order to guide future prevention and control measures

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Effectiveness of Neuraminidase Inhibitors for Preventing Staff Absenteeism during Pandemic Influenza

Vernon J. Lee* and Mark I. Chen*

We used a deterministic SEIR (susceptible-exposed-infectious-removed) meta-population model, together with scenario, sensitivity, and simulation analyses, to determine stockpiling strategies for neuraminidase inhibitors that would minimize absenteeism among healthcare workers. A pandemic with a basic reproductive number (R_0) of 2.5 resulted in peak absenteeism of 10%. Treatment decreased peak absenteeism to 8%, while 8 weeks' prophylaxis reduced it to 2%. For pandemics with higher R_0, peak absenteeism exceeded 20% occasionally and 6 weeks' prophylaxis reduced peak absenteeism by 75%. Insufficient duration of prophylaxis increased peak absenteeism compared with treatment only. Earlier pandemic detection and initiation of prophylaxis may render shorter prophylaxis durations ineffective. Eight weeks' prophylaxis substantially reduced peak absenteeism under a broad range of assumptions for severe pandemics (peak absenteeism >10%). Small investments in treatment and prophylaxis, if adequate and timely, can reduce absenteeism among essential staff.

Concerns regarding the advent and impact of the next influenza pandemic have led >120 countries to develop pandemic preparedness plans (*1*). Studies have shown that treatment with neuraminidase inhibitors and prophylaxis of selected subpopulations are cost-effective strategies to limit the pandemic's impact on the healthcare system (*2*,*3*). However, supplies of neuraminidase inhibitors are limited, and countries may not have the financial resources to purchase large stockpiles. Policymakers will thus have to determine priorities for treatment and prophylaxis.

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One priority is to maintain essential services during the pandemic's peak—to ensure business continuity and mitigate the resultant damage. Absenteeism of essential staff from work should be minimized to prevent service disruption when most needed. This is particularly crucial for healthcare workers (HCWs) because they may have an increased risk for exposure and illness while facing a surge in demand for healthcare services.

A recent study proposed that hospitals should consider stockpiling neuraminidase inhibitors for treatment and prophylaxis (*4*). To provide policy guidance to reduce the pandemic's impact on HCWs, this study analyzed the use of neuraminidase inhibitors in minimizing absenteeism by simulating an HCW population in a transmission dynamics model.

Methods

Model Structure and Dynamics

We used a deterministic, modified SEIR (susceptible-exposed-infectious-removed) meta-population model to evaluate strategies for minimizing absenteeism among HCWs during an influenza pandemic. The model consisted of 2 distinct populations in Singapore: the general population and an HCW population (Figure 1A). Singapore's mid-year population in 2005 was 4.35 million, and the public HCW population of 20,000 represented essential staff that required protection. Oseltamivir was the neuraminidase-inhibitor modeled because of its effectiveness in treatment and prophylaxis, good safety profile, and common use in national stockpiles (*5*–*8*). Standard treatment regimen was 75 mg, twice per day for 5 days, and prophylaxis required 75 mg once per day for as long as planned.

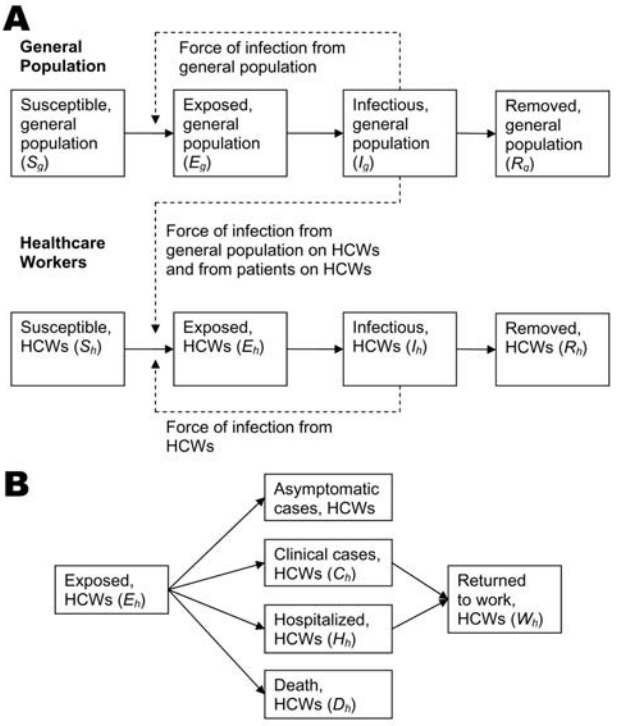

Figure 1. A) Modified SEIR (susceptible-exposed-infectious-removed) model for transmission of pandemic influenza within the general population and healthcare worker (HCW) subpopulation. B) Absenteeism among exposed HCWs.

This study assumed that the general population did not receive treatment or prophylaxis with oseltamivir. Three strategies for HCWs were considered: no action (providing symptomatic relief), treatment only (early treatment of all symptomatic HCW infections), and prophylaxis (prophylaxis together with early treatment). Different predetermined prophylaxis substrategies were considered, based on the weeks of prophylaxis; each additional week required 140,000 doses in addition to separate treatment stockpiles. To be conservative, we assumed that prophylaxis stockpiles would last only for the planned duration. Separate analyses explored the effect of stopping prophylaxis after individual clinical infection, with redistribution of prophylaxis doses to other HCWs to prolong prophylaxis beyond the planned duration; however, this strategy is only possible if tests can promptly confirm individual infection and logistics networks allow for redistribution.

We assumed that all persons were susceptible to the pandemic virus and that the general population epidemic occurred as a single wave after introduction of a single infectious case. We ignored the contribution of new introductions after the start of the epidemic. Persons were removed from the susceptible state, after infection, through recovery or death (Figure 1A). Births, deaths from other causes, immigration, and emigration during the period were assumed to be negligible.

We assumed a range of infectious periods similar to those from other studies; we also assumed that the disease was infectious at about the same time a person became symptomatic; i.e., the latent period coincided with the incubation period (*9*,*10*). A range of basic reproductive numbers (R_0), based on these infectious and latent periods, were then used to generate epidemics in the general population with varying rates of transmission. These R_0 then determined the course of the HCW epidemic.

HCWs were assumed to be exposed to influenza from 3 sources and may be more likely to be exposed than the general population (*11*). The first source was exposures from colleagues (HCW-to-HCW transmission) at a proportion (ω); the second was from persons outside the workplace ($1-\omega$). In the absence of published estimates, the base case assumed that 50% of infections were attributed to HCW-to-HCW transmission, with sensitivity analysis performed from 20% to 80%. The third source was from general population case-patients (patient-to-HCW transmission), expressed as the ratio of susceptible HCWs who could be infected by incident case-patients who sought treatment from the healthcare system (H/P). The extent of transmission is dependent on interventions such as barrier precautions (*11*). On the basis of findings from exploratory analysis, increasing the H/P ratio moves the HCW epidemic earlier; at an H/P of 2.08, the HCW epidemic peaks before the start of prophylaxis, negating the outcomes of prophylaxis. Therefore, H/P values >2 do not substantially contribute to the outcomes and study conclusions, and sensitivity analysis was performed for H/P from 0 to 2 (online Technical Appendix, available at www.cdc.gov/EID/content/13/3/449_app.htm). Transmission from HCWs to patients was assumed negligible compared with other sources of infection for the general population, and the general population epidemic was independent of transmission dynamics within the HCW population.

Once infected, an HCW would have 4 outcomes based on absenteeism (Figure 1B). Those with asymptomatic infection were assumed to be fit for work. Absenteeism due to symptomatic infection, hospitalization, and death was determined for the different strategies. The study assumed that all HCWs were absent from work while symptomatic and that prophylaxis reduced HCW-to-HCW transmission (*9*). Each scenario was further analyzed on the basis of different R_0; the disease's incubation and infectious periods were kept constant.

Pandemic Duration and Prophylaxis Initiation

The point of local detection of pandemic influenza depends on various factors and is unknown. Approximately 2,800 cases of influenzalike illness (ILI)

occur per day in Singapore (*2*), of which a small fraction is sampled for virologic surveillance (*12*). The base case assumed that the pandemic influenza subtype would be detected when incident symptomatic cases exceeded 10% of baseline ILI rates. The pandemic duration was defined as the period when incident pandemic influenza cases remained above this stated level. Prophylaxis was given to HCWs at the time of disease detection and continued for the planned duration. We conducted sensitivity analysis for starting prophylaxis on introduction of the first case and when incident cases exceeded 1%–100% of the baseline ILI rate.

Other Input Parameters

The input parameters for analysis (Table 1) were obtained from local sources when available as detailed in a previous study on stockpiling strategies in Singapore (*2*). Other values were obtained from international sources. To account for uncertainties, wide ranges were used for analysis.

HCWs were assumed to be adults 20–64 years of age with a mix of persons at low and high risk for influenza complications similar to that in the general population. Hospitalization and case-fatality rates were estimated for a pandemic of average severity (*2*). To account for the effect of severe pandemics, a scenario using death rates from the 1918 "Spanish flu" (5% average) and correlated hospitalization rates was performed (*19*).

Outcome Variables and Sensitivity Analysis

Outcome variables from the analyses included pandemic duration, peak staff absenteeism, and days with

Table 1. Parameters of neuraminadase inhibitor stockpiling strategies model*

Parameter	Notation†	Minimum‡	Base case‡	Maximum‡	Reference
Input					
General population	N_g		4,350,000		(*13*)
Healthcare staff	N_h		20,000		Estimated
ILI rate, per day	ι		2,800		(*2*)
Transmission dynamics					
Incubation and latent period, d	α	1.0	2.0	3.0	(*9*,*10*)
Infectious period, d	γ	1.5	4.1	7.0	(*9*,*10*)
Reproductive number	R_0	1.5	2.5	6.0	(*9*,*14*)
Transmission probability/d	β	0.37	0.61	2.0	Calculated, R/γ
HCW-to-HCW transmission	ω	0.2	0.5	0.8	See text
HCW infections caused by incident cases of clinical influenza (H/P)	δ	0		2.0	See text
Detection threshold, proportion of baseline ILI rate	ν	Introduction of 1st case	0.1	1	See text
Disease severity and antiviral efficacy					
Hospitalization rate (HCW)/100,000 infected§	η	12.4	88.6	186.7	(*2*)
Length of stay and medical leave if hospitalized, d	ϕ	9.0	12.0	20.0	(*2*)
Case-fatality rate (HCW)/100,000 infected§	μ	1.9	20.3	65.1	(*2*)
Proportion of infected persons without prophylaxis who have symptoms	θ_1	0.50	0.67	0.80	(*9*,*15*)
Oseltamivir efficacy for preventing infection in exposed persons	ε_1	0.28	0.35	0.52	(*9*,*16*,*17*)
Oseltamivir efficacy for preventing disease in infected persons	ε_2	0.5	0.6	0.9	(*2*,*9*)
Oseltamivir efficacy for preventing transmission of infection by infected persons	ε_3	0.6	0.8	0.98	(*9*)
Proportion of infected persons receiving oseltamivir prophylaxis who have symptoms	θ_2	0.07	-	0.2	Calculated, $\theta_2 = \theta_1(1-\varepsilon_2)$
Medical leave without treatment, d	σ	2	4	5	(*2*)
Reduction in medical leave with oseltamivir treatment, d	χ	0.1	1.0	2.0	(*2*)
Reduction in hospitalization or case-fatality rate with treatment	ψ	0.4	0.6	0.8	(*2*,*18*)

*HCW, healthcare workers, ILI, influenzalike illness.
†Notations are used in the equations listed in the Appendix.
‡Base case values are given with the minimum and maximum values used in the model where applicable.
§Based on hospitalizations and deaths among those with clinical influenza.

absenteeism >5%. For parameters relating to disease severity and antiviral efficacy, 1-way sensitivity analysis was performed to determine the effect on outcomes. In addition, Monte Carlo simulation analysis, with 1,000 iterations per scenario, was performed with the range of parameter estimates modeled as triangular distributions. For parameters pertaining to transmission dynamics, separate analyses were performed to determine the effects of variations in HCW-to-HCW and patient-to-HCW transmission. We also tested the outcome effects of assuming different latent and infectious periods. Epidemics with similar R_0 but different latent and infectious periods have different growth rates. To facilitate comparison between epidemics with different latent and infectious periods, both epidemic growth rates and R_0 values were presented. The relationship between latent and infectious period, R_0, and growth rates was described by Mills et al. (*14*) and elaborated in the Online Technical Appendix. Finally, the outcomes were determined for the various strategies upon initiation of prophylaxis at different times.

We used Berkeley-Madonna 8.3 software (University of California, Berkeley, CA, USA) to run the model. Details of the equations are shown in the Appendix; additional methods and results are shown in the Online Technical Appendix.

Results

The epidemic curve for a base-case pandemic with R_0 of 2.5 had a 12-week duration (Figure 2). When no action was taken, peak HCW absenteeism was ≈10%. Treatment only, using 121,000 doses of oseltamivir, decreased peak absenteeism to 8%. Prophylaxis for 4 weeks required 117,000 treatment doses in addition to 560,000 dedicated prophylaxis doses (equivalent to treatment courses for 1.6% of the general population) and led to higher peak absenteeism than treatment only. Eight weeks of prophylaxis required 52,000 treatment doses in addition to 1.12 million dedicated prophylaxis doses (equivalent to treatment courses for 2.7% of the general population) and reduced peak absenteeism to ≈2%; the peak occurred as a secondary increase after termination of prophylaxis. Discontinuing prophylaxis for clinical infections and redistributing stockpiles to prolong prophylaxis in other HCWs did not provide additional outcome benefits because the doses saved were insignificant; >96% were used during the preplanned duration for the relevant scenarios. From the Monte Carlo simulation of peak absenteeism for different strategies in a pandemic with R_0 of 2.5, with varying disease severity and antiviral efficacy parameters, 6 weeks of prophylaxis was sufficient under all scenarios to have a net benefit over treatment only (Figure 3).

One-way sensitivity analyses showed that the following input parameters had the most effect on peak absenteeism: "days of medical leave without treatment," with 15%–96% variation from the baseline outcome, depending on the R_0 and strategy used; "reduction in medical leave with treatment" with 22%–61% variation; "symptomatic proportion in infected persons without prophylaxis" with 19%–25% variation; and "oseltamivir efficacy in preventing disease in infected persons" with 21%–87% variation. Other input parameters had less effect on the outcome.

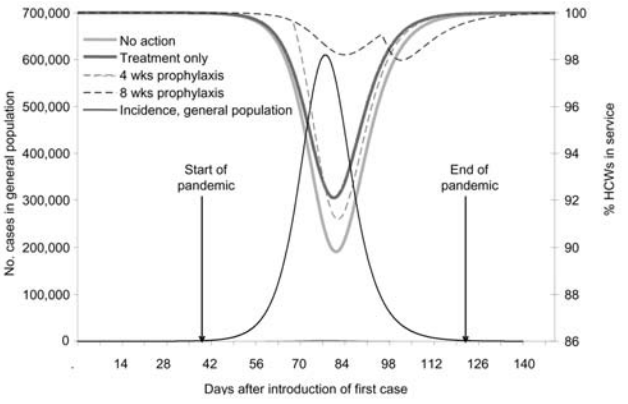

Figure 2. Dynamics of population infections and the effect of different strategies on absenteeism among healthcare workers for a base-case pandemic.

Table 2 shows the outcomes for pandemics with different R_0. If no action was taken for pandemics with $R_0 \geq 2$, absenteeism exceeded 5% for >15 days. In pandemics with lower R_0 (≤2), pandemic durations were longer and peak absenteeism did not exceed 10%. Treatment only in these pandemics reduced peak absenteeism by as much as 25% compared with no action. However, prophylaxis of ≈8 weeks did not accrue substantial benefits over treatment only.

Pandemics with higher R_0 (≥4) were of shorter durations; peak absenteeism was >20% in some scenarios. Treatment only reduced peak absenteeism by >15%, and 6 weeks of prophylaxis was sufficient to reduce peak absenteeism by >75% over no action. Across all R_0, insufficient durations of prophylaxis increased peak absenteeism compared with results for treatment only.

During a pandemic similar in severity to the 1918 influenza pandemic, with a 5% mortality rate and R_0 of 4 (*14*), peak absenteeism reached 20% with no action; hospitalizations and deaths contributed substantially to absenteeism, unlike the situation in less severe pandemics. The 3 strategies—treatment only, 4 weeks of prophylaxis, and 6 weeks of prophylaxis—reduced peak absenteeism by 25%, 43%, and 80%, respectively.

We also tested the adequacy of prophylaxis for a base-case pandemic under different scenarios for HCW-to-HCW and patient-to-HCW transmission. Higher HCW-to-HCW transmission resulted in an increased postprophylaxis epi-

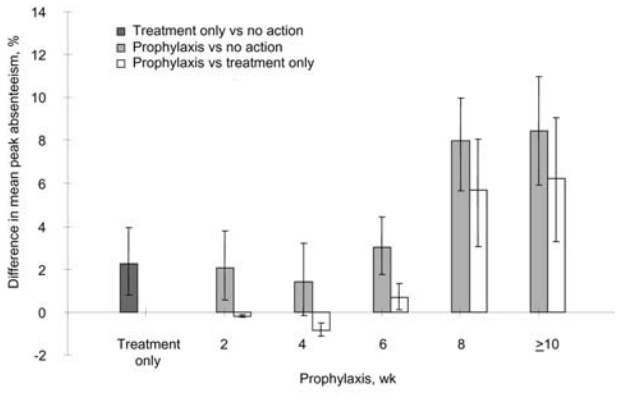

Figure 3. Simulation analysis of the difference in mean peak absenteeism for different strategies in an R_0 = 2.5 (base-case) pandemic (50th percentile shown in solid bars with the 5th and 95th percentiles shown in error bars).

demic peak. The HCW epidemic coincided with the general population epidemic if the patient-to-HCW infections variable was minimized (H/P = 0). Increasing H/P alone shifted the HCW epidemic such that it preceded the general population epidemic and amplified peak absenteeism by as much as 1.4× for the base case. For the prophylaxis strategies, increasing the patient-to-HCW transmission resulted in the distribution of HCW absenteeism away from the postprophylaxis period into the pre- and intraprophylaxis periods, which resulted in lower peak absenteeism up to a point. For H/P >2.0, peak absenteeism occurred before initiation of prophylaxis, negating the effect of longer durations of prophylaxis. Under all HCW-to-HCW and patient-to-HCW transmission scenarios for a base-case pandemic, 6 weeks of prophylaxis provided equal or superior results to treatment only; 8 weeks of prophylaxis was always superior (Online Technical Appendix).

Figure 4 shows the changes in peak absenteeism when latent and infectious periods were varied. For any rate of growth, assuming different latent periods changed peak absenteeism by <1% for most scenarios; assuming longer infectious periods increased peak absenteeism by <3%. However, epidemics with higher growth rates for any latent and infectious periods increased peak absenteeism by >10% when no action was taken. Although changes in the transmission parameters substantially changed peak absenteeism levels for certain scenarios, the overall conclusions remained similar. For epidemics with low peak absenteeism (<10%) and prolonged duration (low growth rate), prophylaxis strategies were less effective than treatment only. In contrast, for epidemics with higher peak absenteeism (>10%) and shorter duration (high growth rate), prophylaxis of ≥6 weeks was superior to treatment only.

Figure 5 shows the adequacy of prophylaxis for a base-case pandemic under different prophylaxis initiation points based on pandemic detection. Earlier detection and prophylaxis initiation resulted in a greater likelihood that shorter durations of prophylaxis would be ineffective. If prophylaxis were initiated on entry of the first pandemic case, 14 weeks of prophylaxis would be required for maximal benefit. Prophylaxis for 6 weeks was more effective than treatment only if it was initiated when incident pandemic cases in the general population exceeded 10% of the ILI rate, whereas 8 weeks of prophylaxis was effective when incident pandemic cases exceeded 1%.

Discussion

During an influenza pandemic, essential services such as healthcare must be maintained, especially during the pandemic's peak, when the maximal number of patients require care, and healthcare services can ill afford absenteeism due to infection. Absenteeism may also occur for reasons such as background illnesses and the need to care for ill relatives. During the severe acute respiratory syndrome epidemic in Singapore in 2003, schools were closed for weeks. Although no study documented the resultant workplace absenteeism, parents may have taken time off to care for their children. The New Zealand government has predicted overall absenteeism levels as high as 40% (*20*), and actual pandemic workplace absenteeism levels will likely exceed those shown in this study.

Table 2. Effects of influenza pandemic prevention strategies on healthcare worker absenteeism

Reproductive no. (R_0)	Pandemic duration, wk	Peak % absent by strategy (days with >5% absent)					
		No action	Treatment only	2 weeks' prophylaxis	4 weeks' prophylaxis	6 weeks' prophylaxis	8 weeks' prophylaxis
1.5	24	2.8 (0)	2.1 (0)	2.1 (0)	2.1 (0)	2.2 (0)	2.3 (0)
2	15	6.7 (17.8)	5.1 (5.4)	5.2 (6.5)	5.5 (9.1)	5.9 (11))	4.6 (0)
2.5	12	10.2 (21.1)	7.9 (16)	8.1 (16.2)	8.8 (16.2)	7.2 (10.8)	2 (0)
3	10	13 (20.6)	10.2 (16.6)	10.6 (16.7)	11.4 (15)	4.7 (0)	2.5 (0)
4	8	17.3 (18.7)	13.9 (15.7)	14.6 (15.4)	10.8 (11.1)	3.7 (0)	3.7 (0)
6	6	22.5 (16.5)	18.5 (13.9)	19.7 (12.9)	5.5 (4.1)	5.5 (4.1)	5.5 (4.1)
Pandemic similar to 1918 "Spanish flu"*		20.2 (28.6)	15.1 (18.3)	15.8 (17.9)	11.6 (13)	4.1 (0)	4.1 (0)

*R_0 =4; mortality rate = 5% (hospitalization set to the ratio of the hospitalization rates to the case-fatality rates in Table 1).

Treatment and timely use of prophylaxis with neuraminidase inhibitors reduce HCW absenteeism compared with no action. As shown in previous studies, treatment provides benefits over no action and should be considered

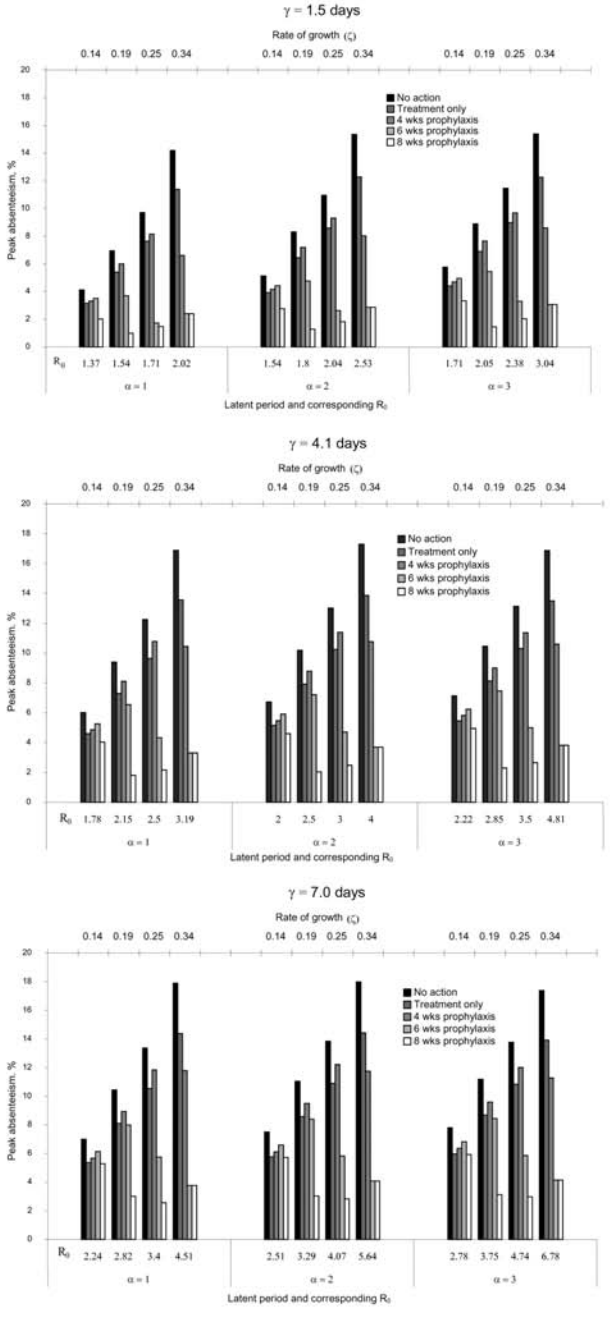

Figure 4. Peak absenteeism with different treatment (Tx) and prophylaxis (Rx) strategies varying rates of growth (ζ)*, latent periods (α), and infectious duration (γ). *ζ is the initial rate of growth of the epidemic curve and is determined by the reproductive potential and the infectious agent's doubling time (T). The latter is related to the rate of growth by the following equation:

$$T = \frac{\ln(2)}{\zeta}$$

in preparedness plans to reduce illness and death (*2*,*3*,*21*). Using prophylaxis to prevent infection results in a secondary increase in infections after prophylaxis is stopped because HCWs remain susceptible at a time when transmission in the general population is ongoing. Insufficient durations of prophylaxis thus result in poorer outcomes than treatment only. For prophylaxis strategies to accrue more benefits than treatment only, the prophylaxis duration must be sufficient to cover the pandemic's peak. Eight weeks of prophylaxis, the maximum safe duration previously studied (*22*), was sufficient to provide a substantial reduction in peak absenteeism under a broad range of assumptions for more severe pandemics where peak absenteeism exceeded 10%. Six weeks of prophylaxis was marginally beneficial, if one assumes that prophylaxis was initiated after incident pandemic cases exceeded 10% of the baseline ILI rate.

An important policy consideration is the timing of prophylaxis initiation. Improved surveillance, critical for early detection, paradoxically increases the likelihood of initiating prophylaxis too early, causing predetermined stockpile durations to be inadequate. Many countries have developed comprehensive preparedness plans to reduce a pandemic's spread. These may prolong the pandemic's duration within the country, which would compound the issue of stockpile adequacy. If prophylaxis is started prematurely, stockpiles will be exhausted before the delayed waves of the pandemic occur and thus will not reduce absenteeism more than would treatment only. Prophylaxis should not be initiated until a certain point in the epidemic curve, but this may be difficult, given public sentiment and pressure. Further studies are needed to determine the ideal time for prophylaxis initiation and the role of surveillance in evaluating the pandemic phases and projected spread.

The current avian influenza outbreaks have increased fear of an imminent severe pandemic. Pandemics of lesser severity place fewer requirements on essential services. Our study showed that such pandemics also result in lower staff absenteeism rates; treatment and prophylaxis may thus be less critical to service continuity. On the contrary, severe pandemics increase the strain because of the numbers of patients, hospitalizations, and deaths and the reduced response capacity of healthcare services. For pandemics with high mortality rates, high growth rates, or high R_0, prophylaxis provides greater benefits than it does for pandemics with lower mortality rates, low growth rates, or low R_0; and the required duration of prophylaxis is shorter.

Our results are subject to several limitations. The true level of transmission in HCWs remains unknown. In a heightened state of alertness, HCWs will be equipped with personal protective equipment, and patient–HCW transmission may be minimized, resulting in lower absenteeism rates (*10*). Another limitation is that effects over the entire

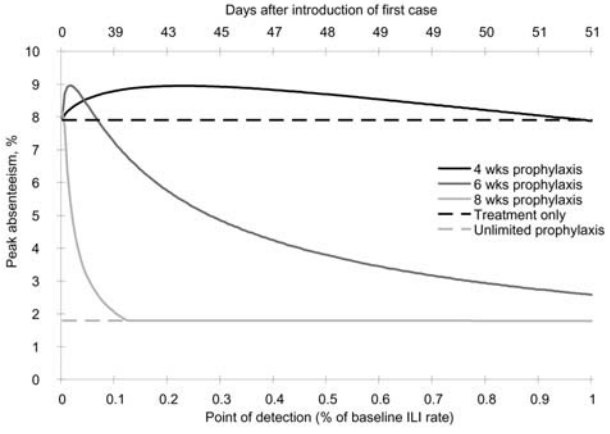

Figure 5. Peak absenteeism observed with different times of initiating prophylaxis, according to point of detection in a base-case pandemic. ILI, influenzalike illness.

HCW population were aggregated. In reality, subsets of HCWs exist with varying levels of exposure. Stochastic variation and nosocomial outbreaks, which were not modeled, may result in higher local absenteeism rates than predicted by this model. Further studies that use individual-based stochastic models may provide improved representation of disease transmission to test other interventions. Studies should also consider modeling the effect of multiple pandemic waves. Finally, the study parameters used were based on historical data; the validity of the projections will depend on how the next pandemic compares with its precedents.

Conclusion

Countries must consider the effects of an influenza pandemic on essential services. Those planning neuraminidase inhibitor stockpiling for treatment and prophylaxis of essential staff should consider the relatively small quantities required. Treatment and 8 weeks of prophylaxis for HCWs in Singapore costs US $2 million, compared with US $400 million for a similar populationwide stockpile and the ≈US $20 million spent for national stockpiling (*2*). In severe pandemics, when the need for protection is greatest, prophylaxis of short duration has a potential role in mitigating the effects. For prophylaxis strategies to succeed, stockpiles must be adequate and their deployment must be timed to cover the pandemic's peak. If adequacy and timeliness cannot be achieved, prophylaxis may result in higher absenteeism than treatment only, which makes the latter strategy a more effective option.

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Appendix

Modified SEIR Model

The model was run across 365 days at time steps of 0.05 days. The equations used in the analysis are shown below; the notations are represented in Table 1.

General Population

For the general population, persons move from the susceptible (S_g) to the exposed (E_g), infected (I_g), and removed (R_g) states as shown in the respective equations below.

$$\frac{d(S_g)}{dt} = -\beta \frac{I_g}{N_g} S_g$$
$$\frac{d(E_g)}{dt} = \beta \frac{I_g}{N_g} S_g - \frac{E_g}{\alpha}$$
$$\frac{d(I_g)}{dt} = \frac{E_g}{\alpha} - \frac{I_g}{\gamma}$$
$$\frac{d(R_g)}{dt} = \frac{I_g}{\gamma}$$

Where β is the transmission probability per day from an average infectious person, N_g is the size of the general population, α is the incubation period, and γ is the infectious period.

HCW Population

Transmission and disease severity parameters are determined by whether HCWs are given treatment and/or prophylaxis. The use of treatment and prophylaxis is indicated by the variables *i* and *j*, respectively. *i = 0* denotes when treatment is not in use, and *j* = 0 when prophylaxis is not in use, and *i = 1* and *j = 1* denote when treatment and prophylaxis are in use, respectively. The use of prophylaxis is conditional to the pandemic having been detected and the stockpile, *P,* not having been exhausted.

Transmission Dynamics

For the HCW population, persons move through the susceptible (S_h), exposed (E_h), infected (I_h), and removed (R_h), states as shown below:

$$\frac{d(S_h)}{dt} = -(\lambda_h + \lambda_g + \lambda_p)(1 - j\varepsilon_1)S_h$$
$$\frac{d(E_h)}{dt} = (\lambda_h + \lambda_g + \lambda_p)(1 - j\varepsilon_1)S_h - \frac{E_h}{\alpha}$$
$$\frac{d(I_h)}{dt} = \frac{E_h}{\alpha} - \frac{I_h}{\gamma}$$
$$\frac{d(R_h)}{dt} = \frac{I_h}{\gamma}$$

where N_h is the size of the HCW population. *j* indicates the use of prophylaxis, so that when *j = 1*, HCWs have a reduced susceptibility to infection due to the efficacy of prophylaxis in preventing infection (ε_1), and are the forces of infection acting on HCWs.

λ_h is the force of infection from HCW-to-HCW transmission within the workplace, and is defined as the following:

$$\lambda_h = \omega\beta(1 - j\varepsilon_3)\frac{I_h}{N_h}$$

where ω is the proportional contribution due to HCW-to-HCW transmission to the force of infection, and ε_3 is the efficacy of oseltamivir in reducing infectiousness, which renders a proportion of HCWs on prophylaxis noninfectious when *j = 1*.

λ_g is the force of infection from exposure of HCWs to the general population during the proportion of their time spent outside the workplace. The force of infection is similar to that in the general community, subject to the proportion of time spent outside the workplace (1 –ω). λ_g is thus defined as

$$\lambda_g = (1 - \omega)\beta\frac{I_g}{N_g}$$

λ_p is the additional force of infection from patient-to-HCW transmission due to symptomatic incident patients as they enter the healthcare system with pandemic influenza (occupational hazard). No discrimination between the probability of acquiring infection in the community healthcare or hospital healthcare setting is represented, because the actual probability of transmission in either setting is unknown. Influenza patients are assumed to be distributed randomly among the HCW population and to have an aggregated probability δ of infecting susceptible HCWs with whom they come into contact, regardless of single or multiple contact episodes or duration of contact. The rate at which new symptomatic infections from the general population will present to the healthcare system at any point in time would be

$$\frac{\theta_1 E_g}{\alpha}$$

Therefore, the force of infection for each HCW, λ_p is as follows:

$$\lambda_p = \frac{\delta\theta_1 E_g}{\alpha N_h}$$

where N_h is the number of HCWs under consideration.

We assumed that the small population of infectious HCWs did not affect the transmission dynamics of the disease in the general population.

Absenteeism

HCWs who are exposed will progress from the exposed state (E_h) to the states of asymptomatic infection, clinical infection (C_h), hospitalization (H_h), or death from the disease (D_h). Only the last 3 states contribute to absenteeism according to the respective durations off work as follows:

$$\frac{d(C_h)}{dt} = \theta_{j+1}(1-(1-i\psi)\eta)\frac{E_h}{\alpha} - \frac{C_h}{(\sigma - i\chi)}$$

$$\frac{d(H_h)}{dt} = \theta_{j+1}(1-i\psi)(\eta-\mu)\frac{E_h}{\alpha} - \frac{H_h}{\phi}$$

$$\frac{d(D_h)}{dt} = \theta_{j+1}(1-i\psi)\mu\frac{E_h}{\alpha}$$

where η is the hospitalized proportion, σ is the duration of medical leave in uncomplicated illness, ϕ is the duration of hospitalization and subsequent medical leave in complicated illness, and μ is the case-fatality proportion. ψ is the reduction in hospitalization or deaths with treatment, and χ is the reduction in medical leave with uncomplicated illness with treatment; both these terms are hence only active for values of $i = 1$. θ_{j+1} is the symptomatic proportion and hence takes the value of θ_1 in the absence of prophylaxis and θ_2 when prophylaxis is used, reflecting the efficacy of prophylaxis in reducing symptomatic disease (ε_2).

The number of healthcare staff in operation at any time is hence given as

$$O_h = N_h - C_h - H_h - D_h$$

The proportion absent at any given time is

$$\frac{O_h}{N_h}$$

We ignored the contribution of new recruitments after the start of the epidemic.

Incidence Rates, Start of Pandemic, and Use and Consumption of Prophylaxis Stockpile

The incident number of symptomatic cases of pandemic influenza in the general population, V_g, is given as

$$V_g = \frac{\theta_1 E_g}{\alpha}$$

The pandemic is deemed to start when

$$V_g > \upsilon\iota$$

where ι is the baseline ILI rate, and υ is the detection threshold. When $V_g > \upsilon\iota$, then the predetermined stockpile, P, which is expressed as the number of days of prophylaxis stockpiled per HCW, begins to be consumed in strategies that use prophylaxis, i.e.,

$$\frac{d(P)}{dt} = -1$$

In a prophylaxis strategy, $j = 1$ when both conditions, $V_g > \upsilon\iota$ and $P > 0$, are satisfied; otherwise, $j = 0$.

Environmental *Burkholderia cepacia* Complex Isolates in Human Infections

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Members of the *Burkholderia cepacia* complex (Bcc), found in many environments, are associated with clinical infections. Examining diverse species and strains from different environments with multilocus sequence typing, we identified >20% of 381 clinical isolates as indistinguishable from those in the environment. This finding links the natural environment with the emergence of many Bcc infections.

The *Burkholderia cepacia* complex (Bcc) is a group of closely related gram-negative bacteria comprising at least 9 species (*1*). They are routinely isolated from the natural environment, where they can have a range of beneficial properties (*2*). However, these bacteria can also frequently cause fatal infections in vulnerable humans, such as those who have cystic fibrosis (CF). Because Bcc bacteria are not normally carried as commensal organisms, the main sources of infection are considered to be patient-to-patient spread (*3*,*4*); hospital settings, including medical devices and contaminated disinfectants; and the environment (*5*,*6*). Therefore, although Bcc species may have an important environmental role in agriculture and biotechnology industries, their use also represents a potential clinical risk to susceptible members of the community (*7*,*8*). All species of Bcc can be isolated from the environment in differing degrees (*2*). Similarly, all current Bcc species have been cultured from CF patient sputum (*2*). Infection control measures have been implemented to reduce patient-to-patient transmission; although effective, these measures have not prevented the emergence of new infection. Thus, the environment could be acting as a constant nonpatient reservoir for infectious Bcc pathogens.

Previous studies have reported the possibility of humans acquiring Bcc directly from natural environments (*9*,*10*). The most recent of these studies reported evidence that a *B. cenocepacia* strain, isolated from soil, was indistinguishable by several typing methods (pulsed-field gel electrophoresis [PFGE] genomic fingerprinting and repetitive extragenic palindromic [rep]–PCR) from isolates of the problematic CF lineage PHDC (*10*).

The Study

To evaluate how widespread the emergence of environmental isolates as causes of clinical infections may be, we used a highly discriminatory and transportable typing method to study isolates from several large bacterial culture collections. Multilocus sequence typing (MLST) is a relatively new technique based upon unambiguous sequence analysis of several housekeeping genes. Unlike previous methods for Bcc strain typing (*10*), MLST is not based on banding patterns but instead relies on the robust comparison of DNA sequence information. This process facilitates both the identification and matching of identical clones as well as their evolutionary comparison to closely related strains.

Using a recently validated MLST scheme (*11*), we analyzed a collection of 381 clinical isolates of all 9 currently reported Bcc species, from 28 countries. A total of 187 distinct sequence types (STs) were identified from clinical isolates within this collection and compared with 233 environmental Bcc isolates (113 STs). We found that 81 clinical isolates (encompassing15 STs) were identical by MLST to a wide range of environmental isolates. This figure represents 21.5% of the clinical isolates examined (for clarity, a subset are shown in the Table; [*12*]).

The resolution of strain identification offered by MLST is such that different isolates sharing the same ST (genotypically indistinguishable at all 7 loci) are defined as clones of the same strain (e.g., for a group of isolates within this collection, we have further validated this identity by cloning and sequencing up to 10 random fragments of DNA). The 15 STs identified from environmental and clinical sources belonged to 6 different Bcc species (Table): *B. cepacia* (4 STs), *B. multivorans* (2 STs), *B. cenocepacia* (3 STs), *B. stabilis* (2 STs), *B. vietnamiensis* (2 STs), and *B. ambifaria* (2 STs). Three *B. cenocepacia* STs also belonged to 2 different *recA* lineages defined within this species: IIIA (1 ST) and IIIB (2 STs).

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Table. MLST analysis of the *Burkholderia cepacia* strains showing their species, source, and geographic location*

Bcc species	ST	Isolate name	Source	Country	Source of isolate	Year of isolation
B. cepacia						
	1	ATCC 17759†	ENV	Trinidad	Soil	1958
	1	LMG 14087	NON	UK	Wound	1988
	10	ATCC 25416^T	ENV	USA	Onion	1948
	10	J1050	NON	USA	Human	Before 1983
	266	BC20	ENV	USA	Water	–
	266	AU6671	NON	USA	Wound	–
	365	HI-3602	ENV	USA	Soil	–
	365	C8509	CF	Canada	Sputum	1999
	365	AU3206	CF	USA	Sputum	–
B. multivorans						
	21	ATCC 17616†	ENV	USA	Soil	Before 1966
	21	AU0453	CF	USA	Sputum	–
	21	C9140	CF	Canada	Sputum	2000
	375	R-20526	ENV	Belgium	River water	2002
	375	IST455	CF	Portugal	Sputum	2000
B. cenocepacia IIIA						
	32	POPR8	ENV	Mexico	Radish	–
	32	5–457	CF	Czech Republic	Sputum	2002
	32	R-16597	CF	Belgium	Sputum	2001
	32	BCC1118	NON	UK	Wound	Before 1994
B. cenocepacia IIIB						
	37	BC-1	ENV	USA	Maize rhizosphere	–
	37	AU2362	CF	USA	Sputum	2000
	122	HI-2424	ENV	USA	Soil	–
	122	AU1054	CF	USA	CF	–
	122	CEP0497	NON	Canada	Leg ulcer	1995
B. stabilis						
	50	LMG 14294†	CF	Belgium	Sputum	1993
	50	R-16919	ENV	Belgium	Industrial	–
	50	LMG 14086†	ENVH	UK	Respirator	1970
	51	HI-2482	ENV	USA	Shampoo	–
	51	ATCC 35254	ENVH	USA	Medical solution	1980
	51	CEP0928	ENVH	USA	Albuterol solution	–
	51	LMG 14291	CF	Belgium	Sputum	1993
	51	LMG 7000	NON	Sweden	Blood	1983
B. vietnamiensis						
	61	J1702	ENVH	USA	Hospital equipment	–
	61	BCC0190	CF	USA	Sputum	–
	61	J1712	NON	USA	Wound	–
	61	J1738	NON	USA	Wound	–
	61	J1742	NON	USA	Wound	–
	67	R-20590	ENV	Belgium	River water	2002
	67	D0774	CF	Canada	Sputum	2003
B. ambifaria						
	81	MVP-C2-4	ENV	Italy	Maize rhizosphere	1996
	81	BCC0250†	CF	Australia	Sputum	1994
	77	AMMD^T	ENV	USA	Soil	1985
	77	AU0212	CF	USA	Sputum	–

*MLST, multilocus sequence typing; Bcc, *B. cepacia* complex; ST, sequence type; ENV, isolated from the environment; NON, isolated from a non-cystic fibrosis (CF) patient; CF, isolated from a CF patient; IIIA or IIIB, isolates belonging to *B. cenocepacia recA* subgroup A or B, respectively; ENVH, isolated from a hospital environment.
†Panel strain (*12*); superscript T, type strain for species.

Conclusions

STs occurring in both clinical and environmental niches were found in 6 of the 9 formally described Bcc; the greatest degree of overlap occurred in *B. cepacia* and *B. stabilis* (Figure). The proportion of STs not shared between clinical and environmental isolates varied for each Bcc species we examined. This finding may reflect the few clinical or environmental isolates for that species or high genetic diversity; a larger sample size is needed to find identical matches. Species dominated by clinical STs (>83% of STs) were *B. multivorans, B. cenocepacia recA* lineage IIIA, and *B. dolosa*. Those species containing mainly environmental STs (>80%) were *B. ambifaria*, *B. anthina*, and *B. pyrrocinia* (Figure). Although this distribution agrees with findings of previous studies (*2*), it also reflects the distribution of isolates within the collections from which isolates were obtained.

Several ST matches between clinical and environmental isolates were of particular interest. MLST ST-10 was shared by *B. cepacia* J1050, a human isolate cultured in the United States (Cleveland, Ohio) and the type strain for *B. cepacia* ATCC 25416, isolated from an onion. This evidence of clonality augments the clonal relationship reported earlier (*9*) between ATCC 25416 and a UK isolate from a CF patient. The *B. multivorans* IST455 isolated from a CF patient's sputum in Portugal, as reported in a previous study (*13)* had the same sequence type (ST-375) as R-20526, which was isolated from the River Schelde in Belgium.

B. cenocepacia recA lineage IIIA isolates with ST-32 (Table) were from 4 independent sources: POPR8 (isolated from a radish in Mexico), BCC1118 (isolated from a UK non-CF patient infection), R-16597 (isolated from a CF patient in Belgium), and 5–457 (isolated from a CF patient in the Czech Republic). ST-32 appears to be a globally distributed, predominantly clinical strain (A. Baldwin, unpub. data). The *B. cenocepacia recA* lineage IIIB isolates identified as ST-122 (Table) include the PHDC strains, predominant in US CF patients (AU1054) and previously found in US soil (HI-2424) (*10*), and CEP0497, which was obtained from a leg wound in a non-CF patient in Canada. Together with a recent report of PHDC strains identified in Europe (*14*), the Canadian isolate in our study adds further weight to the identification of this ST as a globally distributed strain.

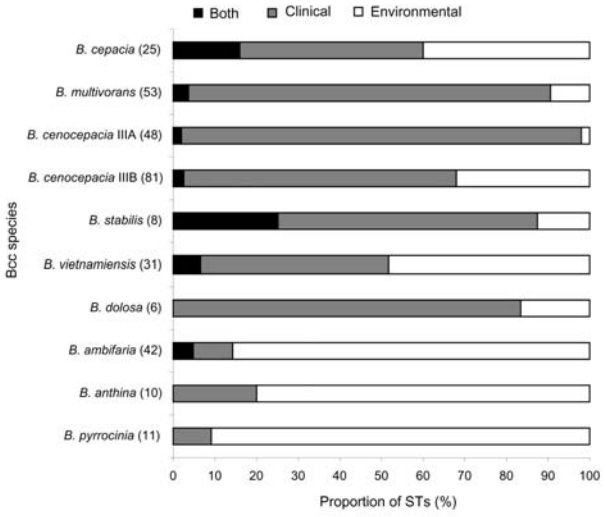

Figure. Proportion of sequence types (STs) within each *Burkholderia cepacia* complex (Bcc) species from clinical, environmental, or both sources. The bar chart shows the proportion of STs derived from the environment (white), clinical (gray), and both sources (black shading). The total number of STs examined for each *B. cepacia* species is in parentheses.

MLST analysis of *B. stabilis* corroborated the high degree of clonality observed by PFGE fingerprint analysis in the original description of this species (*15*). However, MLST was further able to distinguish 8 STs among the 26 isolates examined, which indicates that MLST may be a more effective method than PFGE for epidemiologic analysis of *B. stabilis*. This increased resolution adds to the observation that 2 *B. stabilis* STs are globally distributed and isolated from clinical samples and an array of different niches, including domestic products, medical solutions, industrial process contaminants, and hospital devices.

In summary, >20% of the clinical isolates we examined were indistinguishable by MLST from isolates from environmental sources. This finding suggests that conservation of intrinsic determinants necessary to thrive in environmental niches may confer an ability to colonize susceptible humans. Further work is urgently required to more extensively investigate the emergence of pathogenic members of the Bcc in the natural environment and the risk for infection this may represent.

Acknowledgments

We thank Angela Marchbank and Lynne Richardson for technical support. This publication made use of the Bcc multilocus sequence typing website (http://pubmlst.org/bcc/) developed by Keith Jolley and hosted at the University of Oxford.

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Cryptosporidium hominis Infection of the Human Respiratory Tract

Rubén Mercado,* Gregory A. Buck,† Patricio A. Manque,† and Luiz Shozo Ozaki†

Cryptosporidium oocysts, observed in a natural sputum sample of a patient with HIV, were further studied by using DNA markers to determine the species of the parasite. *C. hominis* was identified as the species infecting the patient's respiratory tract, a finding that strengthens evidence regarding this pathogen's role in human disease.

Intestinal cryptosporidiosis is a common parasitic disease that causes self-limiting diarrhea in immunocompetent persons (*1*). Higher frequencies of *Cryptosporidium* infection are observed in immunocompromised humans, and the main clinical pattern of the infection in these persons is a chronic, life-threatening secretory diarrhea (*2*).

At least 8 species of *Cryptosporidium* are described as infecting humans. *C. hominis* and *C. parvum* are the most frequently observed in intestinal infections in humans (*3*). *C. meleagridis* is also detected both in immunocompetent and immunodeficient patients, although at a lower rate than *C. parvum* (*4*).

Respiratory tract infection by *Cryptosporidium* spp. has been described for immunodeficient persons, most all of whom were coinfected with HIV. However, pulmonary cryptosporidiosis was also described in patients without HIV infection (*5*,*6*). In all cases, no systematic identification of the species of *Cryptosporidium* was pursued except by Meamar et al. (*7*), in which the parasite was identified as *C. parvum*.

We describe the detection and identification of *C. hominis* in the respiratory secretions of a patient with HIV (sample Chile01). We used an oligonucleotide species-specific method and sequencing of parts of the 18S rRNA gene to determine the species of *Cryptosporidium*. Both analyses showed that the species of *Cryptosporidium* present in the pulmonary secretion of this patient was *C. hominis*.

The Study

In September 2004, a 58-year-old man, who received an HIV-positive diagnosis in 1996, was hospitalized with respiratory symptoms characterized by persistent cough. *Cryptosporidium* oocysts were detected in a sputum sample from the patient by using Ziehl-Neelsen stain (Figure 1). An aliquot of ≈10 mL of respiratory secretion was obtained. DNA was extracted as follows: 200 µL fluid was centrifuged and the pelleted material digested overnight at 65ºC with proteinase K in the presence of 10% sodium dodecyl sulfate. We then sequentially extracted the digested material with phenol, phenol-chloroform-isoamyl alcohol, and chloroform-isoamyl alcohol. The DNA was then precipitated with sodium acetate and ethanol, and after centrifugation, the pelleted DNA was dissolved in 50 µL of water.

For molecular typing, we first used the species-specific oligonucleotide PCR assay Lib13, as described by Tanriverdi et al. (*8*), with a new sense oligonucleotide primer based on the genome sequences of *C. hominis* (*9*) and *C. parvum* (*10*). The new primer, Lib13SF02 (5′-TTTTTTCATTAGCTCGCTTC-3′), a fragment of ≈400 bp, was amplified specifically from *C. hominis* DNA with the anti-sense primers Lib13SRT-1 (5′-ATTTATTAATTTA TCTCTTACTT-3′) and from *C. parvum* DNA with Lib13 SRT-2 (5′-ATTTATTAATTTATCTCTTCG-3′) (Figure 2). Amplifications were carried out in a PCR mixture of 10 µL containing 0.25 mmol/L of each dNTP, 300 pmol/L of each oligonucleotide, and 1 unit of Taq DNA polymerase (HotMaster, Eppendorf, New York, NY, USA). Temperature cycling was performed on a GeneAmp PCR System (ABI, Foster City, CA, USA) with initial denaturation performed at 95ºC for 5 min, then 40 cycles at 95ºC for 30 s, 52ºC for 30 s, and 68ºC for 30 s. The mixture was then cooled to 4ºC.

The region from bases 7 to 1036 (numbering according to *C. hominis* sequence GenBank no. L16996) of the 18S rRNA gene was sequenced from DNA fragments amplified using the primers 18SF (5′-GTTGATCCT GCCAGTAGTC-3′) and 18SR (5′-TAAGGTGCTGAAG GAGTAAGG-3′) and cloned into the TOPO TA vector (Invitrogen, Brandford, CT, USA) by using standard techniques. Automated sequencing was performed directly on

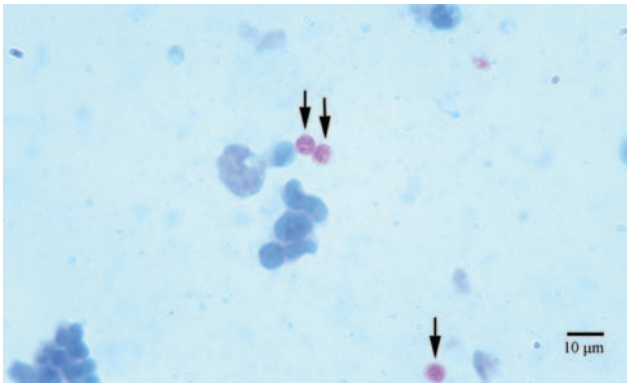

Figure 1. Oocysts (arrows), stained by using the Ziehl-Neelsen method, in sputum from the patient with HIV.

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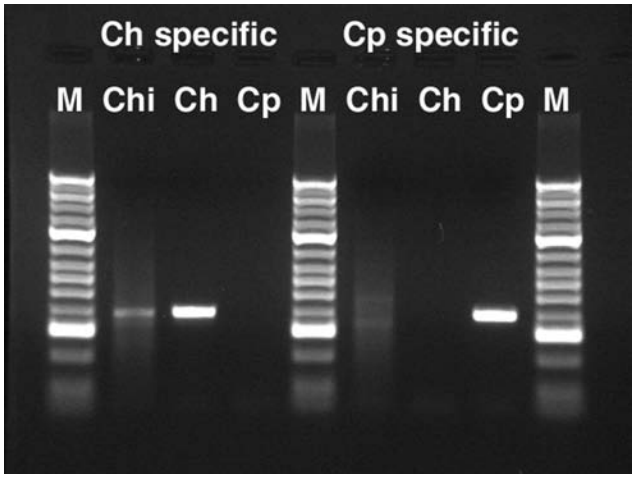

Figure 2. Agarose gel electrophoresis of DNA fragments amplified with *Cryptosporidium* species–specific Lib13 primers (*7*). Ch, *C. hominis*; Cp, *C. parvum*; M, DNA molecular marker (Bioline, Randolph, MA, USA; HyperLadder II, higher intensity bands: 0.3, 1, and 2 Kbp); Chi, sample Chile01.

the amplified fragments or on cloned fragments at the Nucleic Acid Research Facilities of Virginia Commonwealth University. All DNA sequences were analyzed by using Sequencher (Gene Codes Co., Ann Arbor, MI, USA).

Figure 1 shows fuchsia (Ziehl-Neelsen)–stained sputum from the patient. Three *Cryptosporidium* oocysts (arrows) with typical sizes ≈5 μm in diameter are visible. Measurements were performed in a calibrated microscope as described by Mercado and Santander (*11*).

A Lib13 PCR assay (*8*) was performed on the DNA purified from the respiratory secretion material, and the results are shown in Figure 2. With the *C. hominis* specific-primer pair (LIBF02/Lib13SRT1), a fragment of the expected size (≈400 bp) was amplified with the Chile01 isolate DNA (Figure 2, lane 2). With this primer pair, we also obtained amplification with the *C. hominis* isolate TU502 (*12*) DNA (Figure 2, lane 3) but not with the *C. parvum* isolate Moredun (*13*) DNA (Figure 2, lane 4). Conversely, the *C. parvum*–specific primer pair (LIBF02/Lib13SRT2) amplified a fragment only with the *C. parvum* DNA (Figure 2, lane 8). No amplification was observed with the Chile01 (Figure 2, lane 6) or the *C. hominis* DNA (Figure 2, lane 7). DNA sequencing of the amplified fragments confirmed the polymorphism to be that of *C. hominis* (results not shown).

We also analyzed the 18S small subunit rRNA gene by amplifying and sequencing an 18S rRNA fragment from the sputum DNA. Amplification and sequencing was concomitantly performed with the *C. hominis* and *C. parvum* DNA. A polymorphic site exists in *C. hominis* as a stretch of 10 to 12 thymines ($T_{10–12}$), while in *C. parvum*, the sequence is TA:::TATATTTT (*14*). The 18S rRNA polymorphic sequence found in the sputum sample DNA (GenBank accession no. DQ286403) is that of *C. hominis*, with a stretch of 11 Ts (Table). Few other nucleotide polymorphisms were found between the sequences (results not shown), which reflect intraisolate variations (*14*). The results with Lib13 assay and the partial 18S rRNA sequence analysis, therefore, identify the species of *Cryptosporidium* infecting the respiratory tract of this patient as *C. hominis*.

Conclusions

Human cryptosporidiosis is better known as an intestinal disease both in immunocompetent and immunocompromised persons. Little information exists, however, on human pulmonary disease caused by *Cryptosporidium* spp., which reflects either the low prevalence of pulmonary cryptosporidiosis or the lack of testing in immunocompetent hosts. Further, if performed, current diagnostic tests may not be sensitive enough to detect the parasite.

In immunocompetent children with intestinal cryptosporidiosis, respiratory symptoms have been noted more frequently than expected (*5*). Studies are needed in immunocompetent persons, especially children <2 years of age, who belong to a group at high risk for intestinal, and by extension pulmonary, cryptosporidiosis.

More information about which species of this pathogen infect humans and the pathogenic patterns each species produces is needed. As we determined here, *C. hominis* have the capacity to adapt to different physiologic environments, such as intestinal and respiratory tract tissues. Our findings provide additional evidence supporting the role of this species of *Cryptosporidium* as a human pathogen and the need to evaluate the importance of pulmonary cryptosporidiosis as a disease in the immunocompromised host.

Acknowledgments

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Table. Nucleotide sequences at the T polymorphic site of the 18S rRNA of the pulmonary sample of *Cryptosporidium* (Chile01), *C. parvum* (Cp), and *C. hominis* (Ch)

Isolate	18S rRNA sequence at 670–710
Chile01	5′-CATAATTCATATTACTATTTTTTTTTTTA GTATATGAAATT-3′
Cp	5′-CATAATTCATATTACTA:::TATATTTTA GTATATGAAATT-3′
Ch	5′-CATAATTCATATTACTA:TTTTTTTTTA GTATATGAAATT-3′

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Cocirculation of 2 Genotypes of Toscana Virus, Southeastern France

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Toscana virus (TOSV), an arthropodborne phlebovirus transmitted by sandflies, can cause febrile illness and meningitis. The vector of TOSV in France was unknown. We detected TOSV RNA in 2 (female *Phlebotomus perniciosus*) of 61 pools of sandflies captured in southeastern France. Two genotypes of TOSV were identified.

Toscana virus (TOSV) is an arthropodborne virus first isolated in 1971 in central Italy from the sandfly *Phlebotomus perniciosus*, then from *Ph. perfiliewi* (*1*,*2*). In Spain, TOSV has been isolated from phlebotomine flies, but species identification was not performed (*3*). TOSV has not been detected in or isolated from sandflies in France.

Most clinical and epidemiologic studies on TOSV have been conducted in Italy, although data from other Mediterranean countries have recently been published (*4*). TOSV has a tropism for the central nervous system and is a major cause of meningitis and encephalitis in the countries in which the virus circulates. Cases of TOSV infection acquired in the south of France indicate that TOSV circulates in the region (*5*,*6*). The aim of our study was to identify the presence of TOSV in *Phlebotomus* spp. collected in southeastern France and to investigate French TOSV at the genetic level. For that purpose, we compared viral sequences from sandflies and from French patients with other sequence data previously reported from Italy and Spain.

The Study

Sandflies were trapped during 4 days in July 2005 in 2 cities in southern France (Marseille and Nice) by using techniques previously reported (*7*) (Figure 1). Adult flies shelter during the day in dark, quiet, and humid places, and eggs are laid in terrestrial microhabitats rich in organic matter that provides food for larvae, including soils containing herbivorous animal feces. Therefore, traps were placed at dawn in or near animal housing facilities (horse stalls, rabbit hutches, hen houses) or in areas where visceral canine leishmaniasis is endemic (*8*). Each morning, sandflies were collected and identified morphologically, by dissecting genital organs, according to morphologic taxonomic keys (*9*). Sandflies were pooled (up to 30 individual flies) according to trapping origin, species, and gender (Table) in 1.5-mL tubes and processed as described (*7*).

A variety of primers targeting different genes were used: primers specific for TOSV in the polymerase gene (*10*) and primers designed in this study from the alignment of nucleoprotein sequences of selected phleboviruses (retrieved from GenBank) specific for the species *Sandfly fever Naples virus* (SFNV-S1 [5′-CTTYTTRTCYTCYC TRGTGAAGAA-3′], SFNV-R1 [5′-ATGATGAAGAARA TGTCAGAGAA-3′], SFNV-S2 [5′-GCRGCCATRTTKG GYTTTTCAAA-3′], SFNV-R2 [5′-CCTGGCAGRGACA CYATCAC-3′]). The reverse transcription–PCR (RT-PCR) was performed by using the Access RT-PCR kit (Promega, Madison, WI, USA) according to the manufacturer's recommendations. L and S RNA primers were used at 0.8 μmol and 0.4 μmol per reaction, respectively. The cycling program of the RT-PCR consisted of 48°C for 45 min and 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, the annealing temperature for 1 min, and 68°C for 45 s, with a final elongation step at 68°C for 7 min.

Using ground material obtained from pools AK and AR and patients' samples, we injected Vero-E6 cells (protocol available on request). Most sandflies belonged to the *Ph. perniciosus* species (Table, Figure 1), 483 (96.8%) in Marseille, 110 (62.5%) in Nice. Two other species of phlebotomine flies were identified, *Ph. mascitii* (n = 4, 0.8%), and *Ph. ariasi* (n = 3, 0.6%) in Marseille. In addition, 123 *Sergentomyia minuta* were identified and led to the detection of TOSV RNA, as recently reported (*7*). Finally, 61 pools of *Phlebotomus* spp. were organized to be tested for the presence of TOSV RNA.

We detected TOSV RNA by both PCRs in 2 pools (AK and AR) of female *Ph. perniciosus* trapped in the neighborhood of Marseille. AK and AR sequences were distinct from each other (1.4% and 1% nucleotide divergence for S and L RNA sequences, respectively), from other sequences available in GenBank, and from those determined during this study or in previous studies (0.7%–21.5% and 17.4% nucleotide divergence for S and L RNA sequences, respectively) (*7*).

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[1]Equally participated in this work.

[2]Equally participated in the design and supervision of this study.

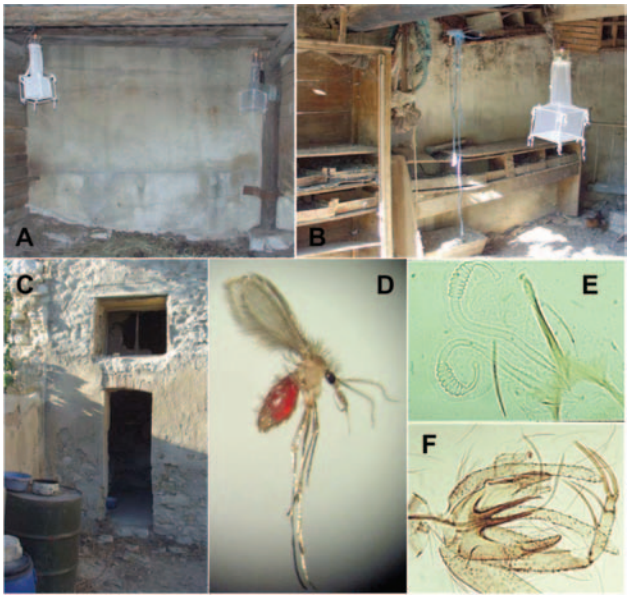

Figure 1. CDC light traps, adapted for sandfly trapping, placed in horse stables (A), near rabbit hutches and henhouses (B), and in quiet places in the shade of human habitations, where dogs sleep (C). Engorged female *Phlebotomus perniciosus* sandfly trapped in a horse stable (D); spermatheca of *Ph. perniciosus* female after dissection (E); genitalia of *Ph. perniciosus* male after dissection (F).

Sequences from 3 TOSV-infected patients who lived in the region of Marseille were included in the genetic analysis. For 2 patients, sequences were obtained after virus isolation in Vero cells: strain IMTSSA/2004 (GenBank accession no. AY766034) (*6*) and strain 1500590 (GenBank accession no DQ975232–975233, this study). Another sequence was determined from direct amplification of a cerebrospinal fluid sample (GenBank accession no. DQ975231, this study). Nucleotide distances (see online Appendix Table, available from www.cdc.gov/EID/content/13/3/465-appT.htm) clearly allowed discrimination of the 2 genotypes previously described as the Italian and the Spanish genotypes by reference to the origin of viruses. Sequences obtained from sandfly and clinical material collected in France indicated that both genotypes are circulating in southern France. Indeed, *Ph. perniciosus* specimens AK and AR and human samples 5501805 and IMTSSA/2004 displayed high identity levels with TOSV of Spanish origin. In contrast, *S. minuta* specimen Smin2005 and human sample 1500590 were more closely related to the Italian strain Iss.Phl3 (online Appendix Table). Phylogenetic analysis of nucleotide sequences (Figure 2A) showed that Spanish, French, and Italian TOSV and SFNV formed a cluster that included members of the species *Sandfly fever Naples virus*, for which genetic data were available. Such analysis confirmed that the 2 French TOSV sequences detected in *Ph. perniciosus* (AK and AR) and the 2 TOSV sequences from clinical samples (IMTSSA2004 and 5501805) grouped with Spanish TOSV (100% bootstrap support), while *S. minuta* specimen Smin2005 and human sample 1500590 clustered with Italian TOSV sequences. Topology analysis indicated that the 4 French TOSV most closely related to Spanish TOSV root the Spanish sequences (Figure 2A). Together, all these sequences displayed a cline topology. Further studies, with more sequences, are needed to investigate this finding. As previously reported (*3*), despite significant variability between TOSV isolates at the nucleotide level (up to 13.2%), phylogenetic analysis of amino acid sequences did not allow discrimination between the Italian and Spanish genotypes (online Appendix Figure, Figure 2B). Indeed, in the nucleoprotein

Table. Capture of sandflies and identification

Trapping region	Species	Sex	No. sandflies	No. pools
Marseille	*Phlebotomus perniciosus*	M	291	17
		F	185	15*
		Unknown	7	3
	Ph. ariasi	M	2	2
		F	1	1
	Ph mascitii	M	1	1
		F	3	3
	Phlebotomus sp.	M	3	3
		F	2	1
		Unknown	4	4
	Total Marseille		499	50
Nice	*Ph. perniciosus*	M	78	5
		F	32	3
	Phlebotomus sp.	M	43	2
		F	23	1
	Total Nice		176	11
Total			675	61

*Two pools (AK and AR) out of these 15 pools were positive. AK sandflies were trapped in the Maillane District (43°17′51" N, 5°29′43" E). AR sandflies were trapped in the Mont d'Or District (43°22′52" N, 5°22′17" E).

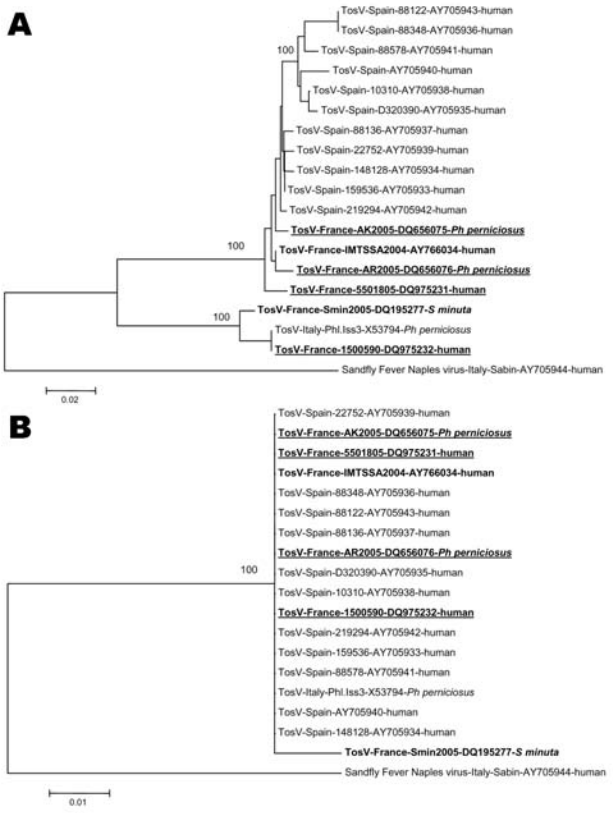

Figure 2. Phylogenetic trees based on nucleotide (A) and amino acid (B) sequences in the nucleoprotein gene of phleboviruses within the species *Sandfly fever Naples virus*. Sequence information corresponds to virus/country of origin/strain/GenBank accession no/host. Sequences representing French TOSV are in **boldface.** Sequences determined in this study are underlined. Sequence alignment was achieved with ClustalX 1.81 with sequences from other phleboviruses retrieved from GenBank. Accession numbers of GenBank sequences used for genetic analyses are indicated. Phylogenetic studies were conducted by using MEGA version 3.0 (*11*). Genetic distances were calculated with the pairwise distance and Jukes-Cantor methods at the nucleotide and amino acid level, respectively. Phylogenetic trees were constructed with the neighbor-joining method. The robustness of the nodes was tested by 500 bootstrap replications.

region considered for analysis, all TOSV amino acid sequences were 100% identical, except that obtained from *S. minuta*, which diverged by 1 amino acid (data not shown).

Conclusions

This study provided genetic evidence, from the analysis of 2 independent genes, that TOSV circulates in populations of *Ph. perniciosus* in southeastern France. The absence of detection of TOSV in the Nice region may be due to the small number of sandflies collected. A similar study aimed at collecting a larger number of sandflies is therefore necessary to address whether TOSV circulates in the region of Nice and whether, if it does, it belongs to the Spanish genotype, the Italian genotype, or another yet unrecognized genotype.

The rate of infection observed with TOSV in sandflies collected during this study (2/675) is of the same magnitude of that reported in Spain (*3*) and lower than rates reported in Italy (*2*). The distribution and respective frequency of sandfly species in France (this study) and Spain (*3*) were different: *Ph. papatasi* (6.8%) and *Ph. sergenti* (8.5%) were cataloged in Spain, not in France, whereas *Ph. mascitii* (5.9%) was identified in France, not in Spain. *Ph. perfiliewi*, known to replicate TOSV in nature in Italy, has not been identified in either French or Spanish studies.

In conclusion, we have provided the first evidence for the circulation of TOSV in *Ph. perniciosus* in the region of Marseille, southeastern France. We also determined that TOSV belonging to the 2 genotypes previously recognized in Italy and Spain circulates and causes human infections in southeastern France.

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Rapidly Fatal *Acanthamoeba* Encephalitis and Treatment of Cryoglobulinemia

Wouter Meersseman,* Katrien Lagrou,* Raf Sciot,* Johan de Jonckheere,† Christine Haberler,‡ Julia Walochnik,‡ Willy E. Peetermans,* and Eric van Wijngaerden*

We describe a 66-year-old woman with therapy-refractory cryoglobulinemia treated with rituximab, plasmapheresis, and steroids; a case of fatal meningoencephalitis caused by *Acanthamoeba* spp. then developed. Such infections are rare and show an unusually rapid course (possibly related to rituximab).

Infection with *Acanthamoeba*, a free-living ameba, is a rare cause of slowly progressive granulomatous amebic encephalitis (GAE) in immunocompromised patients. This form of encephalitis is almost universally progressive and fatal, typically within 2 months of symptom onset (*1*). We describe a patient with cryoglobulinemia refractory to standard therapy who died of GAE after receiving rituximab.

Rituximab is a monoclonal antibody directed against CD20, a surface antigen expressed in cells of B-lymphocyte lineage. It was developed for treatment of B-cell lymphomas. Recently, therapy with rituximab has been extended to a variety of autoimmune diseases in which B cells have been thought to play a role. The drug substantially depletes normal B cells from the peripheral blood and its use leads to a prolonged period of humoral immune dysfunction (*2*).

The Case

In March 2005, a 66-year-old woman was referred to our hospital because of status epilepticus. A diagnosis of hepatitis C had been made in 2003. In January 2005, hepatitis C–related cryoglobulinemic vasculitis with cutaneous and renal involvement developed. The cryoprecipitate contained 586 mg/L of immunoglobulin G (IgG) and 517 mg/L of IgM (normal <10 mg/L). Treatment with prednisolone (1 g intravenously for 3 days, followed by 64 mg orally once a day until day 31) was started in January 2005 (day 1). Despite this treatment, the vasculitis worsened. Plasmapheresis was started on day 10 (8 sessions) and resumed on day 39 (10 sessions). The patient's cryocrit decreased. The prednisolone dose was tapered on day 31 to 16 mg. Increasing palpable purpura on the lower legs was observed on day 46. The decision was made to start rituximab infusions (375 mg/m^2), which she received starting on day 48 on a weekly basis; she received 2 infusions. On day 55, she had a seizure and was transferred to the intensive care unit. She was deeply comatose with a Glasgow coma score of 4/15. Vital signs were normal. Magnetic resonance imaging showed a T2 hyperintense lesion in the right frontal region of the brain (Figure 1). At that time, the lesion was believed to be related to the cryoglobulinemia. Examination of cerebrospinal fluid (CSF) showed a protein level of 228 mg/dL, a glucose level of 53 mg/dL, and 13 lymphocytes/μL. Culture of blood and CSF, serologic tests, and PCR did not show any fungi, viruses, or bacteria. The patient remained in a nonconvulsive status epilepticus despite combination anticonvulsive therapy. She died on day 61.

Postmortem examination showed signs of glomerulonephritis, liver fibrosis, and moderate signs of myocarditis. A formalin-fixed, 1,378-g brain specimen showed no discoloration or meningeal opacification throughout most parts of the brain. Small lacerations were visible in the pedunculus cerebri superior and both hemispheres of the cerebellum. No obvious intraparenchymal bleeding was present. Hematoxylin and eosin–stained sections showed a necrotizing hemorrhagic meningoencephalitis and adjacent recent infarctions. Numerous trophozoites and *Acanthamoeba* cysts were observed within necrotic areas and overlying meninges. Trophozoites were predominantly perivascularly located in nonnecrotic areas and cysts were detectable within blood vessel walls (Figure 2A). For immunohistochemical analysis, 3-μm–thick sections of paraffin-embedded brain tissue were cut and stained with an antibody to *Acanthamoeba* spp. (from rabbits immunized with *Acanthamoeba* genotype T4) at a dilution of 1:2,000. Antigen retrieval was performed by heating sections in 0.01 mol/L citrate buffer (pH 6.0) for 60 minutes. ChemMate kit K5001 (Dako, Glostrup, Denmark) was used for immunostaining (Figure 2B).

We conducted a PCR for detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri* in brain tissue and CSF by using JDP primers for a diagnostic small subunit rDNA fragment as previously described (*3*). *Acanthamoeba* DNA was detected in brain tissue but not in CSF. Typing of the *Acanthamoeba* strain by sequencing the amplified partial small subunit rRNA gene region (ASA.S1) with primer 892c as previously described (*4*) showed that the strain had genotype T4. The sequenced region is identical to that found in the European Molecular

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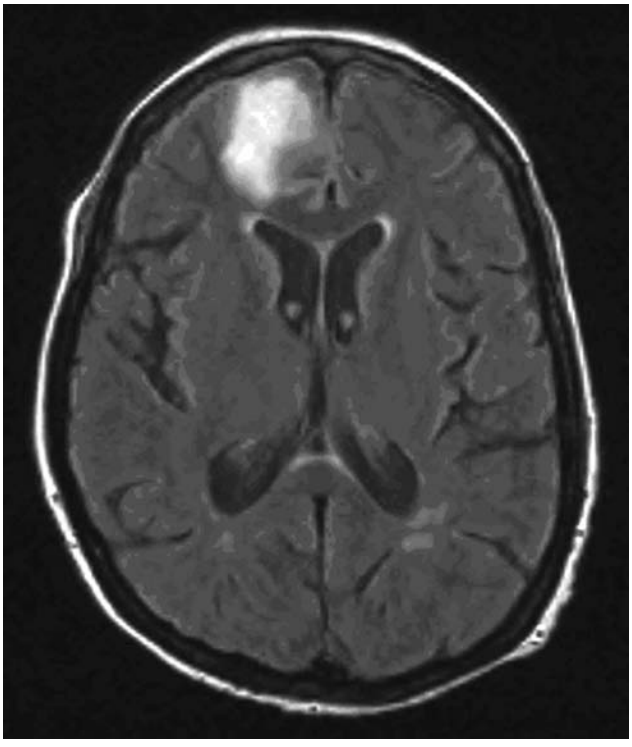

Figure 1. Magnetic resonance image of the patient's brain after the first seizure showing a hemorrhagic lesion in the right frontal lobe.

Biology Laboratory database (accession no. AP07407, Figure 2C).

Conclusions

We report a rapidly fatal case of GAE in a woman with cryoglobulinemia treated with rituximab. GAE is a rare but often lethal subacute cause of meningoencephalitis, typically occurring in immunocompromised patients. *Acanthamoeba* spp. are the most common free-living amebae, are ubiquitous in the environment, and are widespread in water and soil. Low-level antibody titers are found in >50% of asymptomatic patients (*5*,*6*). More than 20 species of these amebae have been described (*1*). In addition to causing keratitis after use of contact lenses, *Acanthamoeba* spp. are responsible for meningoencephalitis in chronically ill, debilitated patients; in patients taking immunosuppressive drugs or receiving chemotherapy; and in patients with AIDS (*1*). GAE shows a chronic course with atypical symptoms of low-grade fever, encephalopathy with cognitive abnormalities, headache, and seizures. This disease is rarely identified in patients before death. CSF analysis shows moderate pleiocytosis, but it rarely contributes to the diagnosis because amebae are generally not found in the CSF. Recently, detection of serum antibodies was successfully used in screening for free-living amebae in patients in California with encephalitis (*7*). However, this process proved to be of value mainly for identifying *B. mandrillaris*, another free-living ameba.

Evidence of *Acanthamoeba* infection was recently found by PCR in brain tissue of a patient with lupus, even

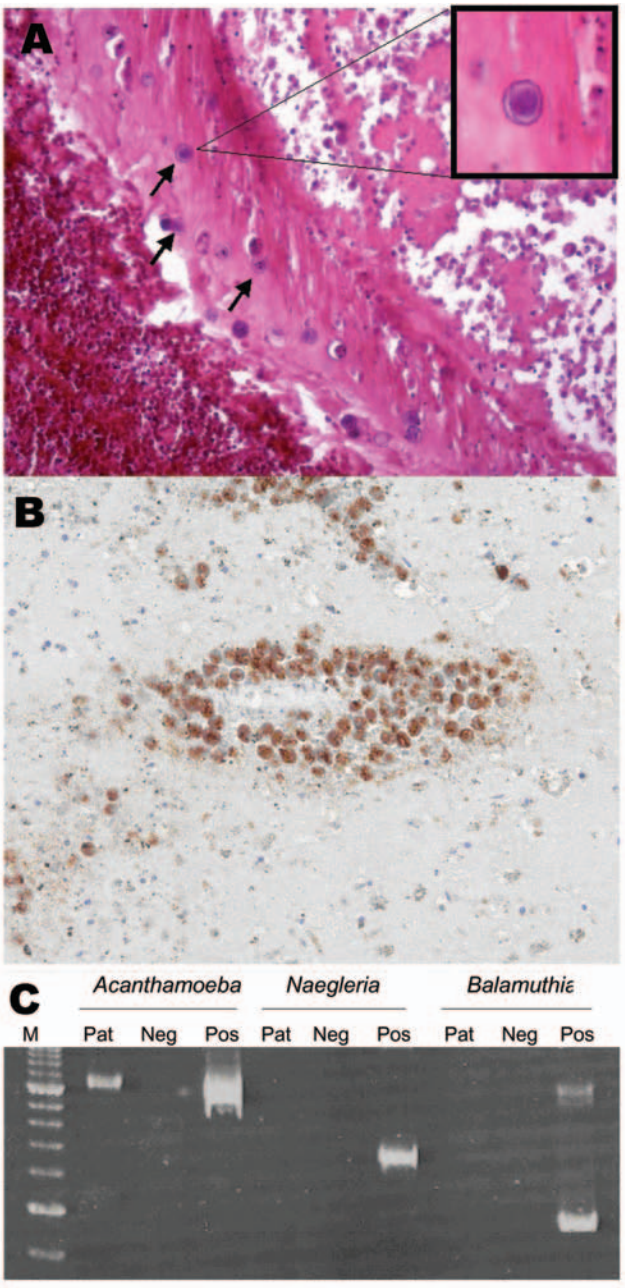

Figure 2. A) Cysts in a vessel wall (arrows) of the patient (hematoxylin and eosin stain, magnification ×250). Inset shows a cyst at higher magnification (hematoxylin and eosin stain, magnification ×800). B) Immunohistochemical staining with antibody to *Acanthamoeba* cysts within vessel walls (magnification ×250). C) Polyacrylamide gel electrophoresis of PCR products for *Acanthamoeba* spp., *Naegleria* spp., and *Balamuthia mandrillaris* using JDP primers for a diagnostic small subunit rDNA fragment. M, molecular mass marker; Pat, patient; Neg, negative control; Pos, positive control.

without isolating the ameba (*8*). A PCR with tissue and CSF is potentially useful in clinical laboratories in identifying the cause of meningoencephalitis without the need for specific antibodies.

Because the patient's cryoglobulinemia was poorly controlled, we decided to begin treating our patient's condition with rituximab. This monoclonal antibody has been shown to be effective in treating cryoglobulinemia resistant to standard therapies with corticosteroids and plasmapheresis (*9*). This antibody against the CD20 receptor on the surface of the B lymphocytes destroys these cells by mechanisms involving complement-mediated and antibody-dependent cytotoxicity (*10*). Levels of B cells remain low for 2 to 6 months, which leads to long-term humoral immune dysfunction. Serious viral infections such as erythrocyte aplasia caused by parvovirus B19, fatal hepatitis B, and cytomegalovirus and varicella-zoster virus infections have been reported after administration of rituximab (*11–14*). The fact that our patient died of *Acanthamoeba* encephalitis is striking because rituximab is believed to interfere with humoral immunity, which is not known to play a major role against free-living amebic infections.

We identified a temporal relationship between the weekly rituximab treatments and meningoencephalitis. Although the patient received corticosteroids and underwent plasmapheresis, GAE developed only after rituximab infusions had begun and the steroid dose was being tapered. It is not clear whether rituximab is the only agent responsible for the GAE, or what contributions were made by the earlier treatments the patient received. We could not identify the source of infection in this patient. In particular, we found no evidence for nosocomial acquisition. No other patient had a diagnosis of *Acanthamoeba* brain infection, and no one receiving rituximab had unexplained meningoencephalitis in 2005 in our institution. Our hypothesis is that *Acanthamoeba* spp. were already present in the brain at the time of the first rituximab infusion, but that rituximab may have precipitated the unusually rapid course of the encephalitis.

Rituximab is obtaining widespread use in hematologic and autoimmune diseases as an adjuvant therapy. The full spectrum of opportunistic infections in patients receiving combinations of immunosuppressive regimens remains to be elucidated and warrants vigilance.

Acknowledgments

We are greatly indebted to Elke Wollants for performing the PCR for *Acanthamoeba* spp. on brain tissue and CSF.

Dr Meersseman is a consultant in the medical intensive care unit at the University Hospital of Leuven. His primary research interests are difficult-to-diagnose infections in the intensive care unit.

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Mouse-to-Human Transmission of Variant Lymphocytic Choriomeningitis Virus

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A case of lymphocytic choriomeningitis virus (LCMV) infection led to investigation of the reservoir. LCMV was detected in mice trapped at the patient's home, and 12 isolates were recovered. Genetic analysis showed that human and mouse LCMVs were identical and that this LCMV strain was highly divergent from previously characterized LCMV.

Lymphocytic choriomeningitis virus (LCMV) belongs to the genus *Arenavirus* in the family *Arenaviridae*. *Mus musculus* mice constitute the reservoir of LCMV in nature (*1*), but hamsters can also carry the virus. Humans usually become infected through direct contact with infected rodents or by inhaling infectious rodent excreta or secreta during occupational exposure (laboratory workers, rodent sellers) or when caring for rodents as pets. Although LCMV infection is usually asymptomatic or mild and self-limiting, it can be severe and manifest itself as meningitis and encephalitis (*2*,*3*). Infection during pregnancy may cause abortion or congenital malformations (*4*). In 2003 and 2005, two episodes of infections occurred in 2 groups of 4 recipients of solid-organ transplants; 7 of the 8 patients died (*5*). Although laboratory evidence of LCMV infection was obtained from the 8 organ recipients and from the hamster handled by donor 2, no laboratory evidence of infection could be obtained from specimens from both donors. Therefore, evidence for transmission from donor to recipient was mainly based on epidemiologic data and on the genetic identity of the virus detected in transplanted organs.

The Study

A 5-year-old boy was admitted to the neuropediatric ward of a Marseille University hospital in August 2004 for aseptic meningitis. Forty-five days later, he was admitted again with fever and meningitis, and his condition rapidly deteriorated with encephalitis and hydrocephalus developing as described (*6*). LCMV infection was suspected on the basis of the clinical signs and a detailed interview of the mother, in which she indicated that "the house was invaded with mice." LCMV was diagnosed based on seroconversion and positive PCR results with confirmatory sequence data (*6*).

Because *M. musculus* is the natural host of LCMV, the Public Health Office of Marseille organized a trapping campaign in the vicinity of the patient's house, which resulted in the capture of 20 mice (*M. musculus*) with glue traps. Most were alive when they were received at the laboratory. They were humanely killed and dissected; kidneys, lungs, heart, spleen, and liver were placed individually in 1.8-mL tubes and stored at −80°C. For each animal, 1 kidney was homogeneized as previously described (*7*). Recovered material was used either for virus isolation on Vero cells (detailed protocol available on request) or for total RNA isolation with the EZ1 Virus Mini Kit v2.0 on the BioRobet EZ1 Workstation (QIAGEN SA, Courtaboeuf, France) and eluted in 75 μL final volume. A total of 10 μL was tested for LCMV RNA as described herein. Three different PCR assays, targeting the nucleoprotein gene, were used. System 1 was a nested reverse transcription–PCR (RT-PCR) that used primers 1817V-LCM (5′-AIATGATGCAGTCCATGAGTGCA CA) and 2477C-LCM-3′ (5′-TCAGGTGAAGGRTGGC CATACAT-3′) for the first round and primers 1902V-LCM (5′-CCAGCCATATTTGTCCCACACTTT-3′) and 2346C -LCM (5′-AGCAGCAGGYCCRCCTCAGGT-3′) for the second round. These primers were derived from those reported by Bowen et al. (*8*,*9*) and designed from the alignment of LCMV sequence data retrieved from GenBank. System 2 was a real-time RT-PCR with primers LCM_TM_NP1 (5′-TCATGTGGCARRATGTTGTG-3′) and LCM_TM_NP2 (5′-AAAAAGAAIAARGARAT CACCCC-3′) together with a FRET probe LCM_ MAR_NP (5′-ATGATGCAATCCATAAGTGCGCAGT-3′). System 3 was a SYBR Green real-time RT-PCR based on primers LCM_SG_NP1 (5′-TTRTCRTCYCTYYTYT CYTTYCTCAT-3′) and LCM_SG_NP2 (5′-CAGGTRA CYTTYGARAAITRRAGRAA-3′). The 3 detailed RT-PCR protocols are available on request. Human cerebrospinal fluid (CSF) samples and mouse specimens were added to Vero cells. After incubation at 37°C for 7 days, cells were tested for LCMV RNA by PCR with PCR system 1.

Results of PCRs and virus isolation are presented in the Table. Criteria to consider that samples contained LCMV RNA or LCMV were 1) virus isolation or 2) positive PCR results for at least 2 systems. Genetic analyses

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Table. Detection of LCMV in mice trapped at patient's home and in patient CSF*

Sample	System 1		System 2	System 3	Virus isolation	Interpreted result†
	First-round PCR	Second-round PCR				
Human						
CSF 1	–	+	+	NT	NT‡	+
CSF 2	–	+	+	NT	NT	+
Mouse						
1	–	+	+	+	–	+
2	–	–	+	+	+	+
3	–	–	–	–	–	–
4	–	+	+	–	+	+
5	–	+	+	–	+	+
6	–	+	+	–	+	+
7	–	+	+	+	–	+
8	+	+	+	+	–	+
9	+	+	+	+	+	+
10	–	–	+	+	+	+
11	–	+	+	+	–	+
12§	–	+	+	+	+	+
13	–	+	+	+	+	+
14	–	–	–	–	–	–
15	–	–	+	+	+	+
16	–	+	+	+	+	+
17	–	+	+	+	–	+
18	+	+	+	+	+	+
19	–	–	+	–	+	+
20	–	+	+	+	+	+

*LCMV, lymphocytic choriomeningitis virus; CSF, cerebrospinal fluid, NT, not tested.
†An animal was considered infected with LCMV if virus was isolated either in Vero cells or results of at least 2 of 3 PCRs were positive.
‡After diagnostic tests were performed from CSF, no material was available to attempt virus isolation.
§Virus strain characterized by full-length genome sequencing (GenBank accession nos. DQ286931 and DQ286932 for S and L RNA, respectively).

and phylogenetic reconstruction were based on sequences flanked by primers 1902V-LCM and 2346C-LCM. These primers amplified a 445-bp PCR product (primers included) and provided a 400-nt sequence (primers excluded) used for analysis. Nucleotide alignments were performed by using ClustalX 1.81 with default parameters (*10*). Alignments included the 16 sequences determined in this study and homologous LCMV sequences retrieved from the GenBank database. Phylogenetic analysis was performed with the Jukes-Cantor algorithm for distance calculation and the neighbor-joining method for cluster reconstruction with the MEGA 2.0 program (*11*). The robustness of nodes was tested by 500 bootstrap pseudoreplications.

As shown in the Table, the 2 human CSF specimens and 14 of 20 mouse samples were PCR positive. The 2 sequences obtained from human CSF specimens were 100% identical to each other. The 14 sequences representing mouse kidney specimens were almost identical (98.5% nucleotide identity) (Figure). Comparison of human and mouse sequences showed genetic identity >98% at the nucleotide level (Figure). This high level of similarity suggests that human LCMV infection was caused by transmission from the mice. All 16 sequences determined in this study either from human or rodent material had 12%–13% nucleotide heterogeneity when compared with LCMV sequences deposited in the GenBank database and with the sequence of LCMV strain manipulated in the laboratory, thus excluding the possibility of laboratory contamination. Finally, a total of 12 strains were isolated from Vero cells; 1 was selected to be characterized by full-length genome sequencing (GenBank accession nos. DQ286931 and DQ286932 for S and L RNA sequences, respectively).

Conclusions

Apart from isolated cases or outbreaks of LCMV infection associated with direct contact with laboratory rodent colonies, evidence for direct epidemiologic links between human cases and wild mice was based on virus isolation and antigenic relationships. Evidence based on genetic analysis of human and mice strains was not previously reported. Field investigations, conducted between LCMV discovery in 1933 and World War II, to search for a source of human cases, reported virus isolation from gray mice (*M. musculus*) trapped in or in the close vicinity of patient's house. The strains isolated from mice and humans

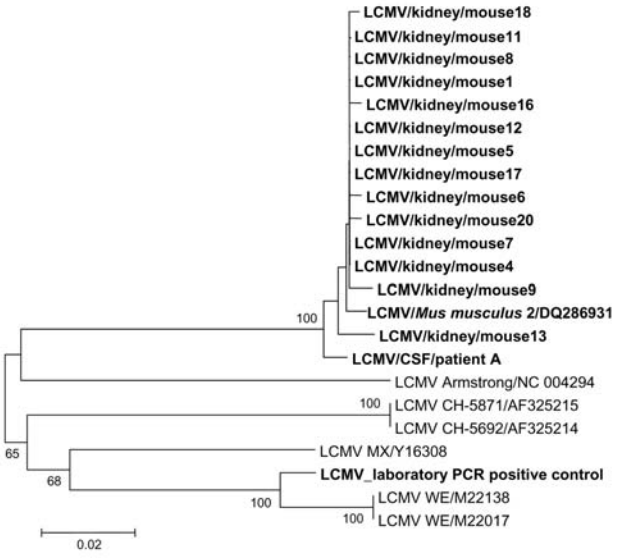

Figure. Phylogenetic tree based on 400-nt sequences amplified by PCR system 1 in the nucleoprotein gene. Lymphocytic choriomeningitis virus (LCMV) sequences characterized in this study were compared with selected homologous LCMV sequences available in the GenBank database. Sequence information corresponds to virus/nature of specimen/host/GenBank accession no. (optional), except for sequences retrieved from GenBank (virus strain/GenBank accession no.). Sequences determined in this study are in **bold type**. The 15 sequences determined from mouse material were almost identical and most closely related to LCMV sequence corresponding to the patient cerebrospinal fluid. These 16 sequences were grouped together and were clearly distinct from other LCMV strains included in the study (Armstrong, CH, MX, WE) and from Traub and Pasteur strains (data not shown).

were similar antigenically and immunologically. However, indisputable evidence of genetic identity was not produced. To our knowledge, this is the first report of human LCMV infection linked to wild mice as assessed by genetic evidence. Rates of infection by LCMV in wild rodent colonies reported in the literature (*12*,*13*) are of similar magnitude as the 70% rate found in this study. Such high rates can explain how clusters of human cases are likely to result from substantial exposure to infectious aerosols. In our study, the mother of the patient was negative for LCMV antibodies; other members of the family refused to undergo serologic testing. Altogether, these sequence data and the evidence of virus isolation from mice provide strong evidence that the LCMV human case resulted from infection with a virus carried by mice infesting the patient's home through direct or indirect contact with mouse excreta. In the case reported by Fischer et al. (*5*), although the LCMV cause is not in doubt, the lack of laboratory evidence (serology, immunohistochemical staining, PCR, virus isolation) of LCMV infection in both donors is intriguing.

This study, together with recent reports of LCMV infection cases, raise concerns regarding the low level of knowledge of LCMV epidemiology that may reflect the fact that LCM was historically more prevalent in rural settings, and that it could be decreasing in the urban populations of industrialized countries. However, the growing proportion of persons living below the poverty threshold in large European and North American cities may recreate conditions compatible with the increased urban circulation of mice, and therefore increase the likelihood of rodent-associated diseases.

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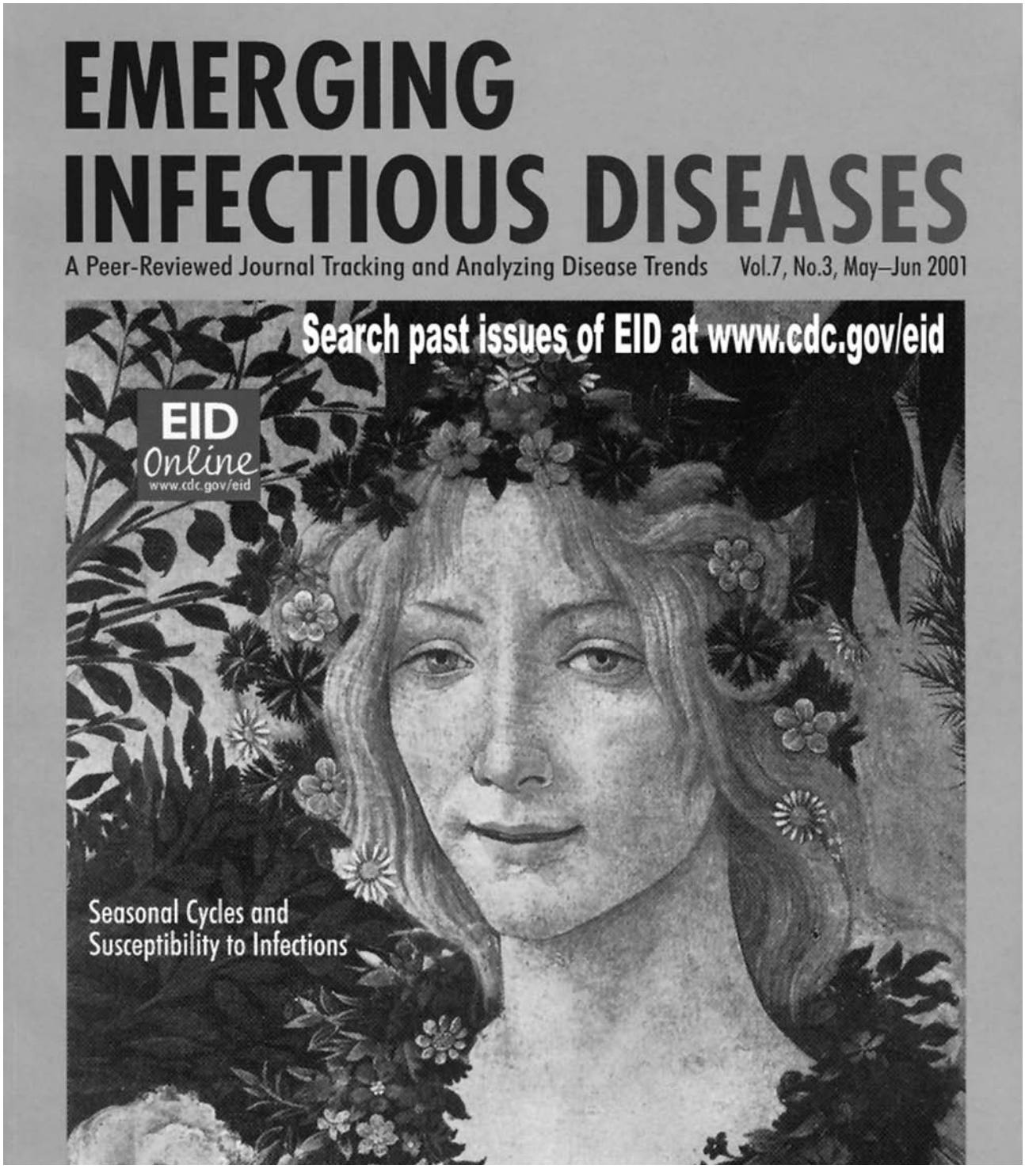

Leishmania donovani and Cutaneous Leishmaniasis, Sri Lanka

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To investigate the relationship of cutaneous leishmaniasis isolates from Sri Lanka to known species, we performed DNA sequencing and microsatellite analyses. We identified *Leishmania donovani* as the agent of Sri Lanka cutaneous leishmaniasis and showed that these parasites are closely related to those causing visceral leishmaniasis in the Indian subcontinent.

Infection with *Leishmania* protozoa can result in cutaneous, mucocutaneous, or visceral leishmaniasis (VL), depending on the parasite, host, and environmental factors (*1*). Globally, the disease results in ≈2 million new cases and 2.4 million disability-adjusted life years each year (*2*). The leishmaniases have received renewed interest because of an upsurge of cases in traditionally leishmaniasis-endemic areas and the emergence of new foci of disease (*3*,*4*). One of the most dramatic examples is a new focus of cutaneous leishmaniasis (CL) in Sri Lanka (*5*), from which >400 cases have been reported since 2001.

Previously, multilocus enzyme electrophoresis (MLEE) characterization of a small number of isolates led to the surprising conclusion that CL in Sri Lanka was caused by *Leishmania donovani* (*5*). However, *L. donovani* typically causes VL, a potentially fatal disease and ongoing public health problem in neighboring India, Bangladesh, and Nepal, as well as in East Africa (*1*,*2*). No cases of VL have been reported in Sri Lanka. Occasional cases of CL due to *L. donovani* have been described in other VL-endemic regions (*6*–*9*). Karunaweera et al. (*5*) examined a limited number of isolates and used a single technique, MLEE. Although this technique is usually reliable for characterizing isolates, important exceptions were found in a recent study on *L. donovani* in East Africa (*10*). Therefore, we further investigated Sri Lanka CL by examining more isolates and using 2 molecular techniques.

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The Study

Suspected clinical diagnoses of CL were confirmed by demonstrating the presence of *Leishmania* amastigotes in skin lesions, promastigotes in cultures, or both (*5*). Ethical approval was obtained from the Ethics Review Committee, Faculty of Medicine, University of Colombo. PCR, performed as described (*11*), confirmed 15 primary isolates as members of the genus *Leishmania.* Eight of the Sri Lanka isolates originated from Welioya (northeast), 1 from Jaffna (north), and 2 from Galle (south).

DNA sequencing of a single-copy gene was used to identify the *Leishmania* species (*10*). The 6-phosphogluconate dehydrogenase (6PGDH) gene was chosen because it shows a high degree of sequence polymorphism among *Leishmania* species (*12*), is well represented in sequence databases, and is known to differentiate the main zymodeme from *L. donovani* in India (MON-2) from that elsewhere (*13*). Primers for conserved regions of 6PGDH were designed by using full-length gene sequences of the *L. major* FV1 (MHOM/IL/1980/Friedlin) and *L. mexicana* BEL21 (MHOM/BZ/1982/BEL21) reference strains. Primers 6PGDH-F (AAT CGA GCA GCT CAA GGA AG) and 6PGDH-R (GAG CTT GGC GAG AAT CTG AC) were designed to generate a 997-bp amplicon incorporating the 822-nt partial 6PGDH sequence that is represented for multiple *Leishmania* species in GenBank. The partial sequences of 6PGDH genes were obtained from 11 Sri Lanka isolates from patients with CL, 2 India isolates from patients with VL, and 2 additional known *L. donovani* strains. These 15 new sequences and 10 publicly available sequences for species belonging to the genus *Leishmania* were used to construct a classification (Figure 1). Of 17 *L. donovani* and *L. infantum* sequences, 14 were >99% identical and could not be separated; the remaining 3 stocks were from India and Bangladesh (Ind-1, Ind-2, and BG1) and clustered together with 58% bootstrap support. Thus, *L. donovani* from Sri Lanka formed a strongly supported group with *L. donovani* and *L. infantum* from Europe and Africa. This group was quite distinct from the group that includes *L. major* and *L. tropica*, which are the parasite species most closely related to *L. donovani* and *L. infantum* and which both cause CL in Africa and Asia. This analysis provided convincing evidence that all 11 Sri Lanka isolates examined were *L. donovani* or *L. infantum.*

Strains of *L. donovani* from Sri Lanka were typed as zymodeme MON-37 by MLEE (*5*). This differs from the predominant India zymodeme (MON-2) in the mobility of 1 isoenzyme, 6PGDH. Therefore, the sequences were further analyzed to investigate the sequence variation underlying the isoenzyme identification. Translation of the 822-nt sequences showed 1 amino acid change that was consistent with the results of MLEE. A single nucleotide difference at position 976 was responsible for the occur-

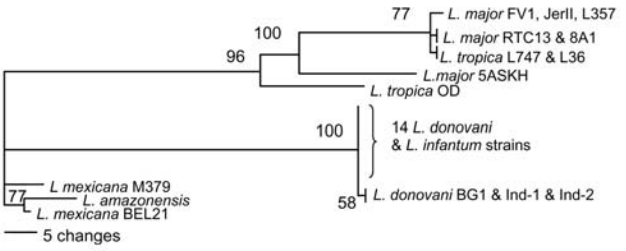

Figure 1. Classification of *Leishmania* species according to the partial DNA sequence of the 6-phosphogluconate dehydrogenase gene constructed with PHYLIP (http://evolution.genetics.washington.edu/phylip.html) using parsimony. Numbers at branch points are bootstrap values compiled by using 100 replicates. Isolates examined and the accession numbers of their 6PGDH sequences in the GenBank/EMBL/DDBJ database are as follows: 11 Sri Lanka isolates, L59, L60, L75, L78, L80, L284, L304, L355, L330, L301, L348 (AJ888888-AJ888898); 2 India isolates, Ind-1, Ind-2 from splenic aspirates of visceral leishmaniasis patients in Muzafapur, Bihar (MHOM/IN/2004/Ind-1 and MHOM/IN/2004/Ind-2, AJ888900, AJ888901); 3 previously identified *L. donovani* isolates BG1 (MHOM/BD/1997/BG1, AJ888899), LEM719 (IMAR/KE/1962/LRC-L57; LEM719, AJ888902), LV9 (MHOM/ET/1967/HU3;LV9, AY168567); and *L. infantum* JPC (MCAN/ES/1998/LEM935;JPC;M5, GeneDB LinJ35.2940). Also analyzed were sequences from the following isolates: *L. tropica* (AY045763, AY168568), *L. major* (FV1, AF242436; 8A1, AF242436; RTC13, AY706106; JerII, AY706105; 5ASKH, AY706107), *L. mexicana* (M379, AY217723; BEL21, AY386372), and *L. amazonensis* (PH8, AY168562).

rence of an uncharged asparagine (codon AAC) in MON-2 or a negatively charged aspartic acid (codon GAC) in MON-1, MON-18, and MON-37 sequences. This single change would explain the lower mobility of the MON-2 6PGDH isoenzyme, similar to the situation previously reported for glutamate oxaloacetate transaminase isoenzymes in East Africa *L. donovani* strains (*10*).

To more closely analyze the relationships of the *L. donovani* and *L. infantum* strains, we performed microsatellite analysis (*10*). These data were combined with a dataset comprising 40 previously examined *L. donovani* and *L. infantum* isolates (Figure 2). The Sri Lanka isolates clustered together and close to a group containing *L. donovani* isolates from India, Bangladesh, and Nepal. *L. infantum* isolates formed a distinct cluster, as did the *L. donovani* isolates from Sudan and Kenya. This analysis reconfirms recent observations (*10*,*14*) that *L. donovani* isolates tend to cluster on a geographic basis, which suggests that strains of this parasite are geographically distinct. Also, although the Sri Lanka isolates form 1 or possibly 2 distinct groups, they are most closely related to *L. donovani*, which causes VL in India, and distant from *L. infantum* parasites.

Conclusions

The results of this study led us to conclude that in Sri Lanka, CL is caused by *L. donovani*, which affects the epidemiology and clinical management of leishmaniasis. CL in Sri Lanka can no longer be regarded as a minor problem; an explosion of cases in the past 5 years, undoubtedly an underrepresentation of the true incidence of disease, has not included a single case of VL. However, the possibility that VL will emerge should be considered because subclinical infection is frequent in VL-endemic areas (*15*). The clinical management of CL is often self-cure, which may be preferable to active treatment because self-cure may promote natural immunity to reinfection. Alternatively, antileishmanial drugs may be administered topically or by intralesional injection (*2*). However, *L. donovani* is recognized as one of the great scourges of mankind (*3*,*4*), and if visceral disease does emerge as a problem, more aggressive treatment of CL in Sri Lanka should be considered, e.g., parenteral administration of antimonial compounds, amphotericin, or oral miltefosine. Unfortunately, no drugs are currently registered for the treatment of leishmaniasis in Sri Lanka, and cryotherapy is the only available option in most healthcare centers. Better availability of drugs to treat CL in Sri Lanka is needed, but their introduction must be carefully monitored and critically evaluated.

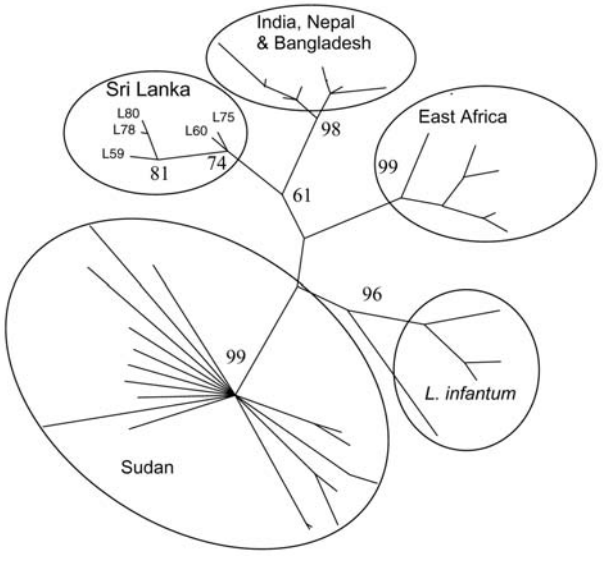

Figure 2. Classification of *Leishmania donovani* and *L. infantum* isolates constructed by using microsatellite data with parsimony in PAUP (Sinauer Associates Inc., Sunderland, MA, USA). Numbers at branch points are bootstrap values compiled by using 100 replicates. Isolates formed geographically based groups (circled). Sri Lanka isolates L59, L60, L75, L78, and L80 are indicated. The tips of other branches are from a dataset of other previously analyzed isolates, including all those identified as *L. donovani* or *L. infantum* and isolates from the Indian subcontinent (*10*).

Our study also raises questions about how infection with apparently identical or very similar parasites can result in radically different types of disease. We speculate that the answers likely lie with the nature of the parasites, the genetics of the human population, or the contribution of sandfly vectors. The data presented here demonstrate the overall close genetic similarity among all *L. donovani* isolates examined. However, some critical genetic difference in Sri Lanka parasites may exist and render them less virulent than *L. donovani* from elsewhere. Clearly much work remains to be done, including PCR or serologic investigation of possible subclinical VL, to understand the factors behind the emergence of Sri Lanka CL due to *L. donovani.* Future studies must be a priority as the number of cases continues to increase.

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Dr Siriwardana qualified from the Faculty of Medicine, University of Colombo, and is currently completing postgraduate PhD training in medical parasitology. Her main research interest is in leishmaniasis.

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Depression after Infection with West Nile Virus[1]

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Previous reports have noted depression after West Nile virus (WNV) infection. We further measured this outcome and found that 31% of patients reported new-onset depression and 75% of these had Center for Epidemiologic Studies Depression scores indicative of mild-to-severe depression. Physicians should be aware of neuropsychiatric consequences of WNV in patients.

West Nile virus (WNV) was identified for the first time in the Western Hemisphere in New York City in 1999; since then, a dramatic westward and southward spread of WNV activity has occurred in the United States (*1*,*2*). In 2002, WNV was identified in the Houston, Texas, metropolitan area, resulting in 105 human cases (*3*).

Long-term clinical sequelae after infection are still being defined. A year after the outbreak of WNV in New York City, 38% of patients subjectively reported depression (*4*). Another 1-year follow-up in Colorado noted that 23% of patients reported anxiety and depression (*5*). In Houston, we have been conducting a prospective study that involves both subjective and objective measurements of physical, neurologic, and cognitive functioning of patients identified with symptomatic WNV infections. We describe the subjective and objective evaluations of depression in these patients.

The Study

Clinical WNV patients, confirmed by immunoglobulin M ELISA and identified through local surveillance in the Houston metropolitan area from 2002 through 2004, were invited to participate in a study to determine long-term clinical sequelae. Those consenting to participate were interviewed. No patient was denied participation on the basis of age, sex, race, or ethnicity. Excluded patients included those who were residing outside of the Houston area, deceased, or lost to follow-up. This study was reviewed and approved by the University of Texas Health Science Center Institutional Review Board (HSC-SPH-03–039).

Initial interviews were conducted after hospitalization, and follow-up interviews were conducted every 6 months until the patient reported being back to pre-WNV infection functioning. For the 1-year follow-up, interviews were conducted during each December at the end of transmission season. Interviews were mainly conducted over the telephone, but a small proportion were conducted in person. In all of these interviews, a higher than expected proportion of patients reported depression immediately after their illness. To better assess this quantitatively, we incorporated the Center for Epidemiologic Studies Depression (CES-D) scale (*6*) into both initial and follow-up interviews. This scale is a commonly used method for objective measurement of clinical depression. The tool is composed of 20 questions focused on self-report of depressive moods and behavioral changes experienced by the patient during 1 week. The resulting scores were interpreted as follows: 1) <15, the patient is not experiencing depression; 2) 15–21, the patient may be experiencing mild-to-moderate depression; 3) ≥22–60, the patient may be experiencing major depression. In addition to the objective measurement using the CES-D scale, we also asked patients if they were experiencing depression since their illness and if they had a previous history of depression, with yes/no responses elicited. Barthel Index scores were used to quantitatively evaluate level of physical functioning and disability in patients; a score of 100 points indicated no physical disability. Because patients also commonly reported a change in personality immediately after WNV infection, we assessed this finding subjectively and asked those reporting a change to describe what they were experiencing.

Data were analyzed by using NCSS statistical software (Kaysville, UT, USA). With the Kruskal-Wallis 1-way analysis of variance on ranks, we analyzed CES-D scores and WNV outcome; CES-D scores and sex, age, and depression (CES-D score of ≥15); and physical functioning (Barthel Index) and depression.

A total of 65 patients were interviewed; 38 (58%) cases had encephalitis when initially evaluated, 19 (29%) had meningitis, and 8 (12%) had fever. The mean age of patients was 55 years (range 12–86 years). Most patients were white, non-Hispanic (80%), followed by black (11%) and white, Hispanic (9%).

One year after infection with WNV, 26 patients reported experiencing depression. Of these, 6 reported a history of depression before infection. Of the 20 patients considered to have new-onset depression, 13 (65%) had a clinical diagnosis of WNV encephalitis (Table), and 10 (50%) were male. The mean CES-D scores for those who reported no depression was 5.5 (range 0–19) compared with a mean score of 22 (range 0–44) for those who reported depression. There was no statistical difference in CES-D

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[1]A portion of this study was presented by K. Murray at the 2006 National West Nile Virus Conference in San Francisco, California.

Table. Subjective and objective measurements of depression and personality changes 1 year after clinical illness from WNV infection*

	All patients (%)	West Nile encephalitis		West Nile meningitis/ West Nile fever	
		Male (%)	Female (%)	Male (%)	Female (%)
Measurements					
New onset depression since WNV	20/65 (31)	8/27 (30)	5/11 (45)	2/12 (17)	5/15 (33)
Mean CES-D score for those reporting depression (range)	22 (0–44)	17 (7–30)	23 (0–37)	38 (32–43)	22 (0–44)
CES-D score ≥15	15/20 (75)	5/8 (63)	4/5 (80)	2/2 (100)	4/5 (80)
Taking antidepressants	7/20 (35)	3/8 (38)	1/5 (20)	1/2 (50)	2/5 (40)
Report antidepressants helping with symptoms	4/7 (57)	2/3 (67)	1/1 (100)	0/1 (0)	1/2 (50)
Receiving counseling	2/20 (10)	0/8 (0)	1/5 (20)	1/2 (50)	0/5 (0)
Report counseling helping with symptoms	2/2 (100)	0	1/1 (100)	1/1 (100)	0
Personality change since WNV	29/65 (45)	12/27 (44)	6/11 (55)	4/12 (33)	7/15 (47)
Anger/irritability/aggression	19/29 (66)	8/12 (67)	4/6 (67)	3/4 (75)	4/7 (57)
Decreased socialization	8/29 (28)	3/12 (25)	1/6 (17)	1/4 (25)	3/7 (43)
Increased sensitivity/cries easily	3/29 (10)	0/12 (0)	1/6 (17)	0/4 (0)	2/7 (29)
Feelings of hopelessness	1/29 (3)	1/12 (8)	0/6 (0)	0/4 (0)	0/7 (0)

*WNV, West Nile virus; CES-D, Center for Epidemiological Studies Depression.

scores between those who had encephalitis and those who had meningitis or fever (p = 0.19) or between those with West Nile neuroinvasive disease (encephalitis or meningitis) compared with those with fever (p = 0.55). On the basis of CES-D scores for those self-reporting depression since their illness with WNV, 5 (25%) patients were classified by CES-D as not depressed, 6 (30%) were classified as having mild-to-moderate depression, and 9 (45%) were classified as having major depression. Of the 39 patients who self-reported that they had not had depression since their WNV illness, 4 had a CES-D score of ≥15. No statistically significant associations were found between loss of physical functioning (Barthel index scores <100; p = 0.39), sex (p = 0.89), or age (p = 0.47) and depression (CES-D scores of ≥15).

Seven (35%) patients reported taking antidepressants for their symptoms; 4 of these patients reported at least some improvement. CES-D scores of 6 of these 7 patients were >15; 4 showed evidence of major depression. Two patients reported seeing a counselor for depression, including 1 who was also taking antidepressants. Both patients reported that the counseling was helpful; however, based on their CES-D scores, both were classified as having major depression at the time of the interview.

Personality changes were reported subjectively among 29 (45%) of the 65 patients (Table); 18 (62%) of these were in patients who had encephalitis, and 16 (55%) of these patients were male. When asked to describe their personality change, 19 patients (66%) reported an increase in anger (being short-tempered and irritable); 8 patients (28%) reported being less social; 3 patients (10%) reported increased sensitivity; and 1 patient (3%) reported feelings of hopelessness.

Conclusions

Depression and personality changes after WNV infection have been briefly observed in previous studies (*4*,*5*); however, this study is the first known to objectively evaluate this outcome. As evidenced by subjective and objective measurements, depression is an important outcome in patients with a clinical diagnosis of WNV infection: indeed, 75% of those reporting no previous history of depression had high CES-D scores. Understanding the pathology of this outcome and determining whether the depression is situational (a result of prolonged recovery) or caused by chemical changes in the brain related to inflammation are critical. Depression was not associated with loss of physical functioning, sex, or age.

Depression after encephalitis, regardless of etiology, is not uncommon. Depression and personality changes in humans have been previously reported as a neuropsychiatric consequence of Lyme disease, Nipah virus, tickborne encephalitis virus, and Saint Louis encephalitis virus infections (*7*–*10*). After the encephalitis lethargica pandemic from 1917 to 1926, depression, mania, and obsessive-compulsive disorder were observed in postencephalitis patients (*11*). These observations led to the understanding of the role of the basal ganglia in mood, personality, and obsessional syndromes. In a mouse model experiment, infection of the brain with Venezuelan equine encephalitis virus resulted in a serotonin presynaptic deficit and postsynaptic hyperreactivity of the serotonin system (*12*).

Inflammation of the brain can result in an alteration in the neurotransmitter serotonin, which may lead to the development of mood disorders (*13*). Capuron et al. identified a significant proportion of patients in whom depression developed after cytokine therapy for cancer. The neurotoxic inflammation induced by cytokines resulted in

decreased levels of tryptophan, the amino acid precursor for serotonin. This decrease was positively correlated with the development and severity of depressive symptoms in patients.

The long-term clinical sequelae of WNV neuroinvasive disease need to be further defined. By understanding potential outcomes and determining whether certain interventions such as physical therapy, counseling, and antidepressant drug therapy can improve recovery, we can better understand prognosis and potential treatment interventions. Physicians should note that depression and personality changes could be important neuropsychiatric consequences in patients with clinical WNV infection.

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Dr Murray is an assistant professor of epidemiology at the University of Texas Health Science Center at Houston, School of Public Health, Center for Infectious Diseases. She assisted in the investigation of the WNV outbreak in New York City in 1999. Her current research is focused on the clinical investigation of WNV in Houston, including long-term clinical sequelae in patients who had neuroinvasive disease.

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Detection of G12 Human Rotaviruses in Nepal

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Of 731 stool specimens collected from children with diarrhea in Kathmandu, Nepal, from August 2004 through July 2005, 170 (23.3%) tested positive for rotavirus. Reverse transcription–PCR, including a revised G12-specific primer set, identified 56 (32.9%) as G2P[4] and 39 (23.0%) as G12 with P[6], P[8], or P[4].

Globally, ≈700,000 children each year die of rotavirus diarrheal disease, and most such deaths occur in developing countries (*1*,*2*). To reduce this substantial burden of rotavirus diarrhea, 2 live, attenuated oral vaccines have been licensed in >60 countries and are being introduced into the areas where they are most needed (*3*). Nepal is a small, poor, landlocked, subtropical country in Asia where the severe diarrhea is common. During 2003–2004, before Nepal was formally integrated into the Asian Rotavirus Surveillance Network (*4*), a surveillance study was initiated, which showed the emergence of G12 strains against a background of predominant G1P[8] strains (*5*). Since the prevalence of G12 strains was unusually high (20%), and such strains may not be adequately covered by existing vaccines, there was an urgent need to continue surveillance and characterization of rotavirus strains by developing a G12-specific primer pair and to continue to monitor the prevalence of G12 strains in this population.

The Study

Stool specimens were collected from children with acute diarrhea attending the rehydration clinic at Kanti Children's Hospital, Kathmandu, Nepal, from August 2004 through July 2005. Rotavirus diarrhea was identified by a commercially available ELISA (Rotaclone, Meridian Bioscience, Inc., Cincinnati, OH, USA). From the rotavirus-positive specimens, genomic RNA was extracted by using a QIAamp Viral RNA Mini kit (QIAGEN Sciences, Germantown, MD, USA), and the purified RNA preparations were used to determine G and P types by reverse transcription–PCR (RT-PCR) as described by Uchida et al (*5*) and by Gouvea et al. (*6*). For the detection of G12 strains, a new primer pair was designed based on the nucleotide sequence of the VP7 gene of strain Arg720 (*7*). The forward primer, G12F, was 19 mer, corresponding to nucleotide positions 169–187 (5′-GTT GTT GTC ATG CTG CCA T-3′), and the reverse primer, G12R, was 20 mer whose sequence was complementary to nucleotide positions 471–490 (5′-A GTA CAG TAC CAA ATT TCA T-3′). A G12 strain in the stool specimen was identified by the presence of a 322-bp band after electroporesis on agarose gel after PCR typing was done on the first-round RT-PCR product under the same thermal cycling conditions, including an annealing temperature of 42°C. Sequencing was performed on the VP7 gene amplification products with primers Beg9 and End9 (Genomic Research Center, Shimadzu Corporation, Kyoto, Japan).

Of 731 stool specimens tested by Rotaclone, 170 (23.3%) were positive for rotavirus antigen. When the distribution of rotavirus-positive cases was examined, 64% occurred in infants ages 3–23 months, whereas only 2.9% occurred in infants <3 months of age. Although samples were not collected in March and April 2005, because of political instability, rotavirus likely circulated among the children in Kathmandu throughout the year. However, a seasonal variation was found in the occurrence of rotavirus diarrhea with a clear peak in January; the rotavirus detection rates varied monthly from 7.3% to 58.6%.

Of 170 rotavirus-positive specimens that underwent molecular genotyping, 4 G and 3 P genotypes were found; G2 and G12 together accounted for 62% and P[4] and P[6] together accounted for 74% of their respective genotypes (Table). A total of 10 different G and P genotype combinations were detected, which together accounted for 76% of rotavirus-positive specimens. Ten (6%) mixed infections, as defined by the specimens containing more than 1 G or P genotype, were found, and 31 (18%) specimens remained untypeable. The most common combinations were G2P[4] (33%) and G12P[6] (17%). No G4 strains were detected, nor were any P[9] strains found (which are often associated with G3 or G12 VP7 specificity). Since neither G3 nor G9 strains occurred in the preceding season in Kathmandu (*5*), their sudden appearance in Nepal needs continued attention.

The G12 primer pair developed in this study successfully amplified 2 established G12 strains, L26 (*8*) and Se585 (*9*), and did not amplify any known G1–G11 and G13–14 strains (Figure 1). Of 39 stool specimens that were found to contain G12 rotavirus by the typing reaction with our G12 primer pair, we selected 12 specimens from those

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Table. Relative frequencies of various combinations of G and P types of human rotaviruses, Kathmandu, Nepal, August 2004 through July 2005

Human rotavirus types	No. isolates (%)						Total, n (%)
	P[4]	P[6]	P[8]	P[9]	Pmix	PNT	
G1	0	5 (2.9)	9 (5.3)	0	2 (1.2)	2 (1.2)	18 (10.6)
G2	56 (32.9)	5 (2.9)	1 (0.6)	0	1 (0.6)	4 (2.3)	67 (39.4)
G3	0	10 (5.9)	0	0	0	0	10 (5.9)
G4	0	0	0	0	0	0	0
G8	0	0	0	0	0	0	0
G9	5 (2.9)	0	0	0	0	1 (0.6)	6 (3.5)
G12	2 (1.2)	29 (17.1)	7 (4.1)	0	0	1 (0.6)	39 (23)
Gmix	1 (0.6)	6 (3.5)	0	0	0	0	7 (4.1)
GNT	6 (3.5)	1 (0.6)	0	0	0	16 (9.4)	23 (13.5)
Total	70 (41.2)	56 (33)	17 (10)	0	3 (1.7)	24 (14.1)	170 (100)

samples that produced the full-length VP7 amplification product for nucleotide sequencing. Each of these VP7 sequences was identified as G12 because the predicted amino-acid sequence was 97%–100% identical with that of prototype G12 strain Se585 in any of antigenic regions A, B, and C (*10*). GenBank accession nos. for G12 VP7 genes were AB275291 (05N054), AB275292 (05K021), AB275293 (05N128), AB275294 (04N605), AB275295 (05N140), AB275296 (05N040), AB275297 (05N065), AB275298 (05K101), AB275299 (05N138), AB275300 (05K046), AB275301 (05K066), and AB275302 (05N145).

Conclusions

G12 strains, including those in the first human cases of infection in the Philippines in 1990 (*8*), had only been detected in sporadic cases, yet from geographically diverse locations, including the United States (*9*), Japan (*11*), Brazil (*12*), South Korea (*13*), and Thailand (*14*). However, recent investigations found that that 17% of rotaviruses collected from children with acute diarrhea during 2003–2005 in eastern India and 7.9% of those collected during 1999–2002 in Argentina were of serotype G12 (*7*,*15*). These findings were followed by our previous study, which showed that 20% of rotaviruses collected during 2003–2004 in Nepal were serotype G12 and were equally distributed between children <15 years of age and adults (*5*). The present study (2004–2005) confirms and extends these observations. Twenty-three percent of rotaviruses from the same region in the period immediately following the 2003–2004 study were serotype G12, and most (76%) were combined with P[6] and others with either P[8] or P[4], even though the dominant strains changed from G1P[8] during 2003–2004 (71%) to G2P[4] during 2004–2005 (33%). The detection of G1P[8] decreased to a minimum of 5.3% of all rotavirus-positive strains. The increasing trend of G12 strains in Nepal appears stable and may reflect the overall trend in the Ganges region at large, which justifies further surveillance in Nepal. G12 strains have similarly been detected in multiple seasons elsewhere. In Brazil, G12 strains continued to appear during 1999–2002 (*7*). The emergence and increase in G12P[6] strains may become a challenge to the current rotavirus vaccination strategy, the efficacy of which may depend on the shared G and P serotype specificity of the vaccine strains and wild-type rotavirus strains circulating among children. In this context, the detection of a G12 porcine rotavirus strain with a porcine genetic background in Kolkata, India (*16*), may indicate reservoirs of an unusual yet emerging G genotype in animals.

The increase in G12 strains also requires the development of a rapid detection method by PCR using specific primers that specifically target G12 strains. Although 2 earlier papers have described similar primers, neither amplification conditions nor the validation of their specificity for G12 strains have been described. Since we have encountered cross-reactions with rotaviruses carrying other genotypes, i.e, G10, in particular (data not shown), primers were designed and amplification conditions were optimized so that no cross-reactions occurred with prototype rotavirus strains carrying genotypes G1–11 and G13–14. This primer pair should become a valuable asset for the identification of G12 strains found among the nontypeable specimens in many epidemiologic studies since the primer binding sites are well conserved among G12 strains from diverse locations (Figure 2).

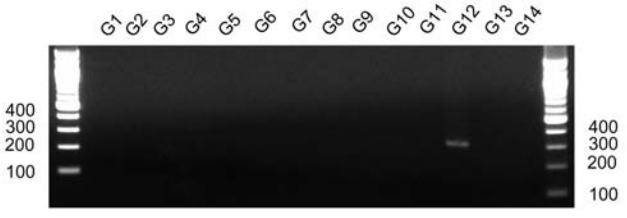

Figure 1. Detection after electrophoresis on a 2% agarose gel of the PCR amplification product with a primer pair G12F and G12R. The prototype rotavirus strain for each G serotype was as follows; G1, Wa; G2, KUN; G3, MO; G4, ST3; G5 OSU; G6, NCDV; G7, PO-13; G8, MW33; G9, 95H115; G10, B223; G11, YM; G12, L26; G13, L338; and G14, FI23. The first and last lanes show molecular mass markers in basepairs.

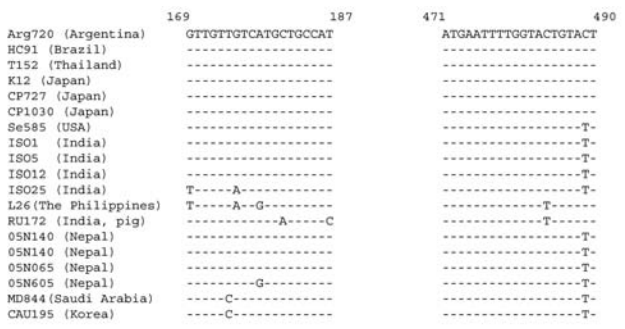

Figure 2. Comparison of the primer binding regions of the VP7 genes of G12 rotavirus strains detected in various geographic locations. Primers were designed based on the Arg720 sequence. The sequence of the forward primer is as shown in the figure; the sequence of the reverse primer is complementary to that shown in the figure.

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Clostridium difficile in Retail Ground Meat, Canada

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Clostridium difficile was isolated from 12 (20%) of 60 retail ground meat samples purchased over a 10-month period in 2005 in Canada. Eleven isolates were toxigenic, and 8 (67%) were classified as toxinotype III. The human health implications of this finding are unclear, but with the virulence of toxinotype III strains further studies are required.

Clostridium difficile is an important spore-forming human pathogen associated with serious enteric diseases worldwide (*1*–*3*). Recently, the epidemiology of *C. difficile*–associated diarrhea (CDAD), appears to have changed; increased illness and relapse rates have been reported (*1*,*3*). Much of this change has been attributed to the emergence of 1 toxigenic strain, classified according to PCR as ribotype 027/toxinotype III and pulsed-field gel electrophoresis (PFGE) as NAP1 (*2*).

Toxigenic strains of *C. difficile* typically produce 2 major toxins, A and B, although a small percentage produce only toxin B (*3*). Certain strains may also produce a binary toxin (known as CDT), whose clinical relevance is under investigation. PCR ribotype 027 strains produce all 3 toxins and have a mutated toxin regulatory gene, *tcdC*, which is thought to be associated with increased toxin production in vitro (*2*).

C. difficile is also associated with enteric diseases in animals, including horses, dogs, and pigs (*4*,*5*). Recent reports indicating that human and animal isolates are often indistinguishable (*4*,*6*) and that PCR ribotype 027 has been isolated from a dog (*7*) have created concerns regarding potential public health implications. *C. difficile*, including PCR ribotype 027 (*4*), has also been isolated from dairy calves, beef calves, veal calves, and adult cattle in Ontario (A. Rodriguez-Palacios et al., unpub. data).

The presence of *C. difficile* spores in bovine feces indicates the potential for contamination of retail meat products. Although contamination does not necessarily mean foodborne transmission, the possibility of *C. difficile* being a foodborne pathogen should be investigated. We therefore evaluated the prevalence of *C. difficile* contamination of retail ground meat samples and characterized the isolates.

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The Study

A convenience sampling scheme was used whereby meat samples (beef, n = 53 and veal, n = 7) were purchased from 5 grocery retailers in Ontario (4 stores, 57 samples) and Quebec (1 store, 3 samples), Canada. The number of meat packages purchased per month was 12, 2, 4, 4, 2, 2, 1, 11, 21, and 1, from January to October 2005, respectively.

C. difficile were isolated by using *C. difficile* culture agar supplemented with *C. difficile* moxalactam norfloxacin (CDMN) and 5% horse blood (CM0601, SR0173E, and SR0048C, Oxoid, Basingstoke, United Kingdom) (*8*). *C. difficile* broth was prepared by mixing the ingredients of CM0601, except for the agar, with 0.1% sodium taurocholate (Sigma-Aldrich, Inc., St. Louis, MO, USA). Briefly, 4–5 g of each sample was added to 20 mL of prereduced CDMN broth and incubated anaerobically at 37°C for 10–15 days. Alcohol shock for spore selection was performed by mixing 2 mL homogenized culture broth and 96% ethanol (1:1 [v/v]) for 50 min. After centrifugation ($3,800 \times g$ for 10 min), the sediment was streaked onto *C. difficile* agar. Up to 2 suspected colonies (swarming, rough, nonhemolytic) were subcultured from each plate. *C. difficile* was presumptively identified on the basis of Gram stain and detection of L-proline aminopeptidase activity (Pro Disc, Remel, Lenexa, KS, USA) and confirmed by identification of the triose phosphate isomerase gene (*9*).

PCR ribotyping and gene identification for toxins A (*tcdA*) and B (*tcdB*), the binding component of CDT (*cdtB*), and the *tcdC* gene were performed as previously described (*4*,*10*). Toxinotyping of selected isolates was also performed (*11*). Antimicrobial drug susceptibility to metronidazole, clindamycin, levofloxacin, and vancomycin was determined for all isolates by using the E-test method (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar (*12*).

C. difficile was isolated from 12 (20%) of 60 meat samples; 11 (20.8%) of 53 ground beef samples, and 1 (14.3%) of 7 ground veal samples (Table 1). Duplicate analysis was performed on 4 samples, and isolation of *C. difficile* was repeatable.

PCR ribotyping showed distinct patterns (Table 1, Figure). The most common ribotype, which accounted for 8 (67%) of 12 isolates, was different from any ribotype previously identified in our laboratory among ≈1,500 human and animal isolates. This ribotype, designated M31, had genes for toxins A, B and CDT; an 18-bp deletion in the *tcdC* gene and was toxinotype III. These are all molecular characteristics of PCR ribotype 027; however, ribotype pattern M31 was different from ribotype pattern 027

Table 1. Description of 12 *Clostridium difficile* strains isolated from retail ground meat samples in Ontario (n = 11) and Quebec (n = 1), Canada, 2005

PCR ribotype*	PCR toxin genes†	Meat code	Ground beef product	Month sampled	Date sample processed	Store/brand code‡
077	A^+B^+, *cdtB*$^-$, classic *tcdC*, toxinotype 0	M01	Regular	Jan	Jun 20	A/1
		M02	Regular	Jan	Jun 20	A/1
M26	A^-B^-, *cdtB*$^-$, *tcdC*$^-$, nontoxinotypeable	M26	Extra lean	May	Aug 10	A/1
M31	A^+B^+, *cdtB*$^+$, type B/C *tcdC*, toxinotype III	M31	Regular patties	Aug	Sep 1	B/4
		M38	Lean	Aug	Sep 1	B/4
		M41	Medium	Sep	Sep 6	C/7
		M43	Veal	Sep	Sep 6	B/6
		M44	Lean	Aug	Sep 6	B/4
		M47	Lean patties	Sep	Sep 6	B/4
		M51	Lean patties	Sep	Sep 6	B/1
		M52	Lean patties	Sep	Sep 6	B/4
014	A^+B^+, *cdtB*$^-$, classic *tcdC*, toxinotype 0	M54§	Regular	Sep	Sep 20	D/2

*077 and 014; nomenclature of Anaerobe Reference Laboratory, University of Wales, Cardiff, United Kingdom; M26 and M31 temporary nomenclature based on the PCR typing method of Bidet et al. (*10*). Eleven (91.6%) of 12 isolates were toxigenic.
†A, toxic gene *tcdA*; B, *tcdB*; *cdtB*, CDTb gene for the binding segment of the binary toxin; – and + indicate absence or presence of the gene. Classic *tcdC*, gene with no deletions; (≈345 bp); type B/C *tcdC*, gene with an ≈18-bp deletion (*4*).
‡Type of processed/packaged product; code 4, ground beef hamburgers sold as a special offer; other codes represent typical commercial ground beef packages.
§Commercial package from Quebec.

(Figure). PFGE with *Sma*I indicated that although this strain was distinguishable from prototypic strains NAP1, it had ≈80% similarity and was classified as NAP1 (B. Limbago, pers. comm.).

Two of the remaining 3 ribotypes had classic *tcdC* PCR fragments and did not have the *cdtB* gene. One group (n = 2), classified as PCR ribotype 077/toxinotype 0, had been isolated from calves, dogs, and humans (*4*). Another isolate from Quebec, classified as PCR ribotype 014/toxinotype 0, had also been isolated from calves and humans (*3*,*4*). The fourth isolate, nontoxigenic ribotype M26, had been isolated from dogs (*6*) but could not be toxinotyped because there was no detectable pathogenicity locus (M. Rupnik, pers. comm.). Overall, 3 (25%) of 12 meat *C. difficile* isolates were indistinguishable from Ontario human isolates.

All meat isolates were susceptible to metronidazole and vancomycin and resistant to levofloxacin and clindamycin (Table 2). These results are in agreement with previous findings for bovine-derived strains (*4*)

Conclusions

This is the first study to identify *C. difficile* spores in retail ground meat intended for human consumption. Previously, a study investigating the role of psychrotrophic clostridia on "blown pack" spoilage of commercial packages of chilled vacuum-packed meats and dog rolls reported 2 incidental isolates of *C. difficile* (*13*). More recently, a *C. difficile* isolate was identified in a commercial turkey-based raw diet intended for dogs (*14*).

The proportion of meat samples contaminated with *C. difficile* in our study (20%, 12/60) seems higher than those in the aforementioned reports. Possible reasons include the use of a more selective culture protocol in this study (*8*) and a potential temporary cluster of isolates with PCR ribotype M31 (Table 1). Those meat samples may have originated from the same larger contaminated batch or were subsequently contaminated at the store level during repackaging of retail products. PCR ribotype M31 was not identified in other samples or stores, which may suggest contamination at the retail level. Because PCR ribotype M31/toxinotype III had not been isolated in our laboratory, contamination during processing is unlikely.

The identification of PCR ribotypes 077 and 014, which are recognized human pathogens (*3*,*15*), is of con-

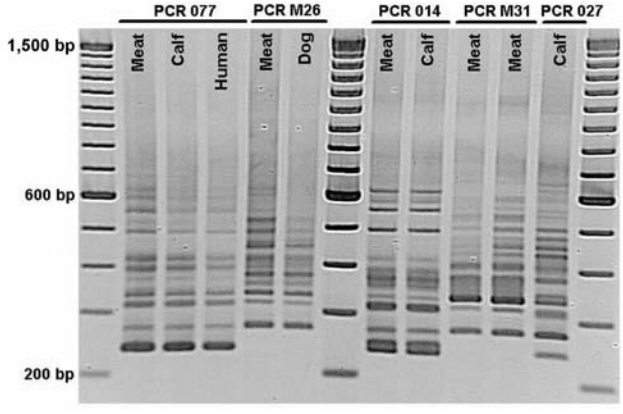

Figure. Comparison of PCR ribotypes of *Clostridium difficile* isolates from meat and of human, bovine, and canine origin in Ontario, Canada, 2005, by using the method of Bidet et al (*10*). PCR 077, 014, and 027 represent international ribotype nomenclature recently reported for calves (*4*). PCR M26 and M31 are temporary ribotype designations. Note that PCR M31 and 027, both NAP1/toxinotype III ribotypes, are different.

Table 2. Drug resistance characteristics of 12 meat-derived *Clostridium difficile* isolates determined by E-test on Muller-Hinton agar after 48 h of incubation*

Antimicrobial drug	MIC_{50}, μg/mL	MIC_{90}, μg/mL	Range, μg/mL	% Resistant isolates
Vancomycin	0.5	0.75	0.5 to 1.0	0
Metronidazole	0.38	0.5	0.19 to 1.0	0
Levofloxacin	32	32	4 to >32	100
Clindamycin	16.0	24.0	8 to >256	100

*Breakpoints used were vancomycin susceptible, ≤4.0 μg/mL; vancomycin resistant >32.0 μg/mL; metronidazole susceptible, ≤8.0 μg/mL; metronidazole resistant, >32.0 μg/mL; clindamycin susceptible, ≤2.0 μg/mL; clindamycin resistant >8.0 μg/mL; levofloxacin susceptible, ≤2.0 μg/mL; levofloxacin resistant, >8.0 μg/mL.

cern, although the actual risk for disease is unclear. Of additional concern is isolation of toxinotype III strains that have many similarities with PCR ribotype 027, an important cause of CDAD in humans (*2*). This similarity was highlighted by classification of this strain by PFGE as NAP1.

The presence of meat-derived PCR ribotypes indistinguishable from human, bovine, and canine ribotypes further supports the potential risk for cross-transmission among species and suggests that ingestion of viable spores might occur. Although proper cooking of meat is emphasized for reducing the risk for foodborne disease, the fact that *C. difficile* is a spore former complicates this issue because spores can survive in ground beef at recommended cooking temperatures (71°C), even when that temperature is maintained for 120 min (A. Rodriguez-Palacios et al., unpub. data).

The clinical and epidemiologic relevance of these microbiologic findings remains unknown. The isolation of *C. difficile* from meat samples does not necessarily mean that CDAD is a foodborne disease. Additional studies are required to determine the prevalence of contamination and its clinical relevance.

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Dr Rodriguez-Palacios recently completed a doctorate degree in veterinary science at the Ontario Veterinary College, University of Guelph. His research interests include infectious diseases of large animals and the study of foodborne pathogens.

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Methicillin-susceptible, Doxycycline-resistant *Staphylococcus aureus*, Côte d'Ivoire

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We report 2 outbreaks of Panton-Valentine leukocidin–positive, doxycycline-resistant, methicillin-susceptible *Staphylococcus aureus* infections in French soldiers operating in Côte d'Ivoire. In a transsectional survey, nasal carriage of this strain was found in 2.9% of 273 soldiers about to be sent to Côte d'Ivoire and was associated with prior malaria prophylaxis with doxycycline.

Staphylococcus aureus strains that produce Panton-Valentine leukocidin (PVL) have been shown to be associated with community-acquired infections such as skin and soft tissue infections and necrotizing pneumonia (*1*). A limited number of PVL-positive, methicillin-resistant *S. aureus* (MRSA) clones have recently emerged globally (*2*) and have been described as causing epidemic community-acquired infections (*3*,*4*). In contrast, PVL-positive, methicillin-susceptible *S. aureus* (MSSA) corresponds to many diverse clones (*5*) that may cause sporadic infections with limited dissemination. We describe severe staphylococcal infections due to a clone of doxycycline-resistant, PVL-positive (doxyR-PVL+) MSSA in soldiers who served in Côte d'Ivoire.

The Study

During 2004 and 2005, 4 soldiers with recurrent cutaneous infections related to doxycycline-resistant MSSA (DoxyR-MSSA) visited the Centre Hospitalier Universitaire in Clermont-Ferrand, France. The soldiers belonged to 2 companies, A and B, based at different places in France (Clermont-Ferrand and Brives, respectively), and had been to Côte d'Ivoire at 2 distinct periods. In October 2005, a health warning was sent to French military authorities. We conducted retrospective interviews of soldiers in company A and performed a transsectional nasal carriage survey of 273 soldiers in company B, who were about to be sent to Côte d'Ivoire. We defined case-patients as soldiers who had had at least 1 cutaneous infection during or after their time in Côte d'Ivoire. Information was collected from company A case-patients by telephone interview and, for hospitalized soldiers, chart review.

When available, staphylococcal strains were sent to the French National Reference Centre for Staphylococci in Lyon, France. Sequences specific for staphylococcal enterotoxin genes (*sea-e*, *seh*, *sek*, *sem*, *sel*, and *seo*), the PVL genes (*luk*S-PV-*luk*F-PV), and accessory gene regulator alleles (*agr* 1–4) were detected by PCR (*6*–*8*). Fingerprinting by pulsed-field gel electrophoresis, *spa* typing, and multilocus sequence typing were performed as described (*9*).

Company A comprised 70 French soldiers who had taken doxycycline, 100 mg per day, for malaria prophylaxis while in Côte d'Ivoire (August–November 2003). During their 3 weeks in training camp, each soldier stayed in 1 of 4 rooms. Of 13 soldiers who stayed in room 3, 8 (61.5%) reported having had at least 1 cutaneous infection while in Côte d'Ivoire, compared with none of 2 soldiers in room 1, 1 (8%) of 13 in room 2, and 7 (18%) of 39 in room 4. Two soldiers (1A and 2A) visited our clinic in November 2004 for treatment of abscesses that required surgical debridement for 1 year. For each patient, doxyR-MSSA was isolated from the abscess in a site acting as a reservoir (nasal or perianal skin). Their conditions were successfully treated with topical application of mupirocin to the reservoir site. No recurrence occurred after a year. Another soldier from company A (patient 3A) was found to be an asymptomatic nasal carrier of doxyR-MSSA. His wife (patient 4A) experienced several recurrent doxyR-MSSA abscesses, including 1 that was debrided at our hospital in January 2004.

In company B, ≈70 soldiers had served in Côte d'Ivoire during October 2004–February 2005 and stayed for 6 weeks in the same training camp that company A stayed in the year before. Two soldiers (1B and 2B) visited our hospital. Patient 1B was hospitalized with furunculosis and osteomyelitis of the left femoral diaphysis 5 months after attending training camp (and 1 month after he returned to France). Surgically removed bone samples contained *S. aureus* isolates that had profile characteristics similar to those obtained from furuncles (Figure). Patient 2B visited our clinic in March 2005 for recurrent axillary

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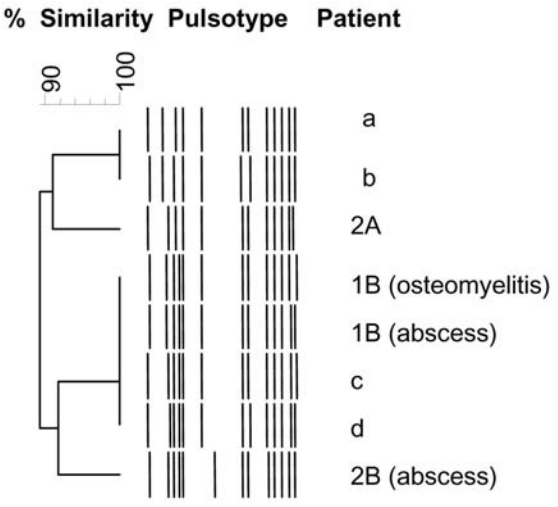

Figure. Dendogram constructed from the schematic representation of the pulsed-field gel electrophoresis types of 4 epidemic methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates included in this study (patients 2A, 1B, and 2B); 1 strain subsequently isolated from an abscess in a soldier belonging to company A, who had been in Côte d'Ivoire in October 2005 (patient b); and 3 MSSA strains isolated from abscesses in soldiers belonging to a company other than A or B (patients a, c, d). Isolates from all patients had the following genetic characteristics: *agr* type 3, positive for Panton-Valentine leukocidin, negative for *mec*A gene, and toxin genes *sea*, *seh*, and *sek*. In addition, isolates from patients a and 1B were sequence type 1 and spa type 590.

abscesses due to the same clone of MSSA. He did not experience recurrence after MSSA eradication from the nasal and axillary reservoirs. In September 2005, recurrent furunculosis developed in a third soldier (3B), who had not gone to Côte d'Ivoire but who was in close contact with soldiers returning from there. In April 2005 and December 2005, doxyR-MSSA–related recurrent abscesses developed in his wife and their 5-year-old child.

To estimate the prevalence of PVL-positive MSSA carriage in soldiers, nasal culture specimens were collected on May 9 and 10, 2005, from 273 soldiers in company B (a total of 1,100 soldiers) who were about to be sent to Côte d'Ivoire. Of these 273 soldiers, 98 (35.9%) were colonized with *S. aureus*. Among them, 28 (10%) were carriers of PVL–doxyR-MSSA and 8 (2.9%) were carriers of PVL+doxyR-MSSA. These PVL+doxyR-MSSA isolates belonged to the same clone as those found in patients 2A, 1B, and 2B. All the MSSA isolates were considered to belong to the same clone as they shared a large core of common genetic characteristics (Figure). All PVL+doxyR-MSSA carriers had been to Côte d'Ivoire and had taken doxycycline for malaria prophylaxis (Table).

Conclusions

We characterized 2 outbreaks caused by the same clone of PVL+doxyR-MSSA. These outbreaks occurred in 2 military companies that served in Côte d'Ivoire at different times and whose soldiers received doxycycline for malaria prophylaxis. This epidemic MSSA clone was responsible for infections traditionally associated with PVL, mainly skin and soft tissue infections, but also deep-seated infections such as severe osteomyelitis. Since these outbreaks, several similar cases affecting different companies who had been to Côte d'Ivoire have been reported to the French military authorities (Figure). Another striking feature was the spread of the clone within families after the soldiers' return from Côte d'Ivoire. This virulent clone has already disseminated to different continents. Analysis of the database of the French National Reference Centre for Staphylococci (which contains the characteristics of ≈5,000 *S. aureus* strains worldwide) found 25 methicillin-

Table. Factors associated with nasal carriage of *Staphylococcus aureus* in 273 soldiers (company B) who were about to be sent to Côte d'Ivoire

Variable	PVL+DoxyR *S. aureus*† (n = 8)	PVL–DoxyR *S. aureus*‡ (n = 34)	DoxyS *S. aureus*§ (n = 56)	p value¶ (PVL+ vs. PVL–)	p value¶ (PVL+DoxyR vs. PVL–DoxyR)
Age, y	24.3	25.9	24.5	NS	NS
Male (%)	87.5	97.1	100.0	NS	NS
Living with health care worker (%)	25.0	26.5	12.5	NS	NS
Living with children <16 y (%)	50.0	61.8	53.6	NS	NS
Hospitalization within 1 year (%)	0.0	17.6	17.8	NS	NS
Fight sport practice# (%)	0.0	5.9	7.1	NS	NS
Previous mission in malaria-endemic area (%)	100.0	91.2	37.5	<0.001	NS
Previous mission in Côte d'Ivoire (%)	100.0	88.2	30.4	<0.001	NS
Previous doxycycline intake (%)	100.0	88.2	32.1	<0.001	NS

*PVL, Panton-Valentine leukocidin; DoxyR, doxycycline resistant; DoxyS, doxycycline susceptible; NS, not significant.
†All methicillin-susceptible *S. aureus* (MSSA).
‡28 MSSA and 6 methicillin-resistant *S. aureus* (MRSA).
§55 MSSA and 1 MRSA, all PVL-negative.
¶Fisher exact test.
#Military exercises that involve physical contact.

susceptible isolates with identical toxin gene content and *agr* type from unrelated patients from Africa, Polynesia, and France (data not shown).

Our study has limitations. The investigation conducted in company A was retrospective, so we could not control for other pathogens. Recall bias may have occurred, and the incidence of cutaneous infections in company A could have been underestimated. However, epidemiologic links between case-patients were well established. Only case-patients who visited our hospital were documented, but they enabled recognition of the outbreaks. Recent similar cases in soldiers who visited our hospital for PVL+doxyR-MSSA cutaneous infections after their return from Côte d'Ivoire confirm that the strain is still disseminating and is strongly associated with a stay in that country (Figure).

The infecting MSSA is remarkable in its resistance to doxycycline, which may favor selection of a preexisting PVL+doxyR-MSSA in carrier soldiers who are given a prophylactic dose of doxycycline. Because the 2 companies had no contact with each other before, during, and after their stay in Côte d'Ivoire, we think that the *S. aureus* clone persists in Côte d'Ivoire. Transmission may have occurred through the persistence of the strain in the environment of the training camp in Côte d'Ivoire, where hygiene conditions were poor (4 rooms, 3 showers, 3 lavatories for ≈70 soldiers). However, the relatively high prevalence of PVL-positive MSSA carriage found in our study suggests that nasal carriage may play an important role in transmission of these highly virulent microorganisms.

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Intermediate Vancomycin Susceptibility in a Community-associated MRSA Clone

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We describe a case of treatment failure caused by a strain of USA300 community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) with intermediate susceptibility to vancomycin and reduced susceptibility to daptomycin. The strain was isolated from the bone of a 56-year-old man with lumbar osteomyelitis after a 6-week treatment course of vancomycin for catheter-associated septic thrombophlebitis.

A 56-year-old man with a history of type 2 diabetes and chronic kidney disease was seen at San Francisco General Hospital in November 2005 because of hyperkalemia and volume overload. On day 4 of hospitalization, a fever of 39°C and cellulitis in the right arm associated with a peripheral intravenous line developed. Two blood cultures were drawn, the line was removed, and therapy was initiated with oral cephalexin. One of the 2 blood cultures subsequently grew methicillin-resistant *Staphylococcus aureus* (MRSA) that was susceptible to tetracycline and trimethoprim-sulfamethoxazole. The patient was treated with oral trimethoprim-sulfamethoxazole and discharged to home to complete a 10-day course. His right upper extremity cellulitis subsequently resolved, but he returned to the hospital in January 2006 with volume overload and symptoms consistent with uremia. A tunneled right internal jugular hemodialysis catheter was placed on January 11, hemodialysis was initiated, and he was discharged to home.

On February 20, he was seen in the emergency department of another facility with nausea and altered mental status. He was afebrile but hypotensive, and tenderness at the entrance site of the hemodialysis catheter was noted. Two blood cultures were positive for MRSA. The patient's catheter was removed, and intravenous vancomycin was started. Transthoracic and subsequent transesophageal echocardiograms were negative for endocarditis, but evidence of thrombosis in the superior vena cava was seen. Multiple blood cultures were positive for MRSA through March 1, after which they became negative. The vancomycin MIC for serial isolates remained unchanged. The patient was treated with vancomycin for a 6-week course, beginning March 1. Anticoagulation with coumadin was also initiated. After clearance of his blood cultures, a right subclavian tunneled hemodialysis catheter and a left upper extremity arteriovenous fistula were placed. Vancomycin trough levels were assayed on multiple occasions during the 6-week course; all were ≥20 μg/mL.

On April 24, the patient was seen by his primary care provider for worsening bilateral knee and back pain and difficulty walking. A lumbar spine radiograph demonstrated cortical irregularity at L4-L5, indicative of discitis. He was admitted to the hospital, and vancomycin was initiated. Magnetic resonance imaging of the lumbar spine on April 25 demonstrated findings consistent with osteomyelitis and discitis at L4-L5. A needle biopsy of the L4-L5 lesion was performed on April 27. That evening, the patient was noted to be febrile and had an episode of emesis. Later in the evening, he was apneic and without a pulse, with fixed and dilated pupils. Cardiopulmonary resuscitation was performed. When neurologic function did not return during the next 4 days, supportive care was withdrawn, and the patient died May 1. Multiple blood cultures from this hospitalization remained negative, but results of a culture of lumbar fluid from a biopsy specimen on April 27 were positive for a vancomycin-intermediate *S. aureus* (VISA) isolate, with a vancomycin MIC of 8 μg/mL.

The antimicrobial susceptibility profiles of the blood isolates from November and February and the lumbar isolate from April are shown in the Table. All MIC susceptibilities were determined by broth microdilution methods per Clinical and Laboratory Standards Institute guidelines. The November and April isolates were tested with MicroScan overnight panels (Dade Behring, Deerfield, IL, USA); the February isolate was tested by using a noncommercial tray. Confirmatory testing of the vancomycin MIC of the April isolate was determined by E-test, which returned an MIC of 6 μg/mL initially and 4 μg/mL on repeat testing. The isolate also grew on vancomycin (6 μg/mL) agar screen plates. Susceptibilities to daptomycin, linezolid, and tigecycline were performed on the February and April isolates by E-test. Notably, the daptomycin MIC of the April isolate was reproducibly 2 μg/mL, an increase from 1 μg/mL for the February isolate. On blood agar plate, the April lumbar isolate was noted to be weakly β-hemolytic with small colony size. With multiple subcul-

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Table. Antimicrobial susceptibility profiles of blood isolate from November 2005, blood isolate from February 2006, and lumbar isolate from April 2006*

Antimicrobial drug	MIC (μg/mL) and CLSI interpretation		
	November	February	April
Nafcillin	>2 R	16 R	>2 R
Clindamycin	2 I	≤0.25 S	≤0.25 S
Erythromycin	4 I	>8 R	>4 R
Trimethoprim-sulfamethoxazole	≤0.5/9.5 S	≤0.25/5 S	≤0.5/9.5 S
Tetracycline	≤1 S	≤0.5 S†	≤1 S
Rifampin	≤1 S	≤0.25 S	≤1 S
Ciprofloxacin	>2 R	>4 R	>2 R
Levofloxacin	>4 R	ND	>4 R
Gentamicin	≤1 S	≤0.5 S	2 S
Vancomycin	≤2 S	2 S	8 I 4–6 I‡
Daptomycin	ND	1 S	2§
Linezolid	ND	2 S	2 S
Tigecycline	ND	0.125 S	0.125 S

*CLSI, Clinical and Laboratory Standards Institute; R, resistant; I, intermediately resistant; S, susceptible; ND, not done.
†Susceptibility to doxycycline performed instead of to tetracycline.
‡Confirmatory susceptibility by E-test and growth on vancomycin (6 μg/mL) agar screen plates.
§Interpreted as nonsusceptible by the Centers for Disease Control and Prevention. Formal CLSI breakpoints for daptomycin resistance have not been established.

tures in the absence of vancomycin this morphotype reverted to the full β-hemolysis and large colony size typical of *S. aureus*, intermediate susceptibility to vancomycin was lost, and daptomycin susceptibility was regained.

Pulsed-field gel electrophoresis with *Sma*I digestion was performed on the 2 blood isolates from November and February and the lumbar isolate from April. All 3 isolates shared an identical pattern with the USA300-0114 control strain (Figure). Sequencing of the protein A gene polymorphic region (*spa* typing) (*1*) of the lumbar isolate obtained the sequence YHGFMBQBLO, typical for clonal cluster 8. PCR was positive for the presence of *mecA*, ACME (arginine catabolic mobile element, a signature gene cluster of USA300), and Panton-Valentine leukocidin genes and negative for the presence of the *vanA* gene, when primers and sequences previously described were used (*2*–*4*).

The community MRSA clone USA300, first identified in 2001, has emerged as a notable cause of colonization and disease in San Francisco (*5*). This clone has been remarkable for its rapid spread and propensity to cause severe infection, particularly skin and soft tissue and pulmonary infection. Recent sequencing of its genome demonstrated possible contributors to its virulence, most notably Panton-Valentine leukocidin and ACME (*2*). The USA300-0114 subclone has been the predominant one during the community MRSA epidemic in San Francisco (*6*) and is becoming widely prevalent in communities throughout the United States (*7*). Surveys of clinical MRSA isolates throughout the city of San Francisco have shown an explosive increase in the prevalence of disease caused by USA300 and displacement of other clones in both inpatient and outpatient settings since 2001, blurring the clone's distinction as community associated.

The first report of clinical VISA infection was from Japan in 1997 (*8*). Molecular typing of this and subsequent VISA isolates showed these to be derived from prevalent hospital-acquired clones primarily belonging to clonal cluster 5 (*9*). The accessory gene regulator (*agr*) group II genotype present in clonal cluster 5 strains has been speculated to predispose to emergence of the VISA phenotype. USA300 is an *agr* group III clone (*6*). The occurrence of the vancomycin-intermediate phenotype in USA300 suggests that development of this phenotype may simply reflect the prevalence of clones in a particular population, rather than a causal relationship to *agr*.

The mechanism of reduced vancomycin susceptibility in VISA is thought to be mediated by an increase in the number of false targets because of a thickened cell wall (*10*), perhaps aided by altered expression of penicillin-binding proteins 2 and 4 (*11*). VISA and hVISA, strains of *S. aureus* that contain subpopulations of daughter cells dis-

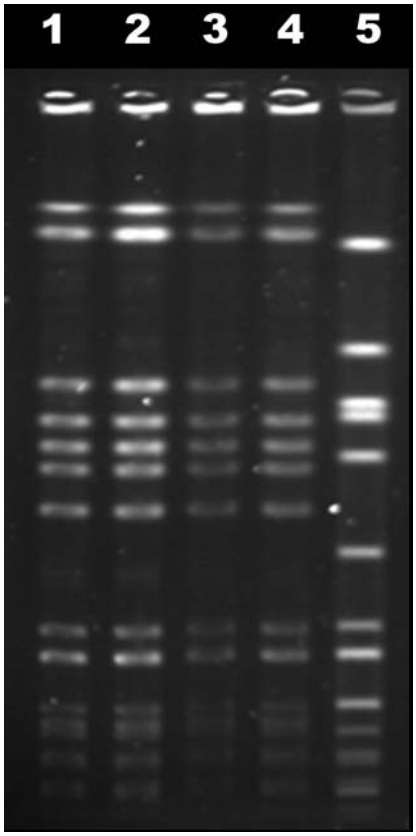

Figure. Pulsed-field gel electrophoresis profiles of November and February blood isolates (lanes 1 and 2), April lumbar isolate (lane 3), reference USA300-0114 isolate (lane 4), and internal control g195a (lane 5).

playing intermediate sensitivity to vancomycin but for which the MICs for vancomycin fall within the susceptible range, can be difficult to detect in the microbiology laboratory because the phenotypes are unstable and can be lost on subsequent passages (*12*); this situation was demonstrated in our case. Reduced susceptibility to daptomycin in vancomycin-intermediate isolates has been described previously, perhaps because of reduced diffusion of the molecule through the thickened cell wall, although this has not been proven (*13*). The clinical importance of the reduced daptomycin susceptibility seen in vancomycin-intermediate isolates, however, is unclear at this time.

Our patient experienced clinical failure of vancomycin therapy despite high serum drug levels, which speaks to the difficulty with which highly invasive *S. aureus* infections are successfully treated with vancomycin, particularly in patients receiving hemodialysis. While most VISA isolates reported in the United States have been isolated from patients receiving hemodialysis, chronic renal disease and hemodialysis have not been definitively identified as risk factors for infections caused by VISA or hVISA (*14*). The frequency of nasal colonization with hVISA was low in hemodialysis patients monitored from 1999 to 2002 in the San Francisco Bay Area (*15*). However, as the prevalence of USA300 increases and prompts further use of vancomycin, intermediate vancomycin susceptibility in USA300 may become more common among both community and hospital isolates.

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Staphylococcal Cassette Chromosome *mec* in MRSA, Taiwan

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To determine the predominant staphylococcal cassette chromosome (SCC) *mec* element in methicillin-resistant *Staphylococcus aureus*, we typed 190 isolates from a hospital in Taiwan. We found a shift from type IV to type III SCC*mec* element during 1992–2003, perhaps caused by selective pressure from indiscriminate use of antimicrobial drugs.

The high prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA), which accounts for as much as 80% of all *S. aureus* isolates causing nosocomial infections in Taiwanese hospitals since 1998, has greatly affected infection control and medical treatment in Taiwan (*1*). At National Taiwan University Hospital (NTUH), a 2,200-bed major teaching hospital in northern Taiwan, MRSA has become a common nosocomial pathogen since the early 1990s. The annual number and incidence of nosocomial MRSA infections, as well as the number of available nonduplicate isolates for the past 12 years at NTUH, are shown in the Figure.

In a previous study, we used pulsed-field gel electrophoresis typing of 140 randomly selected nosocomial MRSA isolates and samples from our collection of nosocomial isolates obtained from 1992 to 1996 to identify 3 major pulsotypes (A, B, and C) (*2*). Pulsotype A was predominant among all MRSA isolates in 1992 (accounting for 50%) and 1993 (52%), pulsotype B was predominant in 1994 (59%) and 1995 (49%), and pulsotype C was predominant in 1996 (83%) (*2*). Pulsotype C remained the predominant clone until 2003 (J.-T. Wang et al., unpub. data).

S. aureus acquires methicillin resistance through a mobile staphylococcal cassette chromosome (SCC) that contains the *mecA* gene complex (SCC*mec*) (*3*). Until now, 5 major types of SCC*mec* element have been characterized and studied (*3*–*5*). However, longitudinal studies of SCC*mec* elements in nosocomial MRSA isolates in a hospital have seldom been reported (*6*). We analyzed SCC*mec* elements in predominant nosocomial MRSA clones at NTUH from 1992 through 2003. Because the predominant MRSA clone at NTUH from 1996 through 2003 was pulsotype C, MRSA isolates obtained from 1997 through 2002 were not studied.

The Study

We analyzed all 140 MRSA isolates we obtained during a previous study (*2*) and 50 other isolates selected from our collection of nosocomial MRSA isolates obtained in 2003. Characteristics of these 190 isolates are shown in Table 1.

Pulsed-field gel electrophoresis patterns were interpreted according to procedures previously reported (*7*,*8*). Thirty-four isolates belonged to pulsotype A, 49 to pulsotype B, 69 to pulsotype C, 6 to pulsotype D, 2 to pulsotype E, 3 to pulsotype F, 11 to pulsotype K, 5 to pulsotype L, 8 to pulsotype M, and 3 (all isolated in 2003) to 3 minor pulsotypes. All isolates were tested by SCC*mec* element typing and multilocus sequence typing (MLST) (*9*) and were analyzed for the Panton-Valentine leukocidin (PVL) gene (*10*) and drug susceptibility to erythromycin, clindamycin, gentamicin, amikacin, ciprofloxacin, levofloxacin, tetracycline, trimethoprim-sulfamethoxazole, rifampin, and vancomycin by using the disk diffusion method (*11*). SCC*mec* element typing was determined by previously described PCR methods (*3*–*5*).

Results of these analyses are shown in Table 2. MRSA isolates of the same pulsotype have the same MLST pattern and SCC*mec* types. Isolates with pulsotype A are sequence type 254 (ST254); those with pulsotype B are ST241; those with pulsotypes C, K, and L are ST239; and those with pulsotypes D, E, F, and M are ST59, ST 254, ST30, and ST5, respectively. All MRSA isolates with pulsotypes A, D, E, and F have the type IV SCC*mec* element. However, only isolates with pulsotypes D and F, as well as 2 isolates from 2003 with 2 minor pulsotypes, have the PVL gene. Isolates with pulsotypes B and C have the type III SCC*mec* element, and isolates with pulsotype M have the type II SCC*mec* element.

Results of MLST and typing of SCC*mec* elements of the 3 isolates with 3 minor pulsotypes obtained in 2003 are

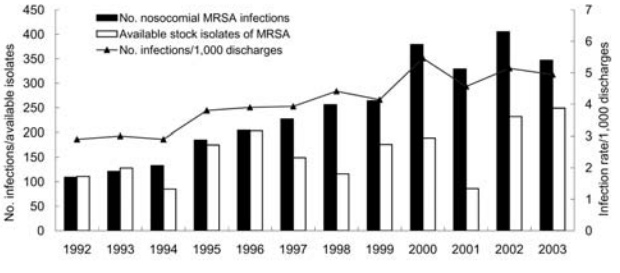

Figure. Number and cumulative incidence of nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) infections per 1,000 discharges and number of available nonduplicate MRSA isolates at National Taiwan University Hospital, 1992–2003.

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Table 1. Pulsotypes, characteristics, and sources of 190 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, Taiwan, 1992–2003

Year, clinical syndrome (no. isolates)*	Pulsotype (no. isolates)	Source specimen (no. isolates)†	Site of isolation (no. isolates)‡
1992			
NI (9)	A (4), B (3), F (1)	Bl (3), Ur (2), Sp (2), Pu (2)	ICU (6), ward (3)
NC (8)	A (3), B (4), F (1)	Sp (4), Wo (4)	ICU (3), ward (5)
IEOH (5)	A (4), B (1)	Sp (4), Pu (1)	ICU (3), ward (2)
1993			
NI (10)	A (1), B (8), F(1)	Bl (4), Ur (1), Sp (2), Pu (3)	ICU (8), ward (2)
NC (14)	A (11), B (3)	Sp (5), Wo (5), Ns (3), Ct (1)	ICU (9), ward (5)
IEOH (1)	A (1)	Sp (1)	Ward (1)
1994			
NI (9)	A (4), B (4), D (1)	Bl (7), Sp (1), Pu (1)	ICU (4), ward (5)
NC (8)	A (1), B (6), C (1)	Sp (4), Wo (3), Ns (1)	ICU (5), ward (3)
1995			
NI (9)	B (3), C (5), E (1)	Bl (2), Sp (3), Pu (4)	ICU (6), ward (3)
NC (25)	A (1), B (13), C (9), D (2)	Sp (14), Wo (5), Ns (6)	ICU (19), ward (6)
IEOH (1)	B (1)	Sp (1)	Ward (1)
1996			
NI (23)	A (1), B (1), C (20), D (1)	Bl (15), Sp (3), Pu (5)	ICU (16), ward (7)
NC (11)	A (1), C (10)	Sp (7), Wo (2), Ns (1), St (1)	ICU (11)
IEOH (7)	A (1), B (2), C (4)	Bl (2), Sp (5)	ICU (5), ward (2)
2003			
NI (24)	C (10), D (2), K (5), L (2), M (3), other (2)	Bl (16), Sp (4), Pu (4)	ICU (10), ward (14)
NC (26)	A (1), C (10), K (6),L (3), M (5), other (1)	Sp (14), Wo (12)	ICU (12), ward (14)

*NI, nosocomial infection acquired at National Taiwan University Hospital (NTUH); NC, nosocomial colonization at NTUH; IEOH, MRSA infection detected at NTUH within 48 h after transfer from another hospital.
†Bl, blood; Ur, urine; Sp, sputum; Pu, pus; Wo, wound; Ns, nostril; Ct, catheter tip; St, stool.
‡ICU, intensive care unit.

shown in Table 2. The correlation between SCC*mec* element typing and MLST in this study corresponds to findings of previous reports (*6*,*12*–*14*). Enright et al. identified ST254-IV MRSA isolates in Germany and the United Kingdom (*12*), and Chongtrakool et al. identified ST239-III and ST241-III MRSA isolates in several Asian countries (*14*).

Conclusions

We demonstrate that the predominant MRSA clone at NTUH in early 1990s had the type IV SCC*mec* element. However, the predominant MRSA clones at NTUH from 1994 to 2003 had the type III SCC*mec* element. These findings differ from those of Wisplinghoff et al., who reported that that the SCC*mec* element in predominant MRSA clones at their institute changed from type III in 1984 to 1988 to type I in 1989 to 1998 (*6*). Differences between our findings and those of Wisplinghoff et al. might be caused by differences in location and epidemiologic characteristics.

MRSA isolates of pulsotypes B and C are more resistant than isolates of pulsotype A to certain antimicrobial drugs, especially fluoroquinolones and trimethoprim-sulfamethoxazole; MRSA isolates with pulsotype C are more resistant to clindamycin but less resistant to rifampin than those with pulsotype B (Table 2). From 1993 through 2000, annual use of fluoroquinolones increased ≈3× at NTUH; however, use of trimethoprim-sulfamethoxazole, clindamycin, and rifampin did not change (*15*). Therefore, the shift of predominant MRSA clones, which also led to the shift in types of SCC*mec* elements at NTUH, might be caused by selective pressure from antimicrobial drugs, especially fluoroquinolones.

The MRSA clone (pulsotype A) that predominated in 1992 and 1993 at NTUH has the type IV SCC*mec* element. Although the first study of MRSA with the type IV SCC*mec* element reported that this element was found in community-acquired MRSA (CA-MRSA) (*5*), some studies have reported MRSA isolates with this element in a hospital environment (*12*). However, to our knowledge, these reports did not demonstrate that MRSA isolates with the type IV SCC*mec* element became predominant among all MRSA isolates in an institution, especially before the mid-1990s.

Furthermore, 4 ST59 MRSA isolates obtained in 1994 and 1996 and 3 ST30 MRSA isolates obtained in 1992 and 1993 have the type IV SCC*mec* element and PVL gene. Recently, ST59 MRSA isolates were found to cause CA-MRSA infection in Taiwan (*13*). Among these ST59 CA-MRSA isolates, some had the type IV SCC*mec* element, and others had the type V SCC*mec* element (*13*). Although the type IV SCC*mec* element could be transferred to CA-

Table 2. Characteristics of 190 methicillin-resistant *Staphylococcus aureus* isolates, Taiwan, 1992–2003*

P (no. isolates)	SCC*mec* type	MLST	PVL	Year of isolation (no. isolates)	Drug susceptibility rate, %†										
					OX	EM	CL	GM	AM	CP	LV	TC	TS	RF	VA
A (34)	IV	254	N	1992 (11), 1993 (13), 1994 (5), 1995 (1), 1996 (3), 2003 (1)	0	0	8.8	0	0	20.1	32.3	5.9	91.2	0	100
B (49)	III	241	N	1992 (8), 1993 (11), 1994 (10), 1995 (17), 1996 (3)	0	0	40.8	0	0	4.1	4.1	0	2.0	18.4	100
C (69)	III	239	N	1994 (1), 1995 (14), 1996 (34), 2003 (20)	0	0	5.8	2.9	0	1.4	1.4	2.9	1.4	95.7	100
D (6)	IV	59	Y	1994 (1), 1995 (2), 1996 (1), 2003 (2)	0	0	0	16.7	33.3	66.7	100	33.3	100	100	100
E (2)	IV	254	N	1992 (1), 1995 (1)	0	0	50	0	0	50	50	0	100	0	100
F (3)	IV	30	Y	1992 (2), 1993 (1)	0	0	0	33.3	66.7	100	100	33.3	100	100	100
K (11)	III	239	N	2003 (11)	0	0	9.1	0	0	0	0	0	0	100	100
L (5)	III	239	N	2003 (5)	0	0	0	0	0	0	0	0	0	100	100
M (8)	II	5	N	2003 (8)	0	0	0	0	0	0	0	100	100	25	100
Other‡ (3)															
	I	5	N	2003 (1)	0	0	0	0	0	0	0	100	100	0	100
	IV	59	Y	2003 (1)	0	0	0	0	100	100	100	100	100	100	100
	IV	59	Y	2003 (1)	0	0	0	0	0	100	100	0	100	100	100

*P, pulsotype; SCC, staphylococcal cassette chromosome; MLST, multilocus sequence typing; PVL, Panton-Valentine leukocidin; N, no; Y, yes.
†OX, oxacillin; EM, erythromycin; CL, clinidamycin; GM, gentamicin; AM, amikacin; CP, ciprofloxacin; LV, levofloxacin; TC, tetracycline; TS, trimethoprim-sulfamethoxazole; RF, rifampin; VA, vancomycin.
‡Including 3 pulsotypes each containing only 1 isolate.

MRSA clones with other genetic backgrounds, our finding supports the possibility that ST59 MRSA isolates with the SCC*mec* element type IV in Taiwan may originate from hospital strains but transfer into CA-MRSA strains.

Chongtrakool et al. recently reported the results of SCC*mec* typing of 615 MRSA isolates obtained in 1998 and 1999 from 11 Asian countries (*14*). The ST239-III, ST241-III, ST254-II, and ST5-II MRSA isolates were prevalent in many Asian countries. However, the ST254-IV, ST30-IV, and ST59-IV MRSA isolates from our study were not found in other Asian countries. In addition, ST254-IV MRSA isolates have been found in Germany and the United Kingdom (*12*). Whether ST254-IV MRSA isolates in our study were transmitted from those in Germany or the United Kingdom by international travel requires further study.

The first MRSA isolate with the type IV SCC*mec* element in our hospital appeared as early as 1992. The SCC*mec* element carried by predominant MRSA clones changed from type IV to type III SCC*mec* element during the period 1992–2003 at NTUH. Because the major MRSA clones isolated in 1994–2003 are more resistant to antimicrobial drugs, especially fluoroquinolones and trimethoprim-sulfamethoxazole, than those obtained in 1992 and 1993, this shift may be caused by selective pressure from indiscriminate use of antimicrobial drugs.

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Clostridium difficile–associated Disease in New Jersey Hospitals, 2000–2004[1]

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Recent emergence of a virulent strain of *Clostridium difficile* demonstrates the importance of tracking *C. difficile* incidence locally. Our survey of New Jersey hospitals documented increases in the rates of *C. difficile* disease (by 2-fold), *C. difficile*–associated complications (by 7-fold), and *C. difficile* outbreaks (by 12-fold) during 2000–2004.

Clostridium difficile, a gram-positive organism, is the most common cause of nosocomial infectious diarrhea in the United States (*1*). In 2005, the Centers for Disease Control and Prevention (CDC) reported on a new, epidemic, toxin gene–variant strain of *C. difficile* on the basis of a study of isolates collected from hospitals in multiple states, including New Jersey. CDC recommended that inpatient healthcare facilities track the incidence of *C. difficile*–associated disease (CDAD), including the clinical outcomes of patients (*2*).

The Study

To estimate the incidence of CDAD in hospitalized patients in New Jersey, we conducted a retrospective survey of acute-care hospitals. An Internet-based questionnaire was distributed to all 81 New Jersey hospitals in early 2005; hospitals that did not respond were contacted by telephone or electronic mail. We collected information on hospital characteristics, the number of CDAD cases, *C. difficile*–positive laboratory test results, *C. difficile*–associated complications, deaths due to any cause within 30 days of diagnosis with *C. difficile* infection, healthcare-associated *C. difficile* outbreaks, recurrent CDAD cases, diagnostic test methods, and surveillance activities. The proportion of community- versus healthcare-acquired cases was not assessed objectively; however, respondents provided their perceptions regarding trends. No individual patient information was obtained.

A CDAD case was defined as a patient with symptoms of diarrhea and at least 1 of the following: positive toxin assay result, diagnosis of pseudomembranous colitis on sigmoidoscopy or colonoscopy, or histopathologic diagnosis. An outbreak was defined as ≥3 cases of healthcare-associated CDAD in the same general area within 7 days. A complicated case was defined as a patient with CDAD in whom toxic megacolon, perforation of the colon, colectomy, or shock requiring vasopressor therapy subsequently developed within 30 days after diagnosis with CDAD. The definition of recurrent CDAD and the method of laboratory diagnoses were determined by each hospital.

Data were analyzed by using EpiInfo version 3.3.2 (CDC, Atlanta, GA, USA) and SAS version 8.02 (SAS Institute, Cary, NC, USA). The medians, means, ranges, frequencies, and totals reported are based on actual responses to the survey questions; hospitals that did not answer a question were excluded from the analysis of responses to that question. Tests for linear trend over the study period were performed by using linear regression. We also examined the association between CDAD rates and staffing levels of infection-control professionals (ICPs) by using a Poisson regression model.

Of the 81 hospitals contacted, 58 (72%), located in 20 of 21 New Jersey counties, responded to the survey. The median bed capacity of participating hospitals was 281 (range 77–683), and the median number of full-time equivalent ICPs per 250 beds was 1.2 (range 0–3).

During 2000–2004, participating hospitals reported a total of 13,394 CDAD cases. The mean annual rate of CDAD increased from 3.7/1,000 admissions in 2000 to 7.7/1,000 admissions in 2004, which represented a >2-fold increase in CDAD rates during the 5-year period ($p<0.05$, Table 1). Of the hospitals that responded, the percentage that did not identify CDAD cases decreased from 55% in 2000 to 25% in 2004. A significant inverse association existed between the number of ICPs per 250 beds and CDAD rates in 2004 ($p = 0.05$, Figure). Table 1 indicates a similar increasing trend in the rates of positive *C. difficile* test results, CDAD outbreaks, complications, annual 30-day crude mortality (deaths due to any cause), and recurrent CDAD infection. Hospitals differed widely in their case definitions for recurrent cases. Most used an arbitrary period (from first episode to CDAD relapse), varying from 6 weeks to 1 year, to define a recurrent case.

Most (60%) respondents thought that the number of cases of community-acquired CDAD increased in 2004 compared with previous years. Smaller proportions of

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Table 1. *Clostridium difficile* infection rates in acute-care hospitals, New Jersey, 2000–2004*

	2000	2001	2002	2003	2004	Mean	Total	2004 rate/ 2000 rate
C. difficile cases	1,585	1,540	2,201	2,974	5,094	2,679	13,394	NA
C. difficile cases/1,000 admissions	3.7	3.2	4.1	4.9	7.7	4.7	NA	2.1
C. difficile–positive test results	2,355	2,759	5,193	8,592	12,445	6,269	31,344	NA
C. difficile–positive test results/1,000 admissions	4.2	4.4	7.8	12.4	18.4	9.4	NA	4.4
C. difficile–complicated cases	2	3	5	12	43	13	65	NA
C. difficile complication rate† (%)	0.1	0.2	0.2	0.4	0.9	0.4	NA	6.8
C. difficile healthcare-associated outbreaks (no. hospitals affected)	2 (2)	6 (1)	10 (7)	11 (7)	25 (12)	11	54	NA
C. difficile healthcare-associated outbreak rate‡	3.7	10.9	18.2	19.6	44	19.3	NA	11.9
Deaths reported within 30 days after diagnosis	0	0	23	50	87	32	160	NA
30-day *C. difficile* crude mortality rate (%)	0	0	1.2	1.9	1.8	1	NA	1.5§
Recurrent *C. difficile* cases	0	1	7	76	171	51	255	NA
Recurrent *C. difficile* rate¶ (%)	0	0.1	0.4	2.7	3.4	1.3	NA	34#

*NA, not available.
†Percentage of *C. difficile* patients in whom complications developed.
‡Outbreaks per 100 acute-care hospitals that responded.
§2004 rate/2002 rate.
¶Percentage of *C. difficile* patients whose infections recurred.
#2004 rate/2001 rate.

respondents perceived increases in the numbers of recurrent cases (55%), healthcare-acquired cases (40%), complicated cases (28%), and deaths (19%) during the same period.

Hospital laboratories most commonly used enzyme immunoassay (EIA) tests for toxins A and B to identify *C. difficile* (88%), followed by stool culture (16%), cytotoxin testing using tissue culture (7%), and EIA for toxin A (7%). Forty (69%) hospital laboratories reported having written institutional policies for correct specimen collection, storage, and transportation of specimens for CDAD diagnosis. Surveillance methods used by hospitals to track CDAD in their institutions are detailed in Table 2.

The survey design had several limitations. First, the analysis was designed to measure the overall incidence of CDAD associated with acute-care hospitalization, regardless of acquisition site, and we did not distinguish between community- and healthcare-associated infections. Second, we did not collect information on nonresponding hospitals and therefore are unable to determine if substantial differences existed between responding and nonresponding hospitals. Third, the rates of CDAD infections were calculated from data provided by the hospitals; hospitals might not have consistently followed the case definitions that were provided for reporting. We also reviewed administrative (i.e., universal billing) data as a secondary data source and found similar trends to those observed in this study. However, this process has multiple weaknesses, including ambiguities in coding and misclassification, which limit its utility for surveillance (*3*). Finally, enhanced awareness of the disease among clinicians and ICPs might have contributed to increased reporting during the 5-year period.

Conclusions

Our results demonstrate that CDAD rates and associated complications rose rapidly among New Jersey hospitals during 2000–2004. How much of the increase reflects rising awareness and how much is a true increase in incidence is unclear. Nevertheless, the trend is dramatic and consistent with published reports in the United States, Canada, and Europe that evaluated CDAD rates during earlier periods (*4–7*).

Our observation that a higher ICP staffing level was associated with lower CDAD rates is consistent with previous studies demonstrating that a higher ICP-to-bed ratio is associated with reduction in rates of healthcare-acquired infections (*8–10*). We recommend that hospitals ensure that their infection-control programs employ sufficient personnel and other resources to implement adequate infection-control practices, with the goal of decreasing CDAD rates in their institutions.

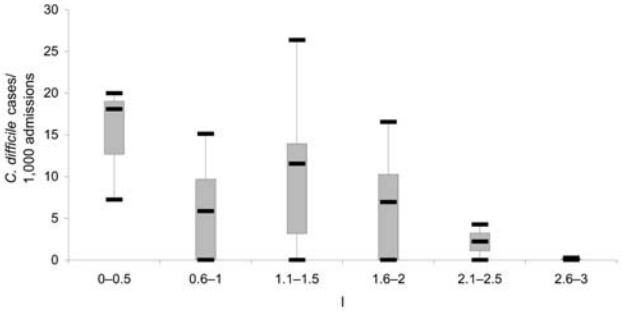

Figure. Boxplot of *Clostridium difficile* rates by number of infection control professionals per 250 beds, 2004. Each box shows the median, quartiles, and extreme values.

Table 2. *Clostridium difficile* surveillance activities conducted by hospitals, New Jersey, 2000–2004

Surveillance activities	No. (%) hospitals
Monitors *C. difficile*–positive laboratory results	55 (95)
Makes a distinction between community- and healthcare-acquired *C. difficile* cases	44 (76)
Uses a standard *C. difficile* case definition for surveillance	35 (60)
Monitors clinical outcome of patients with *C. difficile* infection	28 (48)
Physicians notify infection-control professional of *C. difficile* diagnoses	18 (31)

In terms of surveillance activities, almost all participating hospitals tracked *C. difficile* laboratory results. However, a relatively low percentage of hospitals routinely monitored CDAD complications and deaths. Given recent reports of the emergence of hypertoxin-producing *C. difficile* strains that are more treatment-resistant and potentially more virulent than other strains (*2*), we recommend that hospitals implement or continue comprehensive surveillance programs to track the incidence of both healthcare-acquired and community-acquired CDAD, as well as patient outcomes. Surveillance of these entities will allow ICPs to identify quickly changes in CDAD incidence and severity that could be associated with the introduction of new, more virulent strains. In addition, rapid changes in incidence and detected outbreaks should be reported to public health officials.

Despite the survey's limitations, the estimates provided from this substantial sample of acute-care hospitals are useful for hospitals to develop appropriate CDAD policies and can serve as comparison data for future infection prevention and control efforts in New Jersey and other states. Indeed, given the recent increase in the extent of *C. difficile* death and illness in North America and Europe, the findings in this study show that CDAD is an emerging problem, worthy of substantial investment in effective infection-control and monitoring systems.

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Drug-resistant Nontyphoidal *Salmonella* Bacteremia, Thailand[1]

To the Editor: Despite improved public health, serious infections with nontyphoidal *Salmonella enterica* remain a major clinical and public health concern in Thailand and worldwide (*1*,*2*). Life-threatening *Salmonella* infections resistant to fluoroquinolones, extended-spectrum cephalosporins, or both, have been increasingly reported (*3*). Use of antimicrobial drugs for disease prevention and growth promotion in food animals has been implicated in this increase in drug resistance (*4*). Because of extensive global travel, such increases affect the medical community domestically and internationally (*5*). We report a pilot survey of drug resistance in *Salmonella* spp. in Thailand.

We studied archival nontyphoidal *Salmonella* isolates from bacteremic patients at King Chulalongkorn Memorial Hospital from January 2003 to October 2005 and from bacteremic patients in Thailand sent to the World Health Organization National *Salmonella* and *Shigella* Center in Bangkok during the first half of 2005. The isolates from these archives were nonoverlapping and were kept frozen at –80°C. Isolates were divided into *Salmonella* serovar Choleraesuis and other nontyphoidal *Salmonella* (non-Choleraesuis) because we observed that Choleraesuis isolates show a higher frequency of resistance to fluoroquinolones and extended-spectrum cephalosporins than non-Choleraesuis isolates. A standard Etest method (AB Biodisk, Solna, Sweden) was used to evaluate MICs for nalidixic acid, ciprofloxacin, and ceftriaxone. Susceptibility was defined according to the 2005 criteria for *Salmonella* of the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) (*6*).

Isolates showed high frequencies of antimicrobial drug resistance (Figure). All *S.* Choleraesuis isolates with ceftriaxone resistance also showed high levels of resistance to nalidixic acid (MIC ≥256 μg/mL); most of these also had reduced susceptibility to ciprofloxacin (MIC ≥0.125 μg/mL). Of 73 nalidixic acid–resistant *Salmonella* isolates, 55 (75%) required a ciprofloxacin MIC ≥0.125 μg/mL, 14 (19%) required an MIC of 0.094 μg/mL, and 4 (6%) required an MIC of 0.064 μg/mL. One patient with aortitis caused by ceftriaxone-resistant *S.* Choleraesuis died of a ruptured mycotic aneurysm.

In the food animal industry, the effect of using antimicrobial drugs has long been a subject of concern (*7*–*9*). Evidence from molecular epidemiologic studies (*9*) suggests that these concerns are genuine and that serious problems must be addressed. This concern is also supported by reports of fatal, invasive, nontyphoidal *Salmonella* infections resistant to quinolones or extended-spectrum cephalosporins (*7*,*10*). In Thailand, enrofloxacin, a veterinary fluoroquinolone, is used in animals in the poultry, swine, and seafood industries. Ceftiofur, a third-generation cephalosporin, is used extensively in swine for treatment and prevention of disease and for growth promotion. When compared with previous susceptibility patterns (*5*), current nontyphoidal *Salmonella* infections in humans in Thailand are more resistant to quinolones and cephalosporins. Susceptibility to nalidixic acid correlates well with reduced susceptibility to ciprofloxacin. An alarming increase in ceftriaxone resistance in *S.* Choleraesuis may be associated with inappropriate cephalosporin use in swine farming. Major revisions in current policies for use of antimicrobial drugs in food animals in Thailand are warranted.

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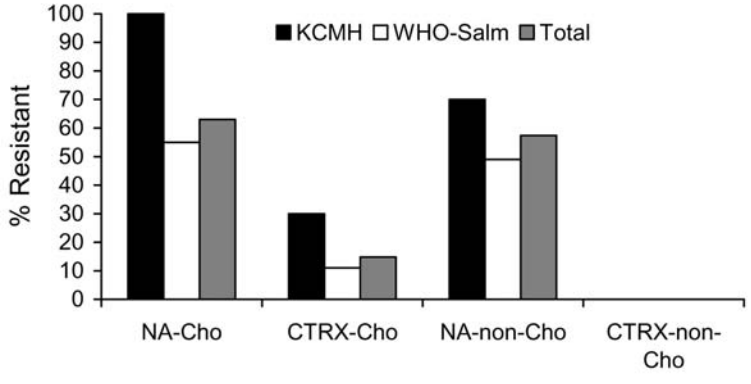

Figure. Percentage of nontyphoidal *Salmonella* isolates resistant to nalidixic acid (NA) and ceftriaxone (CTRX), Thailand. KCMH, King Chulalongkorn Memorial Hospital; WHO-Salm, World Health Organization *Salmonella* and *Shigella* Center. Cho, Choleraesuis; non-Cho, non-Choleraesuis. The analysis included 10 Cho isolates from KCMH, 44 Cho isolates from WHO-Salm, 27 non-Cho isolates from KCMH, and 41 non-Cho isolates from WHO-Salm. Two Cho isolates from WHO-Salm with intermediate MICs for ceftriaxone are also included.

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Fulminant Supraglottitis from *Neisseria meningitidis*

To the Editor: A 68-year-old Caucasian woman with non–insulin-dependent diabetes mellitus, hypertension, and peripheral vascular disease sought treatment at an emergency department after experiencing 2 days of pharyngitis and 1 day of fatigue and dysphagia for solid food. The morning of admission she noted dysphagia for solid food and liquids, dysphonia, severe anterior neck pain, neck swelling and erythema, dyspnea, and a temperature of 102.3°F (39°C). A computed tomographic (CT) scan demonstrated substantial neck soft tissue edema and narrowing of the oropharynx and hypopharynx. She received single doses of intravenous ampicillin/sulbactam, clindamycin, dexamethasone (10 mg), and methylprednisolone (125 mg) before being evacuated by air to our intensive care unit (ICU) at Walter Reed Army Medical Center. Intravenous ampicillin/sulbactam, 3 g every 6 hours, and clindamycin, 900 mg every 8 hours, were continued after the transfer. Two doses of intravenous vancomycin, 1 g every 12 hours, were given before vancomycin was discontinued. Results of laboratory studies were the following: leukocyte count 13.3/mm^3 (71% polymorphonuclear leukocytes, 18% bands) and normal hematocrit, platelet count, blood urea nitrogen and creatinine concentrations, and liver-associated enzymes.

A marker pen was used to track the rapid advance of erythema overnight from her anterior, inferior chin to the top of her breasts (Figure). The infectious disease service was consulted the next morning. When she was examined, her condition had improved; she had normal vital signs, a slightly hoarse voice, and the ability to swallow some saliva. She had no headache or meningismus. The chest erythema was receding. Oral examination demonstrated erythema and an abrasion in the posterior pharynx. Her tongue was not elevated and her uvula was midline. Anterior firm edema without crepitus extended from her chin to the mid-neck. Results of her examination were otherwise unremarkable. The infectious disease consultant recommended restarting a course of vancomycin and discontinuing clindamycin.

A follow-up CT scan with contrast demonstrated anterior cervical soft tissue edema and patent airway with surrounding abnormal thickness and soft tissue density. No abscess or clot was seen. Endoscopic examination in the ICU showed diffuse erythema and generalized supraglottic edema affecting mostly the epiglottis and arytenoids. Dental examination demonstrated no acute pathologic features. Blood cultures at our hospital yielded no growth, and throat culture was negative for group A streptococci.

The patient recovered without requiring intubation (Figure). On the day of discharge, a blood culture from the referring hospital's emergency department was reported to be positive for *Neisseria meningitidis*, serogroup Y. Immediate family members and the otolaryngologists who conducted the endoscopic examina-

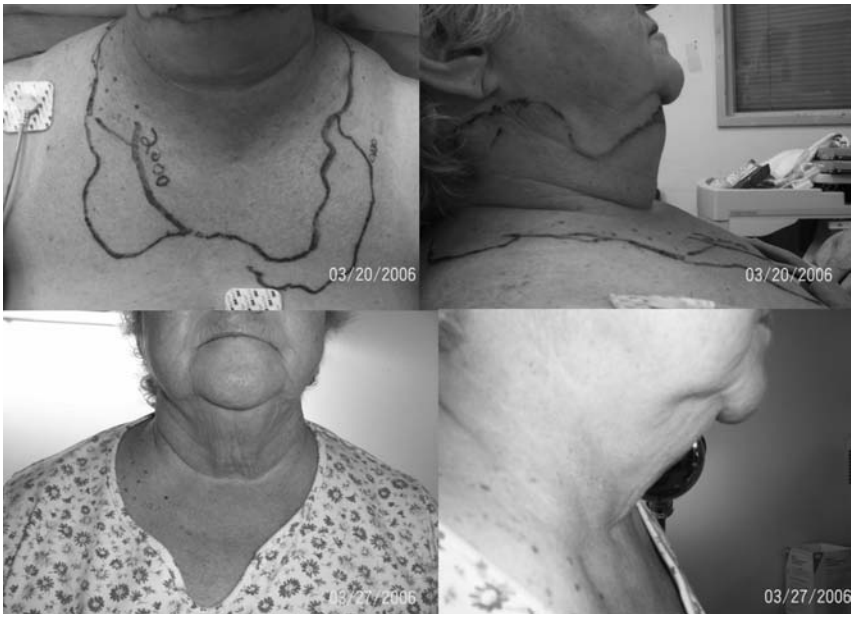

Figure. Top: anterior and lateral views of patient on day 1 of receiving antimicrobial drugs, demonstrating neck erythema and edema. Bottom: anterior and lateral views of patient on day 8 of receiving antimicrobial drugs, demonstrating resolution of neck erythema and edema.

tion were given postexposure prophylaxis. The patient also received terminal prophylaxis. Results of a screening CH50 for terminal complement deficiency were normal.

This patient's condition is consistent with fulminant meningococcal supraglottitis. Supraglottitis implies involvement of the epiglottis and surrounding structures and is more commonly used to describe adult infection than is epiglottitis (*1*). Epiglottitis has become more common in adults than in children since the introduction of the *Haemophilus influenzae* type b vaccine. Other organisms responsible for epiglottitis in adults include *H. influenzae*, *H. parainfluenzae*, pneumococci, *Staphylococcus aureus*, and group A streptococci (*2*).

Despite its propensity to colonize the upper respiratory tract, *N. meningitidis* has rarely been identified as a cause of supraglottitis or other deep neck infections. Only 6 cases have been reported, the first in 1995 (*3*–*8*). Previously reported cases were equally apportioned by sex, and patients were 44 to 95 years of age (*3*–*8*). Including our patient, 3 of 7 were diabetic (*6*,*7*). None showed evidence of meningitis or fulminant meningococcemia, but all had fever, pharyngitis, and airway compromise. Five required airway intervention: 3 intubations and 2 urgent tracheostomies. Two received steroids (*3*,*4*), a 54-year-old man required urgent tracheostomy before receiving steroids, and a 60-year-old man's condition "deteriorated rapidly," but the report does not indicate the interval between receipt of steroids and intubation. Although steroids have been used, their benefit is unproven, and no controlled clinical trials have been conducted (*9*).

Blood cultures have been positive from all reported case-patients. Two isolates were typed as serogroup B, 4 as serogroup Y, and the serotype of 1 was unreported. Meningococcal strains causing supraglottitis appear to be more locally aggressive but cause less disseminated disease, possibly due to decreased tropism for endothelial cells (*8*).

To our knowledge, ours is the second case of meningococcal supraglottitis reported with severe neck edema and cellulitis; a 44-year-old woman in a prior review had features similar to our patient (*8*). An 81-year-old woman with diabetes was noted to have "reddish swelling" on the right side of the neck (*7*), but little was described beyond that. All 3 had serogroup Y infection. We wondered whether serogroup Y might have a propensity to cause cellulitis; however, a review of 10 cases of meningococcal cellulitis included patients with multiple serogroups: C (4 cases), B (2 cases), Y (2 cases), and unknown (2 cases) (*10*).

N. meningitidis may cause supraglottitis more frequently than is recognized (*3*). Timely drawing of blood cultures in relation to administration of antimicrobial drugs is most likely to identify this pathogen in this setting. Because of its public health implications and potential for rapid progression to airway compromise, *N. meningitidis* should be considered among the differential diagnoses of supraglottitis/epiglottitis.

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gyrA Mutations in Fluoroquinolone-resistant *Clostridium difficile* PCR-027

To the Editor: *Clostridium difficile* is the most common cause of bacterial diarrhea in hospitalized patients (*1*). Antimicrobial drug therapy is the most important risk factor associated with the acquistion of *C. difficile,* and several antimicrobial agents including clindamycin, amoxicillin, and cephalo-sporins have been particularly associated with *C. difficile* infection (*2*). Acquisition of resistance to clindamycin is considered 1 mechanism whereby clonal strains emerge and predominate in healthcare environments (*3*). Historically, fluoroquinolone antimicrobial agents were considered low risk for *C. difficile*–associated-disease; however, recent studies indicate a shift in the risk associated with their use (*4*). Furthermore, recent outbreaks in Canada and the United States have been associated with fluoroquinolone exposure (*4*).

Recently, several *C. difficile* outbreaks due to PCR ribotype 027 (PCR-027) and associated with increased disease severity and death have been reported worldwide (*4*). This strain type contains the genes for binary toxin and has an 18-bp deletion and a frameshift mutation in *tcdC* hypothesized to result in deregulated expression of toxins A and B. These strains produce 16× more toxin A and 23× more toxin B in vitro than toxinotype 0 strains (*5*). These isolates demonstrate universal high-level resistance to fluoroquinolones in contrast to that of PCR 027 isolates collected before 2001 (*4*).

We report the mechanism of fluoroquinolone resistance in a cluster (n = 5) of Irish PCR-027 *C. difficile* isolates that were characterized by using toxinotyping and 16–23S ribotyping. Amplification with PCR and sequencing was used to identify the binary toxin gene (*cdtB*) and an 18-bp deletion and a frameshift mutation at position 117 in the *tcdC* gene. Antimicrobial susceptibility to 5 fluoroquinolone antimicrobial drugs was determined with E-tests (AB-Biodisk, Solna, Sweden). The quinolone-resistance–determining region (QRDR) of *gyrA* and *gyrB* was amplified by PCR and characterized. The nucleotide sequence data for partial sequences of the *gyrA* gene were submitted to GenBank and assigned accession nos. DQ821481, DQ821482, DQ821483, and DQ821484.

PCR ribotyping profiles identified 1 cluster of *C. difficile* PCR-027 with clinical isolates that showed indistinguishable profiles to the control 027 strain. PCR identified the *cdtB,* an 18-bp deletion, and a frameshift mutation at position 117 in the *tcdC* gene in all 5 isolates. These strains were universally resistant to the fluoroquinolones tested (ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin, and gatifloxacin, respectively, MIC >32 μg/mL [Table]). Control isolates were susceptible to moxifloxacin and gatifloxacin (MICs 0.3, 0.2 μg/mL, respectively); however, these strains had reduced susceptibility to levofloxacin (MIC 3 μg/mL) and were resistant to ciprofloxacin and oflo-xacin (Table). Sequence analysis determined that all 5 PCR-027 isolates had a single transition mutation (C to T), resulting in the amino acid substitution Thr-82-Ile in *gyrA* (Table). No amino acid substitutions were found in the QRDR of *gyrB* (data not shown).

Mutations in the active site or the QRDR of DNA gyrase and topoisomerase IV have been associated with increased resistance to fluoroquinolones in several bacteria (*6*). This report identifies for the first time a mutation in *gyrA* that is associated with high-level resistance to fluoroquinolones in *C. difficile* PCR-027. In *Escherichia coli,* amino acid substitutions that occur at Ser-83 in *gyrA* have been associated with fluoroquinolone resistance (*6*). Thr-82 in *C. difficile* corresponds to Ser-83 in *E. coli*. Thr-to-Ile amino acid substitutions corresponding to Ser-83 have been associated with fluoroquinolone resistance in several bacteria, including *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Campylobacter jejuni,* and *C. difficile* (*6*). Ackermann et al. described 2 mutations in *gyrA* that resulted in an amino acid substitution corresponding to codon 83 in *E. coli*. Thirteen of the 18 *C. difficile* isolates had the Thr-82-Ile substitution, and 1 strain had a Thr-82-Val substitution (*7*). Dridi et al. described this Thr-82-Ile GyrA substitution in 6 resistant *C. difficile* strains corresponding to 3 serogroups, H1, A9, and 1C (*8*).

Early studies investigating fluoroquinolone antimicrobial agents suggested that most *C. difficile* isolates were susceptible to these drugs.

Table. Characterization of representative isolates, Ireland, 2006

Isolate	Toxigenic status	Ribotype	Fluoroquinolone MIC μg/mL					Amino acid substitution
			Ciprofloxacin	Ofloxacin	Levofloxacin	Gatifloxacin	Moxifloxacin	
1470*	A^-B^+	017	>32	>32	3	0.38	0.25	Thr 82
VPI10463*	A^+B^+	D	>32	>32	3	0.38	0.25	Thr 82
CD 196*	A^+B^+	027	>32	>32	3	0.38	0.25	Thr 82
M216†	A^+B^+	027	>32	>32	>32	>32	>32	Thr-82-Iso
C2191†	A^+B^+	027	>32	>32	>32	>32	>32	Thr-82-Iso
V6-13†	A^+B^+	027	>32	>32	>32	>32	>32	Thr-82-Iso
V6-15†	A^+B^+	027	>32	>32	>32	>32	>32	Thr-82-Iso
V6-20†	A^+B^+	027	>32	>32	>32	>32	>3	Thr-82-Iso

*Control isolates VPI-10463, 1470 CD196.
†Clinical 027 isolates from 3 different institutions investigated in this study.

Antimicrobial drug resistance to this class has increased with fluoroquinolone use, and currently these drugs remain the most frequently prescribed antimicrobial agents in the United States and Europe. Acquired resistance to the newer fluoroquinolone antimicrobial agents is not restricted to ribotype PCR-027, although different amino acid substitutions in the QRDR of *gyrA* and *gyrB* have been described (*7*–*9*). Wilcox et al. have described high-level fluoroquinolone resistance in PCR ribotype-001, an endemic strain type found in several healthcare settings in the United Kingdom (*10*). We have previously described the emergence of a fluoroquinolone-resistant toxin A–, toxin B–positive strain in Dublin (*9*).

We report a mutation in *gyrA* associated with fluoroquinolone resistance in *C. difficile* PCR-027. Antimicrobial drug resistance in *C. difficile* isolates must be monitored because the emergence of universal fluoroquinolone resistance in different *C. difficile* strain types may be a factor promoting outbreaks in hospitals. As exposure to several different fluoroquinolone antimicrobial drugs have been independently associated with *C. difficile*–associated-disease, restricted use of all fluoroquinolones, rather than changing from 1 quinolone to another, may be a necessary step toward preventing and controlling *C. difficile* outbreaks.

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Instructions for Emerging Infectious Diseases Authors

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.

New Strain of Influenza A Virus (H5N1), Thailand

To the Editor: During 2004–2005, 3 major waves of avian influenza outbreaks occurred in Thailand (*1*). The first wave was reported in early January 2004, the second in July 2004, and the third in October–December 2005. In total, 22 persons were infected and 14 died. Recently, a fourth wave began on July 23, 2006. The Thai Ministry of Public Health reported that avian influenza A (H5N1) virus killed 2 infected persons. The first patient, a 17-year-old man in Phichit Province, began to experience symptoms on July 15, 2006, and died on July 24, 2006 (*2*). The second patient, a 27-year-old man in Uthai Thani Province, began to experience symptoms on July 24, 2006, and died on August 3, 2006 (*3*).

The fourth wave of these outbreaks involved chickens and encompassed 2 distinct areas: Phichit Province, identified on July 23, 2006 (*4*), and Nakhon Phanom Province, identified on July 28, 2006 (*5*). We sequenced all 8 gene segments of the 2 viruses isolated from Phichit and 1 virus isolated from Nakhon Phanom and then submitted to GenBank as follows: A/chicken/Thailand/PC-168/2006 (DQ999879–86) and A/chicken/Thailand/PC-170/2006 (DQ999887–94) from Phichit and A/chicken/Thailand/NP-172/2006 (DQ999871–8) from Nakhon Phanom.

Whole genome analysis showed that all 3 samples had undergone minor mutations that are typical of circulating influenza A viruses. Unexpectedly, this outbreak was associated with 2 strains of the virus. The 2 samples from Phichit closely resembled H5N1 strains that had circulated in Thailand during 2004 and 2005. The sample from Nakhon Phanom was newly observed in Thailand and more closely related to H5N1 strains that had been circulating since 2005 in southeast People's Republic of China. The whole genome phylogenetic analysis also showed that the viruses isolated from Phichit belonged to genotype Z, whereas virus isolated from Nakhon Phanom belonged to genotype V, which differs from genotype Z in the PA gene (*6*) (Figure, panel A).

The phylogenetic tree of the hemagglutinin (HA) gene (Figure, panel B) showed that the Phichit samples were similar to the cluster of samples isolated during 2004 and 2005 in Thailand and Vietnam. In contrast, the Nakhon Phanom sample was clustered into the same group with viruses isolated from southeast People's Republic of China, including Zhejiang, Shantou, Hunan, Fujian, Guangxi, and Lao People's Democratic Republic (*7*) with the differences in the cleavage site, SPLR-ERRRK-R/G (underline and dash indicate differences), which had never been found in Thailand. The N-link glycosylation sites (positions 154–156) of the Pichit isolates were NST residues, whereas in the Nakhon Phanom isolate, NNT residues were observed. However, the receptor-binding site of HA (positions 222 and 224) was unchanged.

In the neuraminidase (NA) gene, the new isolates contain 20 amino acid deletions within the stalk region, the same as previously described (*1*). The ESEV residues in the C-terminal and Asp92 of NS1 were observed in the 2006 isolates and in viruses that have been isolated from Thailand, Vietnam, and People's Republic of China. This finding indicates that the new isolates were highly virulent but sensitive to treatment with interferon and tumor necrosis factor-α (*8*). The 2006 isolates contain Glu627 of PB2, identical to the previous isolates from Thailand and Indonesia, which may indicate that the new isolates had less efficient replication capability in

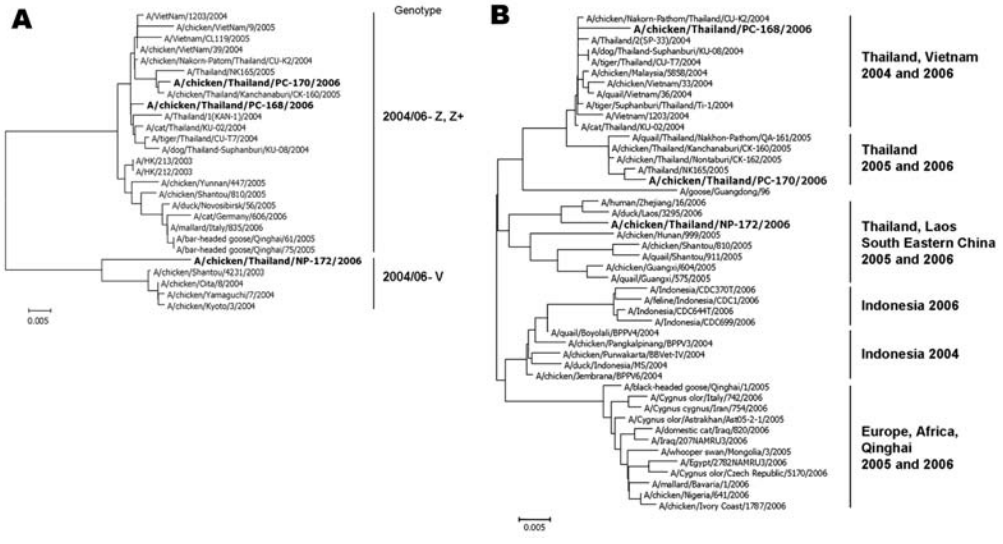

Figure. A) Phylogenetic relationships of the polymerase acid protein gene comparing genotype Z, Z+, and V. B) Hemagglutinin gene of influenza A (H5N1) viruses in Thailand 2006 compared with several other strains worldwide. For a larger reproduction of the phylogenetic relationships, see online figure available at www.cdc.gov/EID/content/13/3/506-G. htm

mammalian hosts (*9*). Drug resistance or sensitivity is based on sequences of M2 and NA. Substitution within residues including L26I, V27A/I, A30S, and S31N of the M2 ion channel protein was used to predict amantadine-resistant mutants, and H274Y of the NA was used to predict for oseltamivir resistance (*10*). The virus observed in 2006 isolates from Phichit was resistant to amantadine but sensitive to oseltamivir, whereas the isolate from Nakhon Phanom was sensitive to amantadine and oseltamivir, which implies that infected patients received different antiviral drugs.

According to previous World Health Organization reports, the HA sequences of most influenza (H5N1) viruses that circulated in avian species during the past 3 years are separated into 2 distinct phylogenetic clades. Clade 1 viruses that circulated in Cambodia, Thailand, and Vietnam were responsible for human infections in those countries during 2004 and 2005. Clade 2 viruses that circulated in birds in People's Republic of China and Indonesia during 2003–2004 and 2005–2006 spread westward to the Middle East, Europe, and Africa. This latter genetic group of viruses has been principally responsible for human infections during late 2005 and 2006 (*11*). The latest wave of the outbreaks in Thailand was caused by viruses closely related to those that caused outbreaks in Thailand in 2004–2005 and to viruses recently circulating in southeast People's Republic of China and other Southeast Asian countries. This finding raises concern for development of new candidate influenza (H5N1) vaccine strains. Geographic spreading, epidemiology, and genetic properties of recently circulating influenza (H5N1) viruses should be considered when developing candidate H5N1 strains of influenza vaccine.

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Kala-azar Control, Uganda

To the Editor: Much of the leishmaniasis in Africa is concentrated in East Africa. In this region, visceral leishmaniasis (kala-azar) is caused by *Leishmania donovani* and is endemic in remote parts of Somalia, Sudan, Ethiopia, Kenya, and Uganda (*1*).

In Uganda, kala-azar is transmitted by the sandfly *Phlebotomus martini,* and transmission is thought to be anthroponotic. Studies in Sudan and Kenya have detected *L. donovani* in domestic animals (*2*,*3*), but whether these play a role in Uganda is

unknown. In Uganda, the disease appears to be restricted to Pokot County, a semiarid lowland area in Nakapiripirit District (see online map in Appendix Figure, available at www.cdc.gov/EID/content/13/3/507-appG.htm). This focus is an extension of a larger focus in West Pokot District in Kenya (*4*). The area is mainly inhabited by the Pokot, a seminomadic tribe of pastoralists. Nakapiripirit is one of the most underserved districts of Uganda, plagued by tribal clashes.

Though kala-azar has been reported in East Africa since the early 1900s, it was not described in Uganda until the 1950s (*5*) and remained largely unnoticed until 1997, when Médecins Sans Frontières (MSF, Swiss Section) began to provide assistance to Amudat Health Centre in Pokot County. In 2000, MSF initiated a kala-azar control program, focusing on passive case detection and treatment.

From January 2000 to February 2006, a total of 3,645 patients suspected of having kala-azar were screened at Amudat Health Centre by using the direct agglutination test or rK39 antigen–based dipsticks (*6*); 2,088 patients with confirmed disease were treated with daily intramuscular injections of sodium stibogluconate or meglumine antimonite, 20 mg/kg bodyweight, for 30 days. Overall, 80% of the patients were <15 years of age, 75% were male, and 70% were from Kenya. From 2000 through 2005, the number of patients treated more than tripled, from 175 to 690 cases per year. Although this increase likely results, at least in part, from greater case detection due to the availability of treatment, we cannot exclude a real increase in disease because kala-azar prevalence in the area is unknown.

Information on local vector behavior and risk factors for infection or disease (e.g., malnutrition and HIV coinfection) is limited, and which potential interventions are appropriate is unclear. A pilot entomologic study in 2004 (J. Stevenson, master's thesis) demonstrated that termite mounds (Figure) are important vector breeding and resting sites and that the practice of sitting on termite mounds while guarding livestock might increase the risk for infection. In contrast, humans may be protected from kala-azar when in close proximity to livestock (i.e., because of diversion of sandflies to alternative hosts) or when lighting fires indoors (smoke acts as a repellant to most biting flies). Ownership of insecticide-treated nets, which could protect persons from sandfly bites and reduce kala-azar transmission (*7*), was low. Although most of the local population had heard of kala-azar, known locally as *termes*, and regarded it as potentially fatal, few were aware of how kala-azar is transmitted (J. Stevenson, master's thesis).

Figure. A large termite mound occupies the central area of this characteristic Pokot compound. The mound provides a resting and breeding site for the sandly vector of visceral leishmaniasis. Photographer: J.H. Kolaczinski.

MSF's treatment of kala-azar is crucial because it reduces the human reservoir and hence transmission. However, current control activities only reach the tip of the iceberg: a large, underlying pool of infected and infectious persons likely exists (*8*,*9*).

Kala-azar in Uganda will not likely be controlled unless the epidemiology of the disease is better understood and preventive activities are undertaken. This knowledge gap is being addressed by a partnership among the Malaria Consortium, MSF, the London School of Hygiene and Tropical Medicine, and the Vector Control Division of the Ugandan Ministry of Health. A case-control study to determine the local risk factors of kala-azar is almost completed and will be followed by seroprevalence studies in several Pokot villages, using a similar approach to recent work in the Baringo District, Kenya (*9*). The results will be used to formulate an integrated control strategy aimed at achieving our ultimate goal of eliminating kala-azar from Uganda.

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Chikungunya Virus Infection in Traveler to Australia

To the Editor: Chikungunya is a mosquitoborne alphavirus in the family *Togaviridae*. Recently, a chikungunya virus epidemic that affected thousands of persons occurred in islands in the southwestern Indian Ocean, including Mauritius and Reunion (*1*). An outbreak is ongoing in India, and cases are being exported to many other countries (*2–4*) The likelihood of importation of exotic infectious agents into Australia increased during events such as the March 2006 Commonwealth Games in Melbourne. Urgent diagnosis of rarely seen infections is a travel health challenge, particularly when serologic tests used for diagnosis in areas with high prevalence are not locally available. Only 1 previously diagnosed case of chikungunya virus infection has been reported in Australia; it involved importation of the virus from Indonesia to Darwin in 1989 (*5*). We report a second case of infection with this virus.

A 59-year-old man came to a hospital emergency department in North Melbourne, Australia, on March 12, 2006, 5 days after traveling from Mauritius for the Commonwealth Games. He reported a 2-day history of acute swelling and erythema of the left leg and associated malaise. Twenty-four hours earlier, severe lumbar back pain and arthralgias that involved the lower limbs had developed, with associated fevers, rigors, and headache.

The patient had a temperature of 39°C, bilateral conjunctivitis, tender and markedly swollen Achilles tendons, a swollen left ankle, and a maculopapular rash that involved the left forefoot and anterior portion of the shin. Initial blood examinations showed leukopenia (leukocyte count 1.8×10^9/L, lymphocyte count 0.2×10^9/L), mild thrombocytopenia (platelet count 105×10^9/L), and abnormal liver function test results (alanine aminotransferase 133 IU/μL, γ-glutamyl transpeptidase 141 IU/μL, bilirubin 7 mmol/L). Malaria blood films and dengue serologic results were negative. A validated, in-house, generic alphavirus reverse transcription–PCR (RT-PCR) showed positive results 24 hours after collection of blood when the patient was admitted.

The patient received supportive treatment and was discharged from the hospital 3 days after admission, at which time leukopenia and thrombocytopenia had improved. The patient had fully recovered on review 1 week after discharge.

For virus isolation, plasma and leukocyte fractions were placed onto Vero E6 cells and incubated at 37°C for 5 days. Cells were observed daily for virus-specific cytopathic effects. A virus isolate was obtained after 4 days of cell culture. A 10-mL volume of supernatant from infected cells was applied to a carbon-coated grid, stained with phototungstic acid, and examined by electron microscopy. This procedure showed virus with morphology similar to Togavirus (data not shown).

Chikungunya virus was identified by a heminested RT-PCR for the non-

structural protein 4 gene. First-round primers were AlphaF1 (GenBank accession no. NC004162, nt 6942–6961, 5′-CSATGATGAARTC HGGHATG-3′) and AlphaR (nt 7121–7141, 5′-CTATTTAGGACCRC CGTASAG-3′). Second-round primers were AlphaF2 (nt 7480–7501) (5′-TGGNTBAAYATGGAGGTIAAG-3′) and AlphaR. Sequencing of the second-round product identified the virus. A 339-bp fragment (GenBank accession no. DQ678928) had 97% identity with the African prototype strain S27 isolated in Tanzania (Tanganyika) in 1953 (AF369024) and 100% identity with viral sequences from Reunion Island in 2006 (DQ443544).

Knowledge of distant epidemics aids clinical recognition of infections not commonly seen in Australia. Websites and electronic bulletins (e.g., Promed) are a conduit of information. On the basis of this case-patient, those in Australia became more aware of the chikungunya virus epidemics affecting the islands of the southwestern Indian Ocean.

Laboratory diagnosis of chikungunya virus infection is usually serologic. However, alphavirus infections not endemic to Australia are unlikely to be diagnosed serologically because specific assays are generally available only for those viruses known to circulate in Australia. Because viremia of alphaviruses is brief, success of RT-PCR depends on early admission and clinical recognition of infection (*6*).

Rapid establishment of a definitive diagnosis had substantial benefits that included management of the febrile patient, reduced need for further investigations, and better prognosis. Infection caused by an introduced arbovirus may have important public health implications in Australia. Because immunity in the Australian population is unlikely, consideration must be given to the potential for transmission of the virus to caregivers and the local community. chikungunya virus is commonly spread by mosquitoes of the genera *Aedes*, including *Aedes aegypti*, *Ae. furcifer-taylori*, *Ae. luteocephalus*, *Ae. albopictus*, and *Ae. dalzieli* (*7*). The Australian Ross River and Barmah Forest alphaviruses are spread by many species of *Aedes* and *Culex* (*8*). Because some local species could transmit chikungunya virus, necessary steps should be taken to ensure containment when a patient is viremic.

This case highlights the potential for exotic viruses to be introduced into Australia by visitors or returning travelers and the utility of molecular testing for their rapid detection. The generic nature of the RT-PCR enabled detection of an alphavirus with subsequent specific identification by sequencing. Rapid identification and differentiation in a public health setting minimized the potential for spread of the virus.

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Avian Influenza A (H5N1) Age Distribution in Humans

To the Editor: A total of 229 confirmed human cases of avian influenza A (H5N1) were reported to the World Health Organization (WHO) from 10 countries of Africa, Asia, and Europe in the 30 months leading up to July 4, 2006 (*1*). WHO has highlighted the skewed age distribution of these confirmed cases toward children and young adults, with relatively few cases in older age categories (*2*). An explanation for this age bias is currently lacking, although a range of behavioral, biological, demographic, and data-related factors may account for the observed pattern (*2*,*3*).

To determine whether the statistical parameters of the case distribution can shed any light on the issue, we reviewed the age profile of patients with confirmed avian influenza A

(H5N1) included in WHO's Situation Updates—Avian Influenza archive (January 13, 2004–May 18, 2006) (*4*). We supplemented our review with case information from an additional WHO source (*5*); to allow for the age structure of reporting countries, we accessed age-specific population estimates for 2005 from the Population Division of the United Nations Secretariat (*6*).

For the period under review, age-related information was available for 169 case-patients with WHO-confirmed human avian influenza A (H5N1) in 10 countries. Information for an additional 47 confirmed case-patients, reported to WHO from Vietnam (n = 39) and Turkey (n = 8), could not be ascertained from the published sources. The mean age of the 169 sample case-patients (77 males and 92 females) was 19.8 years (median 18.0; range 0.3–75.0). Age distribution was as follows: 0–9 years, 26.0%; 10–19 years, 29.0%; 20–29 years, 23.1%; 30–39 years, 16.0%; and ≥40 years, 5.9%. Estimated age-specific case rates per million population were 0.15 (0–9 years), 0.15 (10–19 years), 0.13 (20–29 years), 0.08 (30–39 years), and 0.02 (≥40 years).

Box-and-whisker plots (*7*) (Figure) illustrate the skewed nature of the age distribution of cases by sex (A), year of report (B), and patient outcome (C); the third quartiles of the distributions (Q_3, defined by the box tops) demarcate an age band (30–35 years) above which proportionally few cases (<10%) occurred. The country-level analysis in plot D yields similar findings, although interpretation is limited by the small numbers of cases (<10) in some countries (Azerbaijan, Cambodia, Djibouti, Iraq, and Turkey).

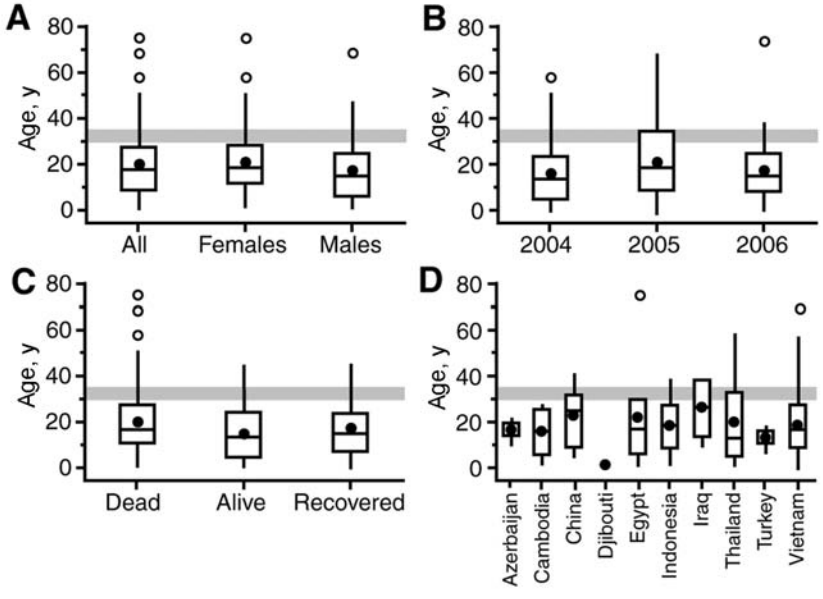

Figure. Age distribution of patients with confirmed cases of avian influenza (H5N1), December 2003–May 2006 (*4,5*). Box-and-whisker plots show the age distribution of patients by A) sex; B) year of report, C) patient outcome, and D) country. The horizontal line and bullet mark in each box give the median and mean age of cases, respectively. Variability in age is shown by plotting the first and third quartiles (Q_1 and Q_3) of the ages as the outer limits of the shaded box. Whiskers encompass all ages that satisfy the criteria $Q_1 – 1.5(Q_3 – Q_1)$ (lower limit) and $Q_3 + 1.5(Q_3 – Q_1)$ (upper limit). Points beyond the whiskers denote outliers. Panel C data are based on the recorded status of patients according to World Health Organization sources, with the category "alive" formed to include patients who were last reported as hospitalized or discharged. The age band 30–35 years is marked on each graph for reference.

Examination of case-patients in the 30- to 39-year age category showed a pronounced "front-loading" effect, with 21 case-patients 30–35 years of age and only 6 case-patients 36–39 years of age.

Subject to multiple selection biases in the identification and reporting of WHO-confirmed human cases of avian influenza A (H5N1) (*2*), our analysis yields 3 noteworthy observations: 1) case counts and case rates suggest similar levels of disease activity in the age categories 0–9, 10–19, and 20–29 years; 2) few cases have occurred above the age band of 30–35 years; and 3) the skewed distribution of cases toward children and young adults transcends sex, reporting period, patient outcome, geographic location, and, by implication, local cultural and demographic determinants.

Behavioral factors increase the risk for exposure in younger persons and have been proposed as 1 determinant of the age distribution of confirmed human cases of avian influenza A (H5N1) (*2*). However, the possible role of biologic (immunologic and genetic) and other factors has yet to be determined (*3*). Such factors may include an age-related bias in case recognition, in which clinical suspicion about the cause of respiratory disease in older persons is lower. Alternatively, we suggest that the 3 observations listed above are consistent with a biological model of geographically widespread immunity to avian influenza A (H5N1) in persons born before 1969, i.e., ≈35 years before the onset of the currently recognized panzootic in domestic poultry. Such a model would account for the similar rates of disease activity in younger age categories, the sudden and pronounced reduction of cases in patients >30–35 years of age, and the age skew that transcends the sociocultural and demographic contexts of countries and continents.

The results of broad serologic surveys for antibodies to influenza A

(H5N1) virus, suggestive of a cohort effect or otherwise, have yet to be published, although anecdotal reports of completed surveys point to a lack of widespread human infection with the virus (*8*). Current evidence indicates that pandemic influenza of humans since 1918 has been restricted to 3 influenza A virus subtypes: H1 (1918–57 and 1977–present); H2 (1957–68); and H3 (1968–present) (*9*,*10*). If an element of immunity to avian influenza A (H5N1) does exist in older populations, its possible association with geographically widespread (intercontinental) influenza A events before the late 1960s merits further investigation.

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Toxoplasma gondii, Brazil

To the Editor: Recently, Jones et al. reported that past pregnancies increased risk for recent *Toxoplasma gondii* infection in Brazil (*1*). They did not, however, control for age. Previous seroepidemiologic studies have shown that age is a main confounding variable in analysis of risk factors for toxoplasmosis (*2*). Age can explain why mothers with more children are at higher risk for toxoplasmosis; the longer persons live in areas with high toxoplasmosis prevalence, the higher their risk for infection.

Also not explored were drinking water–related factors. Our recent study of pregnant women in Quindio, Colombia, found factors that explained attributable risk percent for infection to be eating rare meat (0.26%) and having contact with a cat <6 months of age (0.19%) (*3*). Drinking bottled water was more significantly protective for the group that did not consume undercooked or raw meat (odds ratio 0.06, 95% confidence interval 0.006–0.560, p = 0.008). We think that drinking water–related factors could explain up to 50% of toxoplasmosis infections in our region.

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In response: We thank Dr Gomez-Marin for his letter regarding our article on recently acquired *Toxoplasma gondii* infection in Brazil (*1*). Dr Gomez-Marin states that perhaps age could account for our finding that having had children was a risk factor for recent *T. gondii* infection among women. Studies have shown that age is a risk factor for prevalent *T. gondii* infection; i.e., infection prevalence increases with age (*2*). However, age is not necessarily a risk factor for recent (incident) infection.

Our study of risk factors for *T. gondii* infection was a case-control design to evaluate recent infection, not a cross-sectional study of *T. gondii* infection prevalence in a population. In our study, case-patients with recent infection were similar in age to *T. gondii*–negative control-patients, although among women the mean age of case-patients (33 years) differed slightly from that of control-patients (29 years) ($p = 0.03$, *t*-test). In addition, multivariate analysis comparing the case-patients with control-patients showed that age was not a significant factor. However, when we kept age in the multivariate model for women ($p = 0.87$ for age in the model), the odds ratio for having had children changed little, from 14.94 (95% confidence interval [CI] 3.68–60.73) to 14.01 (95% CI 2.88–68.08). Therefore, we do think that, in this study population, having had children is a risk factor for *T. gondii* infection among women.

Dr Gomez-Marin also states that we did not evaluate drinking water–related factors. However, in our methods section (*1*), we indicated that our questionnaire asked about a comprehensive set of risk factors related to drinking water. Specifically, the questionnaire asked about the types of water (city, private well, and others, including bottled water); chlorination; filtering of water; and ingestion of water from streams, lakes, rivers, ponds, or other sources. Although we evaluated numerous water-related factors, we did not find them to be significant in this study, which applies to 1 area of Brazil. In other areas of Brazil, however, studies in which 1 of our authors (J.L.J.) has been involved have found water to be a risk factor or a source of infection (*2*,*3*).

Again, we thank Dr Gomez-Marin for his letter. We sincerely appreciate his interest and work with toxoplasmosis.

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CTX-M Extended-spectrum β-Lactamases, Washington State

To the Editor: The CTX-M–type β-lactamases are non-TEM and non-SHV plasmid-mediated, class A, extended-spectrum β-lactamases (ESBLs). The CTX-M–type β-lactamases have recently emerged as the most common type of ESBLs, with a global distribution (*1*). In contrast, the CTX-M–type ESBLs are rarely reported in the United States and have not been identified in pathogens isolated from infected patients with gastroenteritis.

We screened 637 *Salmonella* and 126 *Shigella* isolates, collected in the state of Washington during 2003–2004, for CTX-M–type β-lactamases. Of these, 60 *Salmonella* isolates that exhibited an ESBL phenotype were further characterized by PCR for TEV, SHV, CTM-X, and CMY. All were positive for the CMY-2 or TEM-1 β-lactam genes. One *Shigella sonnei* isolate (WA7593), cultured from a fecal specimen in August 2004, tested positive with an ESBL confirmatory disk diffusion panel (ceftazidime 24 mm, ceftazidime/clavulanate 32 mm, cefotaxime 14 mm, and cefotaxime/clavulanate 34 mm; [*2*]). The patient had recently traveled to Pakistan and likely became ill there and returned to the United States while still sick. The transfer of extended-spectrum cephalosporin resistance was tested by conjugation to *Escherichia coli* J53 azi^R (*3*). The MIC for *S. sonnei* WA7593 and its transconjugant, WA7593TC1, were tested by using the E-test (AB Biodisk, Solna, Sweden). Both strains were resistant to cefotaxime and susceptible to ceftazidime and showed almost the same antimicrobial susceptibility patterns as β-lactam antimicrobial drugs (Table).

The type of ESBL produced by these strains was determined by using PCR specific for TEM and CTX-M (*4*,*5*). Both strains were PCR positive for TEM and CTX-M. The TEM type PCR products were then sequenced and identified as TEM-1; no variation was found on the promoter region of $bla_{\text{TEM-1}}$. The entire sequence of $bla_{\text{CTX-M}}$ from WA7593 was then sequenced (*1*), and the product showed 100% homology with $bla_{\text{CTX-M-15}}$ (GenBank accession no. AY960984). The mobile element associated with the transfer of $bla_{\text{CTX-M-15}}$ was investigated by sequencing the flanking regions.

Table. MICs of antimicrobial drugs for *Shigella sonnei* clinical isolate WA7593 and its transconjugant WA7593TC1

Antimicrobial drug	MIC (μg/mL)	
	WA7593	WA7593TC1
Ampicillin	>256	>256
Cephalothin	>256	>256
Cefotaxime	>32	>32
Ceftazidime	4	4
Ceftriaxone	>32	>32
Cefaclor	>256	>256
Imipenem	0.19	0.25
Trimethoprim/sulfamethoxazole	>32	0.032

PCRs were performed with primers from the internal regions of $bla_{\text{CTX-M}}$ gene and primers for insertion sequences IS*Ecp1* and IS*903* (*4*,*5*). Positive PCR products were obtained with primers IS*Ecp1*F and CTX2 (943 bp); no amplified product was produced with primers CTX1 and IS*903*R. Sequencing of a 943-bp amplicon showed that $bla_{\text{CTX-M15}}$ was flanked upstream by an IS*Ecp1*-like element.

The presence of an integron in *S. sonnei* WA7593 and WA7593TC1 was investigated by using integron-specific primers hep35 and hep36 (*2*). Only *S. sonnei* WA7593 produced a PCR product. This finding suggests that the transmission of $bla_{\text{CTX-M15}}$ is not by integron-mediated transfer. A further 162 *Shigella* spp. and 260 *Salmonella* spp. isolated from 2003 through 2005 were also screened for ESBL production; no further isolates were identified.

The presence of a CTX-M–type, ESBL-producing isolate is rarely reported in the United States. The only other reference was from a multistate study in 2001–2002 that identified CTX-M type from *E. coli* isolates from urine, sputum, and blood (*6*). No further reports about CTX-M–producing organisms have been disseminated. Our investigation suggests that CTX-M–type ESBLs may spread throughout the United States through infected travelers. This finding is notable because *S. sonne*i is a common enteric pathogen. Our results further emphasize that travelers from others parts of the world can introduce highly mobile and clinically important resistance mechanisms into the community. The spread of CTX-M ESBLs may be faster and more widespread than previously thought; therefore, CTX-M type should be taken seriously as a surveillance target in the United States, especially in patients with a history of travel outside North America.

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HIV and Hepatitis C Virus Coinfection, Cameroon

To the Editor: Coinfection with HIV and hepatitis C virus (HCV) is now a major public health concern worldwide, owing both to its high prevalence (4–5 million persons of 40 million infected by HIV) and to interactions between the 2 diseases in terms of their diagnosis, natural course, and treatment (*1*,*2*). Although Africa is the continent by far the most badly affected by both HIV and HCV infections, data on coinfection in the general population are lacking. In Cameroon, a central African country, the HCV seroprevalence is among the highest in the world (13.8%) (*3*). We have also reported a high seroprevalence of HIV in a general population of southern Cameroon (7.4%), and especially in young women (22.5%) (*4*). Here, we investigated the prevalence of HIV/HCV coinfection in this population.

A population-based, cross-sectional survey was conducted in September 2001 in 3 villages of the East Province of Cameroon (250 km from Yaoundé, the capital city). The study methods, the baseline characteristics of the participants, and the HIV

seroprevalence have been described in detail elsewhere (*4*). Briefly, all inhabitants >15 years of age were eligible for the survey. After giving their informed consent, the participants were interviewed by using a standard verbal questionnaire, in French or in a local language, during door-to-door visits. Blood samples were collected by peripheral venipuncture, and serum was screened for antibodies to HCV by using an enzyme immunoassay (INNOTEST HCV Ab IV, Innogenetics, Ghent, Belgium). Samples with indeterminate results were retested. All positive and twice-indeterminate samples were confirmed with a third-generation line immunoassay (INNO-LIA HCV Ab III update, Innogenetics). Serologic screening for HIV infection was based on an enzyme immunoassay (Murex HIV-1.2.O, Abbott, Rungis, France). All positive samples were confirmed by using a line immunoassay (INNO-LIA HIV-1+2, Innogenetics).

Among the 484 participants, 256 were women (52.9%), and the median age was 34 years (interquartile range 23–52 years). Most participants (93.6%) were Bantus; the remainder were pygmies. Seven persons refused venipuncture after the interview, and 1 sample could not be analyzed. These 8 persons did not differ from the rest of the study population in term of sex (50.0% women vs. 47.1% women), but they were slightly younger (median, 26.8 years vs. 34.9 years). Of the 476 available samples, respectively 19 (4.0%) had indeterminate HCV serologic results, and 5 (1.1%) had indeterminate HIV serologic results. The overall seroprevalence rates were 21.0% (95% confidence interval [CI] 17.4%–24.9%) for HCV and 7.4% (95% CI 5.2%–10.1%) for HIV. Only 3 patients (0.6%) had positive results for both infections: a man 29 years of age and 2 women ages 36 and 52 years.

The Figure shows the seroprevalence rates of HCV and HIV according to sex and age. Multivariate random-effects logistic regression analyses showed different risk factors for the 2 infections. The HCV seroprevalence was associated with age (<45 vs. ≥45 years, odds ratio [OR] 13.04; 95% CI 6.73–25.30; p<0.001), sex (men vs. women, OR 2.02; 95% CI 1.17–3.47; p = 0.01) and the ethnic group (Bantus vs. pygmies, OR 10.98; 95% CI 1.31–92.42; p = 0.03). In contrast, the HIV seroprevalence was higher in women than in men (OR 10.22; 95% CI 3.19–32.80; p<0.001). No specific risk factors were found in men, whereas women who were unmarried (OR 6.49; CI 2.45–17.17; p<0.001) or school-educated (OR 7.12; 95% CI 1.59–31.78; p = 0.01), or those with a history of sexually transmitted infections (OR 2.92; 95% CI 1.08–7.89; p = 0.03) had higher rates than other women.

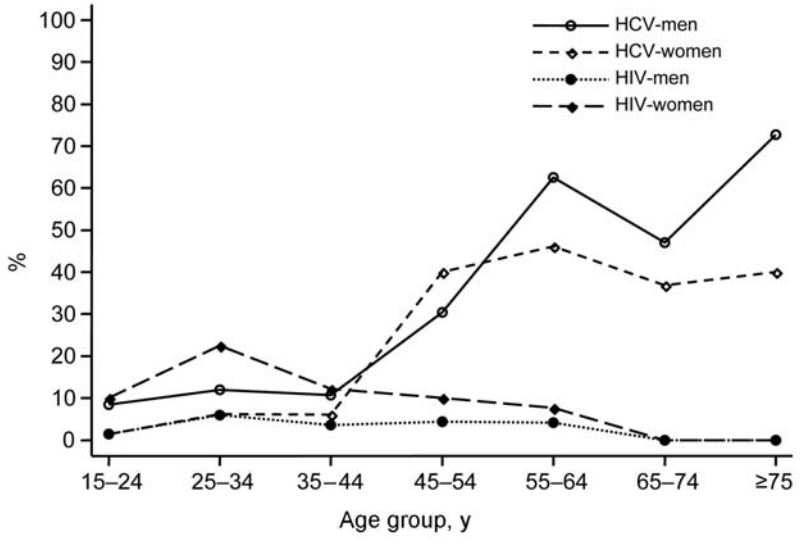

Figure. Seroprevalence rates of hepatitis C virus (HCV) and HIV infection by sex and age in the general population of southern Cameroon, 2001.

HIV/HCV coinfection is therefore rare in this general population, which lives in an area where both HCV and HIV are endemic. This finding could be related to the dissimilar epidemiologic patterns of the 2 infections. Indeed, HIV infection mainly affects young persons, especially young women, while HCV infection is more frequent in older persons of both sexes. We have previously postulated that HIV is likely to be transmitted by the sexual route, in a context of commercial logging and the extensive and complex sexual networks it induces (*4*). In contrast, the route of HCV transmission is unclear. HCV seropositivity was not associated with a history of blood transfusion, injections, surgery, scarification, or tattooing. Intravenous drug use was not investigated in our study but was likely to be infrequent. Although sexual transmission could not be ruled out, especially between regular partners, the shape of the seroprevalence curves and the lack of association with HIV infection, syphilis, or other sexually transmitted infections suggests that this mode of transmission is inefficient, in keeping with other reports (*5*,*6*). Our seroprevalence curves and the study location are consistent with the hypothesis that frequent iatrogenic transmission occurred during mass medical campaigns conducted before 1960 (*7*). The rate of HCV coinfection among the HIV-infected subjects in our study (8.6%) is much lower than the overall rate (25%–30%) in North America and Europe (*1*,*2*), where intravenous drug use is a major risk factor for both

infections (*8*,*9*). This rate was even in the lower range of values found among HIV-infected heterosexual persons in industrialized countries (9%–27%) (*2*). Our results therefore suggest that the high seroprevalence rates of HIV and HCV in Africa will not necessarily result in a high prevalence of HIV/HCV coinfection.

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Amebic Liver Abscess in HIV-infected Patients, Republic of Korea

To the Editor: Amebic liver abscess (ALA) is the most common extraintestinal complication of amebic infection. Although loss of cellular immunity is thought to play a role in infection by the pathogen, whether HIV infection is also a risk factor for invasive amebiasis is controversial (*1–3*). ALA in HIV-infected patients has not been well characterized, although several case series have been reported (*2*,*4*). We report the role of HIV infection status in ALA in an area where ALA is not endemic and the clinical features of ALA in HIV-infected patients.

All patients with ALA at Seoul National University Hospital (SNUH) from January 1990 through December 2005 were identified; some have been previously reported (*5*). SNUH is a 1,600-bed, university-affiliated teaching hospital and the largest referral center for HIV/AIDS in the Republic of Korea. The diagnostic criteria for ALA were radiologic evidence of intrahepatic abscess, trophozoites of *Entamoeba histolytica* in fluid aspirated from an abscess, or absence of bacteria and fungi in aspirated fluid and a titer ≥128 in an indirect hemagglutination assay (IHA) for *E. histolytica*.

Of 31 patients with ALA at SNUH from 1990 through 2005, 10 (32%) were HIV positive. The proportion of HIV-infected patients among patients with ALA increased significantly with time (linear-by-linear association, $p<0.001$) (Figure). Of 10 patients from 1998 through 2005, 8 (80%) were HIV positive. Except for 2 patients with a history of travel to an ALA-endemic area, 88% of the patients were HIV positive.

Median age of the 10 HIV-positive patients with ALA was 34.5 years (range 29–54 years); all patients were male. Four had a homosexual orientation, 4 had a heterosexual orientation, and 2 had an unknown sexual orientation. Fever (100%) was the most common symptom, and abdominal tenderness (90%) and diarrhea (50%) were frequently observed. Median leukocyte count was 9,000/mm^3 (range 3,410–16,700/mm^3), and median CD4 cell count was 279/mm^3 (range 40–370/mm^3). Eight patients had abscesses in the right lobe of the liver and 2 had abscesses in both lobes; 8 patients had 1 abscess and 2 had multiple abscesses. Median size of abscesses was 7.25 cm (range 3–12 cm). In 5 patients, pleural effusion was observed in chest radiographs. IHA titer was ≥128 in 10 patients and ≥512 in 8 patients. Median days to defervescence was 2 (range 1–5 days). In 2 patients, perforation of the abscess into the abdominal cavity was a complication. No patients died or had relapses.

Early in the AIDS pandemic, some studies reported that the prevalence of invasive amebiasis was not increased in patients with HIV infection (*1*,*6*). However, recent reports of ALA associated with HIV infection have increased. Studies in Taiwan demonstrated that invasive amebiasis, including ALA, is on the increase in HIV-infected patients in disease-endemic areas (*2*,*7*).

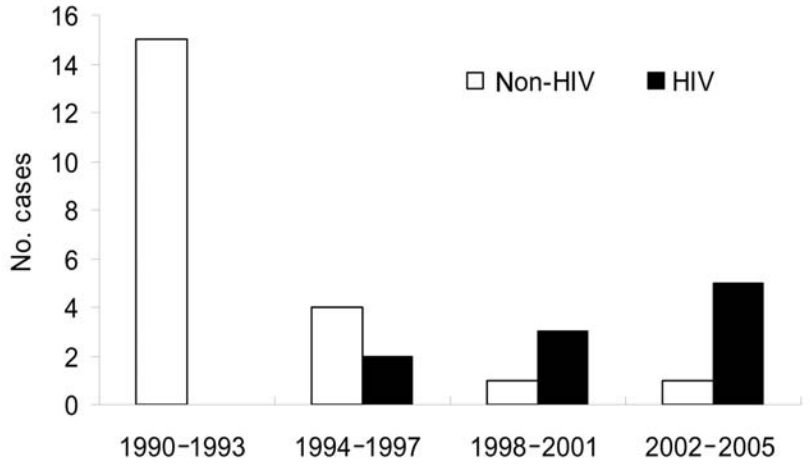

Figure. Number of cases of amebic liver abscess in patients with and without HIV infection at Seoul National University Hospital, Republic of Korea, 1990–2005.

Amebiasis was previously an endemic disease in the Republic of Korea. The positive rate for cysts of *E. histolytica*/*E. dispar* in the general population was 10% in the 1960s (*5*). However, with improvements in sanitation, this rate decreased to 0.5% in 1993 and to nearly 0% in 2004 (*8*). The present study showed that ALA in association with HIV infection is increasing in the Republic of Korea, and that ALA in HIV-negative patients has greatly decreased. In a study in the United States, 38% of patients with ALA with no history of travel to a disease-endemic area were HIV positive (*3*). These results support the view that ALA is an emerging parasite infection in HIV-infected patients in non–disease-endemic areas, as well as in disease-endemic areas.

Immune suppression is an important risk factor for ALA. In animal studies, immune suppression after thymectomy or splenectomy results in an increased incidence of ALA (*9*). Suppressed cellular immunity caused by use of steroids and malnourishment predispose to fatal amebiasis (*10*). In the present study, 90% of patients had CD4 cell counts $<350/mm^3$, which implies that immune suppression by HIV infection may be another risk factor for ALA.

Only 2 patients with pyogenic liver abscess were observed during the study. In these patients, pyogenic liver abscess was diagnosed, despite negative cultures for bacteria and fungi, because trophozoites of *E. histolytica* were not demonstrated in aspirated pus, and results of IHA for amebiasis were negative. However, we cannot exclude amebic liver abscess in these 2 patients because IHA test results can be negative in HIV-infected patients (*2*).

This study suggests that ALA is an emerging parasite infection in HIV-infected patients even in areas where the disease is not endemic. ALA should be considered in HIV-infected patients with space-occupying lesions in the liver, and HIV screening is strongly recommended in patients in areas where ALA is not endemic, especially those with no history of travel to a disease-endemic area.

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Pregnancy and Emerging Diseases

To the Editor: The November 2006 issue of Emerging Infectious Diseases featured 2 perspectives (*1*,*2*) that highlighted the need for strategies to prevent and treat pregnant patients during outbreaks of new or emerging diseases or during bioterrorist attacks. However, neither article discussed implications for a surveillance strategy.

Based on my previous experience at the World Health Organization (WHO) with the severe acute respiratory syndrome (SARS) outbreak, I propose several practical steps for such a strategy: l) systematic identification and reporting of cases in pregnant women, 2) estimation of the number of cases in pregnant women, 3) international clinical networks to share treatment and infection control experience, and 4) standard protocols for sharing clinical and treatment information between nations.

First, cases in pregnant patients should be systematically identified and reported during outbreaks, by including information on pregnancy status and duration of the pregnancy in case-report forms for new diseases. This is important for several reasons. First, case-patients that come to attention in an ad hoc fashion may provide a biased view of outcome, since those with a poor outcome are more likely to draw attention. Second, although pregnancy is not rare, the number of cases in pregnant women in outbreaks of new or emerging diseases in any 1 location may be too small for meaningful analysis.

Unfortunately, during the SARS epidemic, pregnancy status was not included on the international case-reporting forms. Although some countries systematically tested for and recorded pregnancy status, other countries did not. As a result, valuable information was lost, and outcomes for pregnant women could not be properly assessed.

Table. Pregnant patients with severe acute respiratory syndrome (SARS), by country

Country	Estimated no. pregnant SARS patients*	No. pregnant SARS patients reported in scientific literature†	No. SARS cases identified through informal sources‡
China	84§	5	0
Taiwan	6	0	2
Hong Kong	17	12	0
Singapore	5	0	4
Vietnam	1	0	1
Canada	4§	1	0
All other	2§	2¶	0
Total	119	20	7

*National estimate equal 3/4 of the sum over all 5-y age groups of the product of the number of female patients in the 5-y age group by the age-specific fertility rate for that age group.
†Cases were found by author through intensive scientific literature search.
‡Identified through World Health Organization meetings, conference calls and emails.
§The country-specific age-sex distribution of cases was not available for this country; the combined age-sex distribution from all available patients from other countries was used in this estimate. For this reason estimate for this country may be less precise.
¶Both patients were from the United States.

A rough estimate can be made of the number of pregnant women in a particular country likely to have a particular disease such as SARS. Assuming equal attack rates for pregnant and nonpregnant women, the number of pregnant women having a disease can be estimated as equal to three fourths of the sum over 5-year age groups of the product of the number of female patients in the 5-year age group by the age-specific fertility rate for that age group.[1,2,3] While the assumption of equal attack rates does not hold for all infectious diseases, it is a good starting point for a new disease about which there is little information.

Using this method for SARS resulted in an estimate of 119 cases in pregnant women (Table). For most countries, the estimated number of pregnant case-patients was reasonably close to the total number of pregnant case-patients that could be identified in the scientific literature through web searches (column 3) supplemented by cases identified through informal sources, such as emails and at WHO meetings and conference calls (column 4). China was an exception; 84 pregnant case-patients were estimated for China, but only 5 case-patients were identified, all from the same hospital in China.

These estimates have limitations. They do not consider subnational differences in fertility, such as differences for specific ethnic or occupational groups, or rural-urban differences. Nonetheless, they provide a ballpark figure that can be used to assess the extent of pregnancy-related cases.

The estimates were useful during the SARS outbreak in raising awareness of the issues surrounding pregnancy and SARS. As a result of such awareness, WHO formed a clinical network to share clinical experiences regarding treatment of pregnant patients as well as experiences with infection control during obstetrical procedures. Although this network was established rather late during the SARS outbreak, it did result in useful

[1]It is necessary to multiply by 3/4, because live births take 9 months, on average.

[2]The estimated number of pregnant women is based on expected number of live births, and the estimate does not take into consideration fetal loss, premature delivery, or multiple births.

[3]Estimates can be made by using an electronic spreadsheet. Age-specific fertility rates can be drawn from United Nations Population Division data available for all countries.

interchanges between nationals in different countries during conference calls.

The network discussed the establishment of a standardized database for sharing detailed clinical information on the course, outcome, and treatment of pregnant SARS patients. However, this database was never created. Although, fortunately, the scientific literature contains summaries of experiences with 20 case-patients, this is not a substitute for a systematic and reasonably complete database of experiences. To be sure that such a database is established for any new disease, protocols for data sharing should be prepared in advance, and all possible administrative barriers to sharing information should be addressed.

In conclusion, pregnant women are an important group at high risk for outbreaks of new diseases. This situation requires appropriate strategies for surveillance. I have identified several measures that I believe could be used in this regard.

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In response: We thank Ms. Anker for describing some of the surveillance challenges during the severe acute respiratory syndrome (SARS) pandemic and for supporting the idea that during an infectious disease outbreak or public health emergency, pregnant women are a population deserving special consideration (*1*). We also understand and appreciate the challenges she describes in systematically identifying and reporting pregnancies during a public health emergency response such as occurred with SARS. In the aftermath of Hurricane Katrina, we have recognized that it is critical that pregnancy information be collected systematically as part of the medical intake process. This can be accomplished by asking a simple, short question about pregnancy status (i.e.,"Are you or do you think you could be pregnant?") as women are being evaluated and processed as part of an emergency response plan. Urine pregnancy testing may be used to verify pregnancy status for women who are unsure (*2*). Besides helping tailor the provision of healthcare in these settings, pregnancy status information should also be used to track pregnancy outcomes.

We agree with Ms. Anker regarding the importance of pregnancy surveillance efforts. In June 2003 the Centers for Disease Control and Prevention (CDC) announced the first evidence of community-acquired monkeypox in the United States. Because this outbreak was linked to infected prairie dogs often kept as pets by small children, exposed household members included mothers, some of whom were pregnant. Unfortunately, although outbreaks of monkeypox in Africa had previously been well described, these descriptions did not include much information on the natural history of monkeypox among pregnant women (*3*). This example highlights the importance of ensuring that pregnancy information is systematically collected to plan and respond to future outbreaks.

In addition to surveillance of pregnant women in a known outbreak or emergency response, pregnant women should be included in efforts to detect novel disease threats. Unusual patterns of disease among pregnant women may be an early sign of emerging disease in the general population. In their role as frontline physicians, obstetrician-gynecologists and other healthcare providers who care for pregnant women may be among the first to encounter patients with novel infectious disease threats (*4*).

Finally, with Ms. Anker, we support coordinated international efforts to collect and monitor the pregnancy status of affected women during an outbreak or emergency response and are working on ways at CDC to prepare for future threats.

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Escherichia coli Cluster Evaluation

To the Editor: Gupta et al. raise important issues regarding molecular profiling as an epidemiologic tool (*1*). First, since all living organisms are related, the goal of genomic profiling in public health epidemiology is not really to determine "whether such isolates are truly related" (*1*) (they are), but to define the degree of similarity—or, more specifically, to determine whether isolates are sufficiently closely related that the probability of their deriving immediately from the same point source is high enough to warrant epidemiologic investigation. Second, definitive assessment of genetic similarity relationships is challenging because of the limited accuracy and resolving power of conventional methods such as pulsed-field gel electrophoresis (PFGE) analysis (*2*) and the impracticality and expense of better performing technologies. Sequential use of multiple methods (such as PFGE with additional restriction enzymes) will predictably detect additional differences, thereby improving resolving power (*2*). Third, even if genetic similarity could be precisely defined, the relationship between the degree of genetic similarity and the probability of point-source spread is unknown and doubtless varies in relation to pretest probability, depending on the epidemiologic context (e.g., localized vs. multistate clusters). Even <100% similarity may be compatible with point-source spread when genetic drift exists within the reservoir, leading to dissemination of highly similar but nonidentical clones.

Gupta et al. interpret their experience as indicating that, with geographically dispersed isolates, a higher degree of genomic similarity than is reliably provided by single-enzyme PFGE is necessary to improve specificity, thereby avoiding fruitless investigative efforts (*1*). However, whether the subclusters shown by their second-round PFGE were more epidemiologically meaningful than the original cluster remains unclear, nor do we know how representative this experience is. Determination of optimal genetic similarity parameters for geographically distributed epidemiologic surveillance (e.g., through PulseNet) would seem to require more in-depth empirical assessment, possibly incorporating Bayesian likelihood (*3*).

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Novel Hantavirus Sequences in Shrew, Guinea

To the Editor: Hantaviruses, family *Bunyaviridae*, have been known as causative agents of hemorrhagic fever with renal syndrome in Asia and Europe (*1,2*) and hantavirus cardiopulmonary syndrome in the Americas (*3*). Hantaviruses are spread by aerosolized rodent excreta and are strongly associated with their natural hosts, rodents of the family *Muridae*. Based on phylogenetic analyses, hantaviruses have been divided into 3 major groups that resemble 3 subfamilies of their natural hosts (Figure, panel A).

Recently, we found the first indigenous African hantavirus, Sangassou virus (SANGV), in an African wood mouse (*Hylomyscus simus*) collected in Guinea (*5*). Thottapalayam virus (TPMV), isolated from an Asian house shrew (*Suncus murinus*) in India (*6*), is the only known hantavirus to be hosted by a shrew instead of a rodent (*7,8*). We report the recovery of hantavirus RNA of a novel sequence from a shrew, collected in Guinea, West Africa.

During a study of rodentborne hemorrhagic fever viruses performed in Guinea in 2002–2004, 32 shrews of the genus *Crocidura* were collected and screened for hantavirus RNA by reverse transcription–PCR (*5*). An RNA sample designated Tan826 produced a PCR product of the expected size. The animal host was a male *Crocidura theresae* collected in the grassland savannah around the village Tanganya (10°00′02″N, 10°58′22″W) in January 2004. Species identification, following the taxonomic nomenclature (*9*), was performed on the basis of morpho-anatomical characteristics and was supported by molecular analyses.

Partial L segment sequence of 412 nt was determined by cloning and sequencing of the obtained PCR product. Nucleotide sequence comparisons between Tan826 and other representatives of the genus *Hantavirus* showed very low sequence identity values, ranging from 67.7% (Andes virus) to 72.3% (Puumala virus). Corresponding sequences of deduced viral RNA polymerase (137 aa) showed only slightly higher similarity values

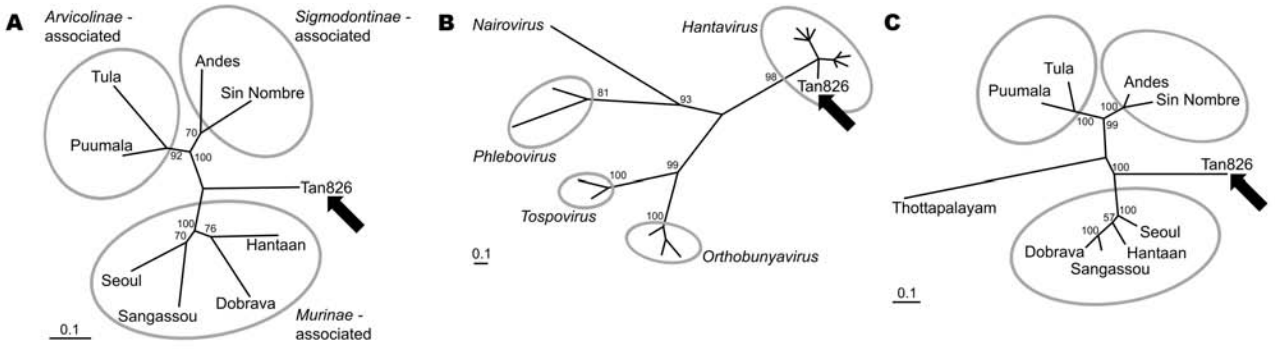

Figure. Maximum likelihood phylogenetic analysis of hantaviruses showing the phylogenetic placement of Tan826 (Tanganya virus, indicated by arrow) based on partial L segment nucleotide (A) and amino acid (B) sequences and partial S segment amino acid sequences (C); GenBank accession nos. EF050454 and EF050455, respectively. The values near the branches represent PUZZLE support values (*4*) calculated from 10,000 puzzling steps; only values ≥70% are shown. The scale bar indicates an evolutionary distance of 0.1 substitutions per position in the sequence. Gray ellipsoids indicate the 3 major hantavirus groups (panels A and C) or different genera of the *Bunyaviridae* family (panel B). A longer version of this caption providing a complete explanation of the analysis used is available online at www.cdc.gov/EID/content/13/3/520-G.htm

of 69.3% (Tula virus) to 76.6% (SANGV). In a maximum likelihood phylogenetic tree (Figure, panel A), Tan826 did not unambiguously cluster with any of the major groups (i.e., *Murinae*-, *Arvicolinae*-, *Sigmodontinae*-associated viruses) and showed equal relatedness to all 3 groups. This exceptional position of the Tan826 sequence within the tree is consistent with its detection in a shrew instead of a rodent host. Because the sequence is only distantly related to other hantaviruses, sequences from additional members of the *Bunyaviridae* family were analyzed. Despite use of a suboptimal dataset of very divergent and short sequences, the phylogenetic placement of Tan862 within the genus *Hantavirus* could be clearly demonstrated (Figure, panel B).

Furthermore, a partial S segment sequence (442 nt, 147 aa of the putative nucleoprotein) was determined to compare Tan826 directly with the shrew-associated TPMV (for which only an S segment sequence was available in GenBank). Rather unexpectedly, the Tan826 sequence showed the lowest similarity to TPMV: 47.5% on nt level and 39.4% on aa level. The identity values to other *Hantavirus* members were also extremely low, 52.2% (Sin Nombre virus) to 62.1% (SANGV) on nt level and 50.6% (Andes virus) to 56.7% (Hantaan, Dobrava virus) on aa level. Corresponding aa sequences were then used for phylogenetic analysis to reduce problems derived from higher sequence diversities. In the resulting evolutionary tree, Tan826 and TPMV did not join any of the 3 major groups but also did not cluster together (Figure, panel C).

Our attempts to obtain more sequence data were hampered by the unique nature of the Tan826 virus sequence, which makes it difficult to design additional effective PCR primers, as well as by the limited amount of available biological material from the shrew. Nevertheless, the sequence and phylogenetic analyses of the 2 partial sequences strongly indicate that they represent a novel hantavirus. The amino acid sequences are highly divergent (≈25%–50%) from those of other hantaviruses and in phylogenetic trees; the Tan826 virus sequence appeared approximately equally related to those of all other hantaviruses. We propose to name the putative new species Tanganya virus (TGNV), after the locality where it was detected.

Detecting the virus in 1 of 32 *Crocidura* shrews, 15 of them *C. theresae*, is not sufficient to define *C. theresae* as a reservoir animal of this novel virus. However, the unique position of TGNV in evolutionary trees supports the idea that a shrew instead of a rodent is the natural host of TGNV. Therefore, it is rather surprising that TGNV did not form a monophyletic group with TPMV. Before this observation becomes either a challenge or support for the hantavirus–host coevolution concept, more extensive sequence data (for comprehensive phylogenetic analysis) and epizootiologic studies (to confirm the natural hosts of both viruses) are necessary.

TGNV represents, after the recently described SANGV (*5*), a second hantavirus from Africa. Its low sequence similarity to other hantaviruses should make this virus serologically distinct from other hantaviruses, as shown for TPMV (*10*). Therefore, human infections by TGNV might be missed when using antibody detection assays based on antigens from conventional hantaviruses.

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Correction, Vol. 13, No. 1

In "Parvoviruses PARV4/5 in Hepatitis C Virus-infected Persons," by J. F. Fryer et al., an error occurred. The originally published title incorrectly included the word "Patient." The correct title is "Parvoviruses PARV4/5 in Hepatitis C Virus-infected Persons."

The updated article is available at http://www.cdc.gov/ncidod/EID/13/1/175.htm

We regret any confusion this error may have caused.

Correction, Vol. 13, No. 2

In "Avian Influenza Risk Perception, Europe and Asia," by Onno de Zwart et al., an error occurred. In the abstract, the last sentence should state, "Risk perceptions were higher in Europe than in Asia; efficacy beliefs were lower in Europe than in Asia."

The corrected abstract appears in the updated article, available at http://www.cdc.gov/EID/content/13/2/290.htm

We regret any confusion this error may have caused.

Biodefense: Research Methodology and Animal Models

James R. Swearengen, editor

CRC, Taylor & Francis, Boca Raton, Florida, 2006
ISBN: 9780849328367
Pages: 331; Price: US $139.95

Since the terrorist attacks of September 11, 2001, biodefense research has been increasingly emphasized. Biodefense: Research Methodology and Animal Models, edited by James R. Swearengen, is a timely and invaluable reference for those performing animal experimentation to develop medical countermeasures and diagnostics against infectious agents and toxins identified as potential biological weapons. Those persons not involved in this field of study who want to educate themselves on biodefense research will also find it interesting and informative. It clearly and concisely provides extensive details about the animal models, both past and present, that have been used to investigate a selected number of disease processes caused by exposure to plausible biological threat agents.

The book is exceptionally well written and furnishes a wealth of information from world-renowned scientists who spearheaded infectious disease research at the United States Army Research Institute of Infectious Diseases. This reference will equip researchers with pertinent information regarding current animal models, so that future work will not repeat experiments already performed, while at the same time minimizing the number of animals projected for future biodefense studies. Most chapters are devoted to a specific biological agent or toxin and supply interesting historical information as well as descriptions about the pathogenesis in humans and in animal models. Specifically, this reference discusses in detail the bacterial agents that cause anthrax, glanders, plague, tularemia, and Q fever; the viral agents that result in Venezuelan and Eastern and Western encephalitis, poxviruses and hemorrhagic fever viruses; and finally, toxin- and superantigen-induced diseases caused by botulinum toxins, ricin, and staphylococcal and streptococcal bacteria. An entire chapter is devoted to the challenges associated with aerobiology. The information in this chapter is well organized and completely outlines the topic, to include transporting and caring for aerosol-challenged animals. The authors adeptly compare the different animal models used and provide reasons why 1 animal model is preferred to another. Biodefense: Research Methodology and Animal Models will certainly benefit scientists designing aerobiology studies or those exploring the infectious agents and toxins discussed in this book. All research laboratories focused on biodefense investigation should seriously consider adding this text to their reference library.

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Francisco Roa (b. 1963). Sands Flowers (1994). (detail) Oil on panel (45 cm x 36 cm). Oglethorpe University Museum, Atlanta, Georgia, USA

The Way Forward Is the Way Back

– Herakleitos of Ephesus, c. 500 BCE

Polyxeni Potter*

"The viewer should see the object as I saw it, but with a lot of room for his own interpretation….The painting is like a very clear mirror which is barely visible but unmistakably there," reflects Spanish painter Antonio López García (b. 1936) (*1*). "I am nostalgic for an art of our times in which a greater number of people can participate" (*1*). Part of a contemporary movement rooted in the traditions of 17th-century Spanish realism, López García, Claudio Bravo, Bernando Torrens, Francisco Roa, and many others have drawn inspiration from the meticulous work of Francisco de Zurbarán, Jusepe de Ribera, and Diego Velásquez to create their own naturalist style (*2*).

Rejecting abstraction, these artists create paintings from a broad range of subjects, choosing, much like their predecessors, a few or even one subject, which they lavish with extreme seriousness and attention, focusing on the "credible detail, that small touch of the familiar," that has long been part of the repertory of Spanish painters (*3*). The characteristic high technical quality is not an end in itself, and the work reaches far beyond mere representation of nature.

In the United States, revival of traditional painting skills in the latter part of the 20th century is credited to professor Richard Lack, who in his essay On the Training of Painters (1969) envisioned atelier training in the contemporary art scene, "Indeed if Western Civilization wishes to retain the art of painting as a living part of its culture, this may be our last hope." He founded the pioneering Atelier Lack in Minneapolis, "a small island of traditional art training surrounded by a sea of hostile opinion" (*4*). Rigorous technique, which had fallen by the wayside in modern times, addressed quality of drawing, color plausibility, truthfulness of light and shadow, highly developed skills of execution, and overall faithful depiction of nature.

The atelier attracted students from all over the world and became the model for similar schools, among them The New York Academy of Art and the Charles H. Cecil Studios in Florence, Italy. The New York Academy of Art won the support of Andy Warhol: "The course of art history," he said, "would be changed if one thousand students could be taught Old Master drawing and painting techniques" (*5*).

In 1982, Lack coined the term "Classical Realism" because as he put it, "Any 20th century painting that suggests a recognizable object, however crudely or childishly rendered, qualifies as 'realistic.' Obviously, the simple word 'realism,' when applied to painting…is no longer meaningful" (*6*). But contemporary American realists are a disparate group, loosely characterized by a realistic approach to representation, which has persisted widely in the post-abstraction era.

A painter in the tradition of Spanish realism, Francisco Roa was born in Guadalajara, Spain, and moved to Madrid at age 18 to study at the

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Universidad Complutense and the Academia Peña. He has received considerable recognition at home, exhibiting in Madrid, Barcelona, and Granada. In 1993, he took his works to Lisbon and Miami, and a year later, to New York and Atlanta.

Extremely careful with detail, Roa, like many of his contemporaries, conveys an individual perception of reality, positioning everyday objects deliberately, proposing his own space and time. Sands Flowers, on this month's cover, first exhibited in the United States in 1994, is a characteristic still life.

Against a vacant background vaguely reminiscent of sand, the artist composes a geometric ensemble to anchor his main object of interest, a glass vessel filled with natural elements past their prime. The board base, stacked on the left, breaks up the horizontal field. The metal circle, weathered and discolored, is slightly off center, the backdrop deliberately smudged. Inside the glass lie red petals, crinkled and lifeless, the detritus of beauty. Crammed in near the top is a hornet's nest, and out of each side, dried blooms jut pathetically, their stems distorted and petals curled.

"All forms of beauty, like all possible phenomena," wrote Charles Baudelaire in his On the Heroism of Modern Life, "contain an element of the eternal and an element of the transitory—of the absolute and the particular" (*7*). In a perfect balance of feeling and form, Roa's poignant scene meets Baudelaire's requirement. For nothing expresses the ephemeral better than flowers. Roses or sand verbenas, they perish all too soon, a metaphor for human transience and fragility.

The eternal and the absolute are elements artists have sought in the formalities of realism and the fragments of abstraction, on the same pathway, one step forward, two steps back; and scientists likewise, for rarely does excellent science or art occur without reference to past knowledge and principle.

In Sands Flowers, Roa probes the precariousness of existence, space and time, life and death. His realistic representation of natural objects in decline provokes speculation—not only on the passage of time and inevitability of death, but for us, also on disease, which unduly hastens the process. In an unending circle, old scourges become new, among them tuberculosis, a hornet's nest of multiple drug resistance, and now extensive resistance to second-line drugs, raising the specter of potentially untreatable disease. Roa sought essence in the staying power of exacting detail. In science too, on the same pathway, sometimes solutions lie simply in the core values: treatment standards, effective precautions, improved technology, better medicines, vaccines, and diagnostic tests (*8*).

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the April issue for the following topics:

Zoonosis Control and Human Benefits

Hantavirus and Arenavirus Antibodies and Occupational Rodent Exposure, North America

Diagnosis of Tuberculosis by Enzyme-linked Immunospot Assay for Interferon-γ

Flinders Island Spotted Fever Rickettsioses Caused by *Rickettsia honei*, Eastern Australia

Symptomatic and Subclinical Infection with Rotavirus P[8]G9, Rural Ecuador

Effectiveness of Interventions to Reduce Contact Rates during Simulated Influenza Pandemic

Spanish Influenza in Japanese Armed Forces, 1918–1920

West Nile Virus Stasis, Houston, Texas, USA

Bluetongue in Belgium, 2006

Influenza Vaccine Effectiveness among US Military Trainees

Cryptosporidiosis Decline after Regulation, England and Wales, 1989–2005

MRSA Transmission between Cows and Humans

Autochthonous Transmission of *Trypanosoma cruzi*, Louisiana

Complete list of articles in the April issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

March 7–9, 2007
6th International Symposium on Antimicrobial Agents and Resistance (ISAAR 2007)
Raffles City Convention Centre
Singapore
http://www.isaar.org

March 20–23, 2007
ISOPOL XVI: 16th International Symposium on Problems of Listeriosis
Marriott Riverfront Hotel
Savannah, GA, USA
Contact: 240-485-2776
http://www.aphl.org/conferences/ISOPOL.cfm

April 30–May 2, 2007
10th Annual Conference on Vaccine Research
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
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May 31–June 1, 2007
2nd International Conference on Avian Influenza in Humans: Recent Developments and Perspectives
Institut Pasteur
Paris, France
http://www.isanh.com/avian-influenza

July 16–18, 2007
Public Health Congress
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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.