EMERGING INFECTIOUS DISEASES

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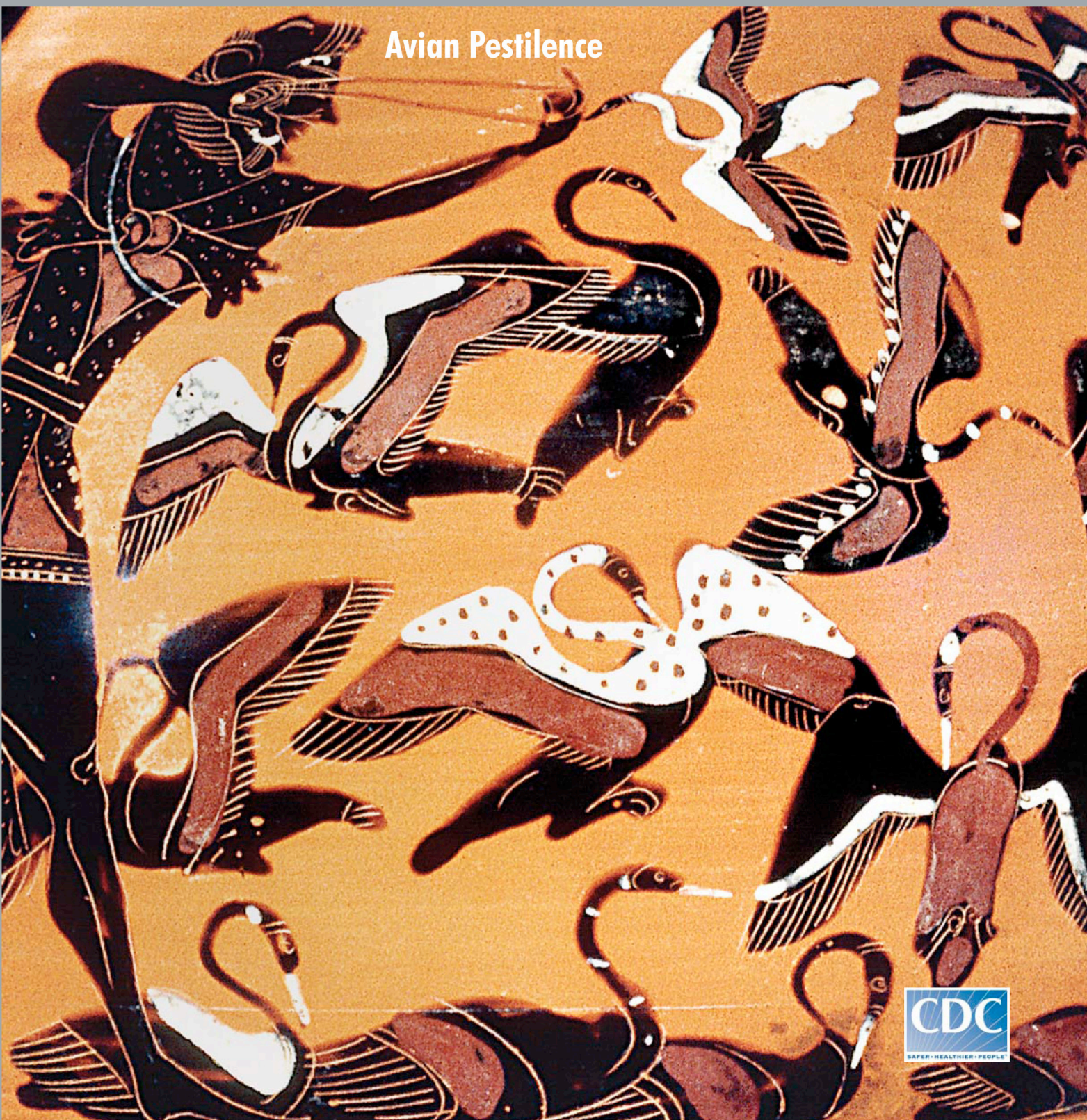
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Vol.11, No.8, August 2005



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Stymphalian Birds
(circa 6th century BC)

Athenian black-figured
amphora (40.6 cm)
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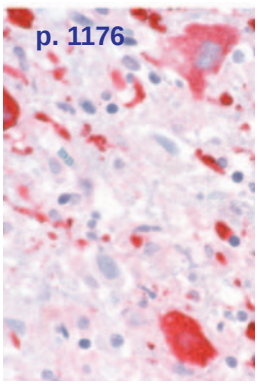

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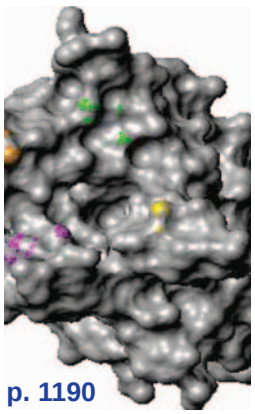

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Epidemiology and Transmission Dynamics of West Nile Virus Disease

Edward B. Hayes,* Nicholas Komar,* Roger S. Nasci,* Susan P. Montgomery,* Daniel R. O'Leary,* and Grant L. Campbell*

From 1937 until 1999, West Nile virus (WNV) garnered scant medical attention as the cause of febrile illness and sporadic encephalitis in parts of Africa, Asia, and Europe. After the surprising detection of WNV in New York City in 1999, the virus has spread dramatically westward across the United States, southward into Central America and the Caribbean, and northward into Canada, resulting in the largest epidemics of neuroinvasive WNV disease ever reported. From 1999 to 2004, >7,000 neuroinvasive WNV disease cases were reported in the United States. In 2002, WNV transmission through blood transfusion and organ transplantation was described for the first time, intrauterine transmission was first documented, and possible transmission through breastfeeding was reported. This review highlights new information regarding the epidemiology and dynamics of WNV transmission, providing a new platform for further research into preventing and controlling WNV disease.

West Nile virus (WNV) was first detected in the Western Hemisphere in 1999 during an outbreak of encephalitis in New York City. Over the next 5 years, the virus spread across the continental United States as well as north into Canada, and southward into the Caribbean Islands and Latin America (1). This article highlights new information about the epidemiology and transmission dynamics of human WNV disease obtained over the past 5 years of intensified research.

Epidemiology

WNV is transmitted primarily by the bite of infected mosquitoes that acquire the virus by feeding on infected birds. The intensity of transmission to humans is dependent on abundance and feeding patterns of infected mosquitoes and on local ecology and behavior that influence human exposure to mosquitoes. Although up to 55% of affected populations became infected during epidemics in Africa, more recent outbreaks in Europe and North America have yielded much lower attack rates (1,2). In the area of most intense WNV transmission in Queens, New York, in 1999, ≈2.6% of residents were infected (most of these were asymptomatic infections), and similarly low prevalence of infection has been seen in other areas of the United States (3,4). WNV outbreaks in Europe and the Middle East since 1995 appear to have caused infection in <5% of affected populations (1,5). These levels of infection are too low to decrease the frequency of epidemics or modulate their intensity through protective immunity.

Data on the incidence of WNV in most of the world are not readily available. WNV transmission has been reported in Europe, the Middle East, Africa, India, parts of Asia, Australia (in the form of Kunjin virus, a subtype of WNV), North America, and parts of Central America and the Caribbean (1,6). In recent years human WNV disease in the Eastern Hemisphere has been reported mostly from areas in the Mediterranean Basin: in Algeria in 1994, Morocco in 1996, Tunisia in 1997 and 2003, Romania in 1996 through 2000, the Czech Republic in 1997, Israel in 1999 and 2000, Russia in 1999 through 2001, and France in 2003 (1,6,7). Enzootics involving horses were reported in Morocco in 1996 and 2003, Italy in 1998, Israel in 2000, and southern France in 2000, 2003, and 2004 (6–8).

In the Western Hemisphere, most human WNV disease has occurred in the United States. Since the virus was detected in New York from 1999 through 2004, 16,706 cases have been reported to the Centers for Disease Control and Prevention (CDC); 7,096 of these were classified as neuroinvasive disease, 9,268 as West Nile fever

*Centers for Disease Control and Prevention, Fort Collins, Colorado, USA

(WNF), and 342 had other or unspecified clinical presentation (reported through June 8, 2005; the proportion of total cases reported that are neuroinvasive disease is artificially higher than what is believed to occur naturally since neuroinvasive disease is more likely to be reported than WNF or asymptomatic infection) (Table 1). Transmission of WNV has spread dramatically from New York to the north, south, and west (Figure 1). From 2002 to 2003, the most intense transmission shifted from the Midwest and south-central states to the western plains and Front Range of the Rocky Mountains. In 2004, most WNV disease cases were reported in California, Arizona, and western Colorado, but foci of highest incidence were scattered across the United States (Figure 1). In the East, WNV transmission recurred for 6 consecutive years with the highest number of human disease cases reported in 2003, indicating that WNV disease has become seasonally endemic. In Canada, transmission of WNV to humans has been documented in Quebec, Ontario, Manitoba, Saskatchewan, and Alberta, and WNV-infected birds have also been found in New Brunswick and Nova Scotia (http://www.phac-aspc.gc.ca/wnv-vwn). Evidence of WNV transmission has been reported from the Cayman Islands, Jamaica, Dominican Republic, Mexico, Guadeloupe, El Salvador, Belize, Puerto Rico, and Cuba, but only 1 human case has been reported from Mexico and 1 from the Cayman Islands (http://www.paho.org/English/DD/PIN/ptoday15_oct03.htm; www.paho.org/English/AD/DPC/CD/wnv.htm; http://www.cenave.gob.mx/von/default.asp; http://www.serc.si.edu/labs/avian/wnv.jsp) (1). The paucity of human cases thus far in Latin America and the Caribbean is surprising, considering the ecologic conditions that favor arbovirus transmission in these areas. WNV isolated from a bird in Mexico in 2003 appeared to be attenuated, but whether viral mutation accounts for the scarcity of human disease remains to be seen (9).

The incidence of WNV disease is seasonal in the temperate zones of North America, Europe, and the Mediterranean Basin, with peak activity from July through October (6,10). In the United States, the transmission season has lengthened as the virus has moved south; in 2003, onset of human illness began as late as December, and in 2004 as early as April (CDC, unpub. data). Transmission of WNV in southern Africa and of Kunjin virus in Australia increases in the early months of the year after heavy spring and summer rainfall (2,11).

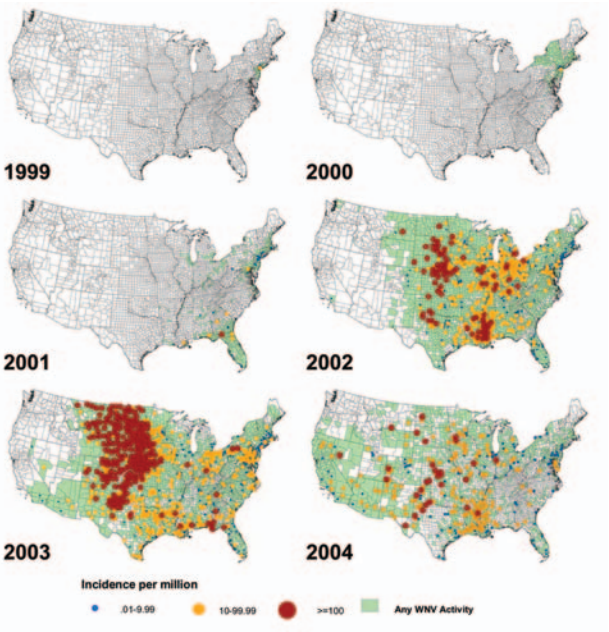

Figure 1. Reported incidence of neuroinvasive West Nile virus disease by county, United States, 1999–2004. Reported to Centers for Disease Control and Prevention by states through April 21, 2005.

In the United States, persons of all ages appear to be equally susceptible to WNV infection, but the incidence of neuroinvasive WNV disease and death increases with age, especially among those 60 to 89 years of age, and is slightly higher among male patients (Figure 2) (10). During 2002, the median age among neuroinvasive disease cases was 64 years (range 1 month to 99 years), compared to a median age of 49 years (range 1–97 years) for WNF cases (10). Of the 2,942 neuroinvasive disease cases, 276 (9%) were fatal (10). Although severe disease occurs primarily in adults, neuroinvasive disease in children has been reported. From 2002 through 2004, 1,051 WNV disease cases among children <19 years of age were reported in the United States; 317 (30%) had neuroinvasive disease; and

Table 1. Human West Nile virus disease cases by clinical syndrome, United States, 1999–2004*

Year	Total cases	Neuroinvasive cases	West Nile fever cases	Other clinical /unspecified	Deaths
1999	62	59	3	0	7
2000	21	19	2	0	2
2001	66	64	2	0	9
2002	4,156	2,946	1,162	48	284
2003	9,862	2,866	6,830	166	264
2004*	2,539	1,142	1,269	128	100
Total	16,706	7,096	9,268	342	666

*Reported to the Centers for Disease Control and Prevention as of June 8, 2005.

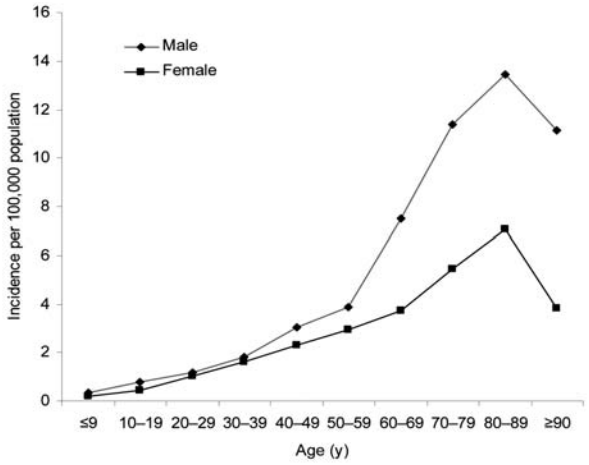

Figure 2. Reported incidence of neuroinvasive West Nile virus disease by age group and sex, United States, 1999–2004. Reported to the Centers for Disease Control and Prevention by states through April 14, 2005.

106 (34%) of these were <10 years (CDC, unpub. data; reported through June 8, 2005). Two (0.6%) pediatric patients with neuroinvasive WNV disease died: an infant with underlying lissencephaly and a 14-year-old boy with immune dysfunction.

The most important risk factor for acquiring WNV infection is exposure to infected mosquitoes. In Romania the risk for WNV infection was higher among persons with mosquitoes in their homes and with flooded basements (12). An analysis of the locations of WNV disease cases during the 1999 outbreak in New York found that cases were clustered in an area with higher vegetation cover, indicating favorable mosquito habitat (13). A study of the outbreak in Chicago in 2002 indicated that human disease cases tended to occur in areas with more vegetation, older housing, lower population density, predominance of older Caucasian residents, and proximity to dead birds, but the effects of these variables were influenced by differences in mosquito abatement efforts (14). Risk factors for infection not related to mosquito exposure include receiving blood transfusions or organ donations, maternal infection during pregnancy or breastfeeding, and occupational exposure to the virus (15–17).

Apart from older age and immunosuppression after organ transplantation, the risk factors for the development of severe neuroinvasive WNV disease have yet to be determined (10,16). Underlying hypertension, cerebrovascular disease, and diabetes have been considered as possible predisposing factors; further study may elucidate the role of these or other host factors that might modify the risk for severe disease or death (12). Genetic predisposition for severe disease has been described in mice but has not yet been elucidated in humans (18). The role of innate and adaptive immune responses in determining outcome deserves further study.

Nonmosquitoborne WNV Transmission

In 2002, intrauterine WNV transmission was documented for the first time (15). A 20-year-old woman had onset of WNV disease in week 27 of gestation. Her infant was born at term with chorioretinitis and cystic damage of cerebral tissue. Intensified surveillance identified 4 other mothers who had WNV illness during pregnancy, 3 of whom delivered infants with no evidence of WNV infection; all 3 infants appeared normal at birth and at 6 months of age (15). The fourth woman delivered prematurely; her infant had neonatal respiratory distress but was not tested for WNV infection. In 2003, CDC received reports of 74 women infected with WNV during pregnancy; most of these women followed up to date have delivered apparently healthy infants (CDC, unpub. data).

Probable WNV transmission through breast milk was also reported in 2002 (15). A 40-year-old woman acquired WNV infection from blood transfused shortly after she delivered a healthy infant. WNV nucleic acid was detected in her breast milk, and immunoglobulin (Ig) M antibody was found in her infant, who remained healthy. No other instances of possible WNV transmission through breast milk have been reported. Until more data are available, and because the benefits of breastfeeding are well documented, mothers should be encouraged to breastfeed even in areas of ongoing WNV transmission.

Transmission of WNV through blood transfusion was first documented during the 2002 WNV epidemic in North America (15). In June 2003, blood collection agencies in the United States and Canada enhanced donor deferral and began screening blood donations with experimental nucleic acid amplification tests. During 2003 and 2004, >1,000 potentially WNV-viremic blood donations were identified, and the corresponding blood components were sequestered. Nevertheless, 6 WNV cases due to transfusion were documented in 2003, and at least 1 was documented in 2004, indicating that infectious blood components with low concentrations of WNV may escape current screening tests (19). One instance of possible WNV transmission through dialysis has been reported (20).

WNV transmission through organ transplantation was also first described during the 2002 epidemic (15). Chronically immunosuppressed organ transplant patients appear to have an increased risk for severe WNV disease, even after mosquito-acquired infection (16). During 2002, the estimated risk of neuroinvasive WNV disease in solid organ transplant patients in Toronto, Canada, was approximately 40 times greater than in the general population (16). Whether other immunosuppressed or immunocompromised patients are at increased risk for severe WNV

disease is uncertain, but severe WNV disease has been described among immunocompromised patients.

WNV infection has been occupationally acquired by laboratory workers through percutaneous inoculation and possibly through aerosol exposure (21,22). An outbreak of WNV disease among turkey handlers at a turkey farm raised the possibility of aerosol exposure (17).

Dynamics of Transmission: Vectors

WNV is transmitted primarily by *Culex* mosquitoes, but other genera may also be vectors (23). In Europe and Africa, the principal vectors are *Cx. pipiens*, *Cx. univittatus*, and *Cx. antennatus*, and in India, species of the *Cx. vishnui* complex (6,24). In Australia, Kunjin virus is transmitted primarily by *Cx. annulirostris* (11). In North America, WNV has been found in 59 different mosquito species with diverse ecology and behavior; however, <10 of these are considered to be principal WNV vectors (CDC, unpub. data) (23,25,26). In 2001, 57% of the positive mosquito pools in the Northeast were *Cx. pipiens*, the northern house mosquito, a moderately efficient vector that feeds on birds and mammals (Table 2). In 2002, *Cx. pipiens* made up more than half of the WNV-positive pools, but *Cx. quinquefasciatus*, the southern house mosquito, generally considered a moderate- to low-efficiency vector, appeared to be the predominant vector in the South. *Cx. tarsalis*, 1 of the most efficient WNV vectors evaluated in laboratory studies, was the predominant vector west of the Mississippi River (CDC, unpub. data) (26).

During 2003, as WNV activity progressed westward, *Cx. tarsalis* became the most commonly reported WNV-positive mosquito species, making up 32% of the positive pools reported, followed by *Cx. pipiens*, *Cx. quinquefasciatus*, and *Cx. restuans* (Table 2). *Cx. salinarius* and *Cx. nigripalpus* may be important vectors in areas where they are abundant (26). During 2004, when large epidemics occurred in the southwestern United States, the most commonly reported WNV-positive species was *Cx. quinquefasciatus*, which made up over half of the positive pools, followed by *Cx. tarsalis* and *Cx. pipiens* (Table 2).

The intensity of WNV transmission is determined primarily by the abundance of competent mosquitoes and the prevalence of infection in mosquitoes. The estimated prevalence of infection, measured as the minimum infection rate (MIR), that is needed to produce epidemics is uncertain. Toward the end of the 1999 New York epidemic, the WNV MIR for all *Culex* mosquitoes sampled in the area was 0.3% with MIRs of individual collections, ranging from 0.07% to 5.7% (27). During the 2000 Staten Island epidemic, the MIRs in mixed *Cx. pipiens/restuans* pools ranged from 0.5% to 1.6% and the MIR in *Cx. salinarius* from 0.3% to 1.2% (28). Relatively low MIRs in *Cx. restuans* (0.2%), *Cx. pipiens* (0.1%) and *Cx. salinarius* (0.1%) in Connecticut during 2000 were associated with an intense epizootic, but apparently a low risk for humans (29). In 2001, moderate to high MIRs in *Cx. quinquefasciatus* (0.5%) and *Cx. nigripalpus* (1.1%) were associated with epizootic and epidemic transmission in Florida (30). In some North American outbreaks, MIRs as high as 15% have been observed (CDC, unpub. data). Vertical transmission of WNV has been experimentally demonstrated in *Cx. pipiens*, *Cx. quinquefasciatus*, and *Cx. tarsalis*, and the virus has been isolated from hibernating female mosquitoes, which may provide a mechanism for persistence of the virus in colder latitudes through the winter and reemergence of transmission in the spring (31,32).

Although both soft and hard ticks can become infected with WNV, they are unlikely to play a substantial role in WNV transmission. In the laboratory, *Argas arboreus* ticks transmitted WNV to chickens, and *Ornithodoros savignyi*, *O. maritimus*, *O. erraticus*, and *O. moubata* transmitted WNV to mice (33). However, of the hard ticks *Amblyomma americanum*, *Ixodes scapularis*, *I. ricinus*, *Dermacentor variabilis*, and *D. andersoni*, the last 4

Table 2. West Nile virus (WNV)–positive mosquito pools, by species, United States, 2001–2004*

2001		2002		2003		2004 (through 11/30/2004)	
Positive pools, n = 612†		Positive pools, n = 3,720†		Positive pools, n = 5,538†		Positive pools, n = 4,755†	
Species	% of pools	Species	% of pools	Species	% of pools	Species	% of pools
Culex pipiens	57.0	*Cx. pipiens*	47.0	*Cx. tarsalis*	31.5	*Cx. quinquefasciatus*	51.4
Cx. restuans	12.4	*Cx. quinquefasciatus*	19.1	*Cx. pipiens*	20.8	*Cx. tarsalis*	20.4
Cx. salinarius	11.4	*Cx. restuans*	9.1	*Cx. quinquefasciatus*	19.1	*Cx. pipiens*	12.7
Culiseta melanura	4.2	*Cx. tarsalis*	7.6	*Cx. restuans*	15.3	*Cx. restuans*	4.4
Cx. quinquefasciatus	2.1	*Cx. salinarius*	3.6	*Cx. salinarius*	4.5	*Cx. erythrothorax*	3.6
Ochlerotatus. triseriatus	2.1	*Aedes albopictus*	2.0	*Ae. vexans*	2.3		
21 other species‡	10.6	23 other species‡	11.5	35 other species‡	6.4	35 other species‡	7.5

*Data were derived from reports submitted by state health departments to the Centers for Disease Control and Prevention's Arbonet surveillance system. Mosquito specimens were collected, identified, and tested in the respective state surveillance systems. Pools were reported as positive if they contained detectable levels of one of the following: infectious WNV, WNV RNA, WN viral antigen.
†Includes only WNV-positive pools reported as monospecific, i.e., excludes mixed pools (e.g., *Cx. pipiens/restuans*) or pools identified only to genus (e.g., *Culex* species).
‡No other species individually comprised ≥2.0% of the WNV-positive pools.

species became infected with WNV, but none transmitted the virus by subsequent bite (33,34).

Dynamics of Transmission: Vertebrate Hosts

Laboratory studies have demonstrated that 74%–100% of *Cx. tarsalis* mosquitoes become infected after consuming blood meals with WNV concentrations of $10^{7.1}$ plaque-forming units (PFU)/mL, while only 0%–36% become infected after consuming a meal containing $10^{4.9}$ PFU/mL (35). The maximum estimated concentration of WNV in human blood tested during screening of blood donors in 2002 was approximately $10^{3.2}$ PFU/mL (S. Stramer, M. Busch, M. Strong, pers. comm.). Thus, it appears unlikely that humans exhibit WNV viremia levels of sufficient magnitude to infect mosquitoes.

Birds are presumed to be the most important amplifying hosts of WNV. In laboratory studies, species in the orders Passeriformes (song birds), Charadriiformes (shorebirds), Strigiformes (owls), and Falconiformes (hawks) developed viremia levels sufficient to infect most feeding mosquitoes, whereas species of Columbiformes (pigeons), Piciformes (woodpeckers), and Anseriformes (ducks) did not (23,36). Certain passerines, including common grackles (*Quiscalus quiscula*), various corvids (crows, jays, magpies), house finches (*Carpodacus mexicanus*), and house sparrows (*Passer domesticus*) were highly infectious to mosquitoes and had mortality rates >40%. Field studies during and after WNV outbreaks in several areas of the United States have confirmed that house sparrows were abundant and frequently infected with WNV, characteristics that would allow them to serve as important amplifying hosts (23,25,37). The importance of birds in dispersing WNV remains speculative. Local movements of resident, nonmigratory birds and long-range travel of migratory birds may both contribute to the spread of WNV (38,39).

Although WNV was isolated from rodents in Nigeria and a bat in India, most mammals do not appear to generate viremia levels of sufficient titer to contribute to transmission (24,40–42). Three reptilian and 1 amphibian species (red-ear slider, garter snake, green iguana, and North American bullfrog) were found to be incompetent as amplifying hosts of a North American WNV strain, and no signs of illness developed in these animals (43). Viremia levels of sufficient titer to infect mosquitoes were found after experimental infection of young alligators (*Alligator mississippiensis*) (44). In Russia, the lake frog (*Rana ridibunda*) appears to be a competent reservoir (45).

Nonmosquitoborne WNV transmission has been observed or strongly suspected among farmed alligators, domestic turkeys in Wisconsin, and domestic geese in Canada (17,46,47). Transmission through close contact has been confirmed in both birds and alligators in laboratory conditions but has yet to be documented in wild vertebrate populations (23,36,44).

Control of WNV Transmission

Avoiding human exposure to WNV-infected mosquitoes remains the cornerstone for preventing WNV disease. Source reduction, application of larvicides, and targeted spraying of pesticides to kill adult mosquitoes can reduce the abundance of mosquitoes, but demonstrating their impact on the incidence of human WNV disease is challenging because of the difficulty in accounting for all determinants of mosquito abundance and human exposure. One study indicated that clustering of human WNV disease in Chicago varied between mosquito abatement districts, suggesting that mosquito control may have some impact on transmission to humans (14).

Persons in WNV-endemic areas should wear insect repellent on skin and clothes when exposed to mosquitoes and avoid being outdoors during dusk to dawn when mosquito vectors of WNV are abundant. Of insect repellents recommended for use on skin, those containing *N,N*-diethyl-m-toluamide (DEET), picaridin (KBR-3023), or oil of lemon eucalyptus (*p*-menthane-3,8 diol) provide long-lasting protection (48). Both DEET and permethrin provide effective protection against mosquitoes when applied to clothing. Persons' willingness to use DEET as a repellent appears to be influenced primarily by their level of concern about being bitten by mosquitoes and by their concern that DEET may be harmful to health, despite its good safety record (49).

To prevent transmission of WNV through blood transfusion, blood donations in WNV-endemic areas should be screened by using nucleic acid amplification tests. Screening of organ donors for WNV infection has not been universally implemented because of concern about rejecting essential organs after false-positive screening results (50). Pregnant women should avoid exposure to mosquito bites to reduce the risk for intrauterine WNV transmission.

Future Directions

WNV disease will likely continue to be a public health concern for the foreseeable future; the virus has become established in a broad range of ecologic settings and is transmitted by a relatively large number of mosquito species. WNV will also likely continue to spread into Central and South America, but the public health implications of this spread remain uncertain. Observations thus far in North America indicate that circulation of other flaviviruses, such as dengue, viral mutation, and differing ecologic conditions may yield different clinical manifestations and transmission dynamics. Over the next few years, research efforts might well be focused in several areas.

Research into new methods to reduce human exposure to mosquitoes is crucial and can help prevent other mosquitoborne illnesses. This should include development of new methods to reduce mosquito abundance, development of new repellents, and behavioral research to enhance the use of existing effective repellents and other personal protective measures against mosquito bites. A better understanding of the dynamics of nonmosquitoborne transmission is essential to prevent disease among infants of infected mothers and recipients of blood transfusions and transplanted organs. Currently available prevention strategies such as the dissemination of knowledge and products for personal protection from mosquito exposure and the application of existing techniques for reducing mosquito abundance in communities at risk of WNV transmission need to be vigorously implemented. National and international surveillance for WNV transmission will be important to monitor spread of the virus and the effect of control strategies. Finally, further research into the ecologic determinants of WNV transmission, including climatic factors and dynamics of reservoir and vector populations, could help in determining geographic areas of higher risk for WNV disease.

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Past Issues on West Nile Virus

Virology, Pathology, and Clinical Manifestations of West Nile Virus Disease

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West Nile virus (WNV) causes epidemics of febrile illness, meningitis, encephalitis, and flaccid paralysis. Since it was first detected in New York City in 1999, and through 2004, >16,000 WNV disease cases have been reported in the United States. Over the past 5 years, research on WNV disease has expanded rapidly. This review highlights new information regarding the virology, clinical manifestations, and pathology of WNV disease, which will provide a new platform for further research into diagnosis, treatment, and possible prevention of WNV through vaccination.

The impressive spread of West Nile virus (WNV) in the Western Hemisphere after its detection in 1999 during an outbreak of encephalitis in New York City has caused >16,000 human disease cases and >660 deaths in North America. Research on the signs, symptoms, and pathogenesis of WNV disease has greatly intensified in the past 5 years. The number of recognized cases of flaccid paralysis due to WNV infection has increased substantially, and research into prognosis and possible therapy has expanded. Genetic variation of the virus has been further characterized and continues to be explored. The pathology and pathogenesis of WNV disease have been described more completely than ever before. Several strategies are being pursued to develop effective vaccines to prevent WNV disease. This article highlights new information about the virology, clinical manifestations, laboratory diagnosis, pathology, and prognosis of WNV illness in humans. The expanded knowledge about WNV disease provides a new platform for future development of diagnostic tests, therapy, and vaccine development.

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Characteristics of West Nile Virus

WNV is an arbovirus in the family *Flaviridae*. Its spherical, enveloped capsid has a diameter of ≈50 nm and contains single-stranded RNA that encodes the capsid (C), envelope (E), and premembrane (prM) proteins, as well as 7 nonstructural proteins that likely contribute to viral replication. The virus has 2 genetic lineages: lineage 1 strains are found in North America, Europe, Africa, Asia, and Australia; lineage 2 strains have been isolated only in sub-Saharan Africa and Madagascar. Lineage 1 strains have been further divided into 4 clades: Kunjin, Indian, A, and B (which includes an Indian isolate) (1). The isolates in clade B, which includes strains from the United States, are all virulent in mice; lineage 2 and other clades in lineage 1 comprise both virulent and attenuated strains (1). Differences in pathogenicity may be related to nucleotides that code for specific regions in the prM, E, or nonstructural proteins of the virus (1,2).

WNV strains from the United States are closely related to strains from Israel, with 99.7% homology in nucleotide sequences, indicating that the strains in the United States almost certainly originated from the Middle East (3). The strain isolated in New York in 1999 is more virulent in American crows (*Corvus brachyrynchos*) than strains from Kenya and Australia (Kunjin virus, a subtype of WNV), and both the New York strain and the Kenyan strain experimentally killed house sparrows whereas the Australian strain did not (4).

Two genetic variants of the North American WNV strain were isolated in Texas in 2002; the major variant differed from the New York 1999 isolate by 0.18% of nucleotides, and the minor variant by 0.35% (1). The 2 variants differed from each other by 0.5% of nucleotides, and their neuroinvasiveness in mice was similar to that of the New York 1999 isolate. In 2003, attenuated WNV

strains were found in birds in Texas and Mexico, providing the first evidence of phenotypic variation of WNV strains in the Western Hemisphere (2,5). The reduced neuroinvasiveness and smaller plaque size of the Texas strains may be due to mutations in nonstructural proteins that result in lower levels of viremia; the attenuated strain from Mexico had a mutation in the E protein (2,5).

Pathogenesis

WNV is thought to replicate at the site of inoculation and then spread to lymph nodes and the bloodstream (6). Viral penetration of the central nervous system appears to follow stimulation of toll-like receptors and increased levels of tumor necrosis factor-α, which increases permeability of the blood-brain barrier (7). WNV directly infects neurons, particularly in deep nuclei and gray matter of the brain, brainstem, and spinal cord (8–10). Collateral destruction of bystander nerve cells may contribute to paralysis (11). Immune-mediated tissue damage may also contribute to pathologic changes in some cases (12). Genetic susceptibility for severe disease in mice has been postulated to involve a deficiency in production of 2′–5′-oligoadenylate synthetase, but this genetic susceptibility has not been elucidated in humans (10). Although most nonfatal WNV infections appear to be cleared by the host immune response, the virus may persist in some vertebrate hosts (10,13).

Clinical Manifestations

The clinical spectrum of symptomatic WNV infection in humans has been further defined during the North American epidemics. About 80% of human infections are apparently asymptomatic (14). Of those persons in whom symptoms develop, most have self-limited West Nile fever (WNF), characterized by the acute onset of fever, headache, fatigue, malaise, muscle pain, and weakness; gastrointestinal symptoms and a transient macular rash on the trunk and extremities are sometimes reported (15,16). A recent follow-up study of WNF patients who sought medical attention found that difficulty concentrating and neck pain or stiffness were also prominent symptoms, and that fatigue and muscle weakness frequently lasted for ≈1 month after onset (16). Of the 98 patients interviewed, 31% were hospitalized, 79% missed school or work because of their illness, and the median time before patients felt fully recovered was 60 days. These patients probably represent the most severe WNF, but even without neurologic manifestations, WNV infection clearly can cause a notable public health problem, Additional nonneurologic clinical manifestations that may rarely occur during WNV infection include hepatitis, pancreatitis, myocarditis, rhabdomyolysis, orchitis, and ocular manifestations (17–24). Chorioretinitis may be more common than previously thought; a study in Tunisia found that 69% of 29 patients hospitalized with WNV disease had chorioretinitis (24). Cardiac dysrhythmias have been observed in some North American patients (Centers for Disease Control and Prevention [CDC], unpub. data) (22).

Neuroinvasive disease develops in <1% of WNV-infected persons, for example, in such forms as meningitis, encephalitis, or paralysis (the proportion of reported cases that are neuroinvasive disease is higher because neuroinvasive disease is more likely to be reported than WNF or asymptomatic infections) (14). The risk for encephalitis increases with age and is higher among organ transplant recipients (25,26). Whether other immunocompromised patients are at higher risk remains unclear, but severe WNV disease has been described in persons with malignancies (9). Whether diabetes, hypertension, and cerebrovascular disease are risk factors also remains uncertain (27). The clinical severity of WNV encephalitis ranges from mild disorientation to coma and death (28,29). Many patients with WNV encephalitis have movement disorders, including severe tremors and parkinsonism (28,29).

In ≈13% of patients with neuroinvasive WNV disease, WNV infection of spinal motor neurons (anterior horn cells) causes acute, asymmetric flaccid paralysis similar to that seen with poliomyelitis (CDC, unpub. data) (18,30,31). Infection of the brainstem and high cervical spinal cord may cause diaphragmatic and intercostal muscle paralysis with resulting respiratory failure and sometimes death. A separate syndrome consistent with acute inflammatory demyelinating polyradiculoneuropathy (Guillain-Barré syndrome) has been infrequently reported (32).

Pathologic Changes

Histologic findings of WNV encephalitis include perivascular inflammation, microglial nodules, variable necrosis, and loss of neurons (Figure panels A, B) (8,9). The deep gray nuclei, brainstem, and spinal cord appear to be most affected (8,9). Patients with flaccid paralysis have perivascular lymphocytic infiltration in the spinal cord, microglial nodules, and loss of anterior horn cells (9). Spinal cord inflammation was seen in 17 of 23 people who died with WNV neuroinvasive disease; inflammation was more prominent in the anterior horns than in the posterior horns of 9 patients (9). Endoneural mononuclear inflammation of cranial nerve roots and spinal nerves can be found in a small percentage of persons. Foci of demyelination, gliosis, and occasional perivascular infiltrates may be found in persons with prolonged clinical courses.

Before 2001, attempts to isolate WNV from postmortem tissues in the United States had been unsuccessful. Recently, the virus has been isolated postmortem from 2 immunosuppressed patients with apparently high viral

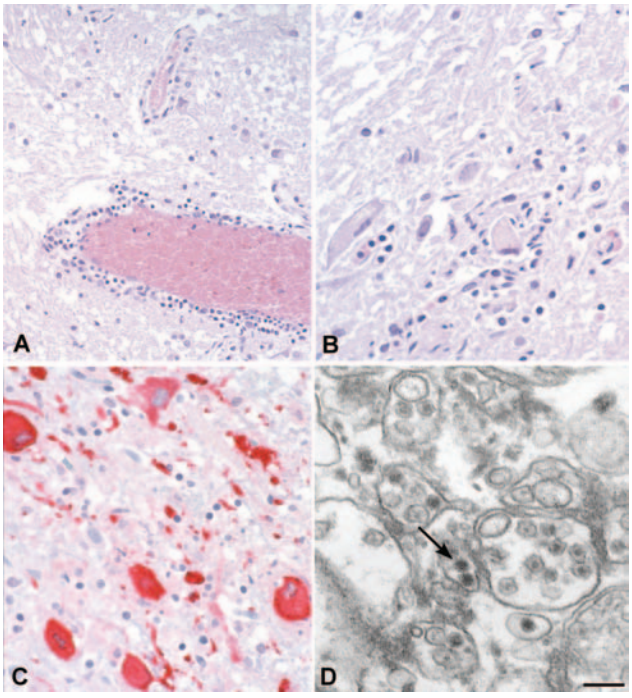

Figure. Histopathologic features of West Nile virus (WNV) in human tissues. Panels A and B show inflammation, microglial nodules, and variable necrosis that occur during WNV encephalitis; panel C shows WNV antigen (red) in neurons and neuronal processes using an immunohistochemical stain; panel D is an electron micrograph of WNV in the endoplasmic reticulum of a nerve cell (arrow). Bar = 100nm.

loads (33). Immunohistochemical (IHC) staining is more sensitive than viral culture, showing WNV antigens in ≈50% of fatal WNV neuroinvasive disease cases; IHC staining is particularly useful in patients who died during the first week of illness when viral antigen concentrations in central nervous system (CNS) tissues are high (9). Viral antigens are usually found within neurons and neuronal processes, predominantly in the brain stem and anterior horns (Figure, panel C). In general, antigens are focal and sparse, except in immunosuppressed patients in whom they can be seen extensively throughout the CNS (9). Visualization of WNV particles by electron microscopy is rare. When found, they are seen within endoplasmic reticulum of neurons (Figure, panel D).

Diagnostic Tests

Routine clinical laboratory studies do not distinguish WNV infection from many other viral infections. Patients with neuroinvasive disease generally have lymphocytic pleocytosis in the cerebrospinal fluid (CSF), but neutrophils may predominate early in the course of illness (28,31). Results of brain magnetic resonance imaging are frequently normal, but signal abnormalities may be seen in the basal ganglia, thalamus, and brain stem of patients with encephalitis, and in the anterior spinal cord in patients with poliomyelitislike syndrome (18,29,31). Clinical features and electrodiagnostic tests can help differentiate poliomyelitislike syndrome from Guillain-Barré syndrome by localizing damage primarily to motor axons, anterior horn cells, or both, with relative sparing of sensory nerves in the former, as opposed to localizing the damage to peripheral myelin or muscle in the latter (18,31,32,34).

Detection of WNV-specific immunoglobulin (Ig) M in serum or CSF provides strong evidence of recent WNV infection. In some patients, IgM antibody against WNV is usually detectable by 8 days after illness onset; however, in patients with WNV neuroinvasive disease, specific IgM is almost always detectable in serum and CSF by the time CNS symptoms begin (35). Among asymptomatic WNV-viremic blood donors who were seronegative at the time of donation, IgM appeared ≈9 days postdonation, and IgG appeared ≈4 days later (M. Busch, pers. comm.). IgM is detectable in serum of ≈36% of patients who have survived WNV encephalitis at 12 months postonset and ≈20% at 16 months postonset; IgM is also detectable in CSF of other patients up to 199 days postonset (36,37). Consequently, detectable IgM may occasionally reflect past rather than recent infection.

Recently developed microsphere immunoassays for WNV antibody appear to be more accurate and efficient than current enzyme immunoassays (EIAs) (J. Johnson, pers. comm.) (38). As with standard EIA, related flaviviral infection may elicit cross-reactive test results. A microsphere assay with nonstructural viral antigens appears to discriminate between primary flaviviral infections that elicit cross-reactive antibody to the E glycoprotein (38).

A ≥4-fold change in virus-specific neutralizing antibody titer (detected by plaque-reduction neutralization test [PRNT]) between 2 serum specimens collected 2–3 weeks apart usually confirms acute WNV infection. Samples with WNV-specific antibody will usually have neutralizing antibody titers to WNV that are >4-fold higher than titers to other epidemiologically relevant flaviviruses included in the assay. However, PRNT may not discriminate between WNV infection and other flaviviral infections in patients with previous flavivirus exposure, because the neutralizing antibody in such cases may broadly cross-react to several related flaviviruses.

WNV infection can also be diagnosed by detecting virus in CSF, serum, or tissues by isolation or nucleic acid amplification tests (NATs). WNV is best isolated in cell culture or suckling mice and identified by indirect immunofluorescence assay with specific monoclonal antibodies or by reverse transcriptase–polymerase chain reaction (RT-PCR). However, WNV is rarely isolated from the blood of patients with neuroinvasive WNV disease

because viremia levels are typically low or absent by the time neurologic symptoms develop. Real-time RT-PCR and nucleic acid sequence-based amplification are the most sensitive NATs, able to detect ≥50 viral RNA copies per mL (≈0.1 PFU/mL), which is ≈1,000-fold more sensitive than culture (39). WNV can be detected in serum by NAT if the specimen is obtained early in infection and is readily detected by NAT, isolation, or IHC staining in brain tissue from persons with fatal cases. The sensitivity of RT-PCR among 28 patients with serologically confirmed neuroinvasive WNV disease was 57% in CSF and 14% in serum (40).

The diagnosis of WNV encephalitis can be supported histopathologically, and there is no pathognomonic lesion. Differential diagnoses include arboviral and other viral encephalitides, rickettsial infections, and various noninfectious diseases. When serum samples and frozen tissues are not available, IHC testing of formalin-fixed tissues with specific monoclonal and polyclonal antibodies is particularly useful.

Prognosis

The clinical course of WNF ranges from a mild febrile illness of several days' duration to debilitating fatigue, aching, and weakness that may last for weeks or months (16,29,41). Although cases of meningitis without alteration of the patient's mental status or other focal neurologic features have a favorable prognosis, persistent headaches and fatigue may be reported (29). Patients with WNV encephalitis or focal neurologic manifestations often have persistent neurologic deficits for months or years (28,29). Of 35 patients hospitalized with WNV disease in New York, only 13 (37%) reported full recovery in physical, cognitive, and functional abilities 12 months after illness onset (41). Many patients with WNV-associated poliomyelitislike syndrome do not recover, but some improvement in limb strength may occur over time (42,43). The overall case-fatality rate for neuroinvasive WNV disease is ≈9% (26).

Clinical Management

Management of severe WNV illness remains supportive. Patients with severe meningeal symptoms often require pain control for headaches and antiemetic therapy and rehydration for associated nausea and vomiting. Patients with severe encephalitis should be observed for development of elevated intracranial pressure and seizures, and patients with encephalitis or paralysis must be monitored for inability to protect the airway. Acute neuromuscular respiratory failure may develop rapidly, particularly in patients with prominent bulbar signs; prolonged ventilatory support may be required (22,30,34).

Ribavirin, interferon-α, WNV-specific immunoglobulin, and antisense gene–targeted compounds have all been considered as specific treatments for WNV disease, but no rigorously conducted clinical trials have been completed. Nonspecific immunoglobulin and plasmapheresis should be considered for patients with Guillain-Barré syndrome but are not indicated for patients with paralysis due to damage of anterior horn cells (30).

Vaccine Development

Two vaccines are available for vaccinating equines: an inactivated WNV vaccine and a recombinant vaccine that uses canarypox virus to express WNV antigens (44,45). An inactivated vaccine is also being studied for use in humans (46). A chimeric live virus vaccine incorporating the genetic sequences for E and prM antigens into a 17-D yellow fever virus backbone has been shown to be efficacious in hamsters and is undergoing initial clinical trials in humans (46). Another chimeric vaccine incorporating WNV genetic sequences into a backbone of attenuated serotype-4 dengue virus–induced protective immunity in monkeys (44). A DNA vaccine that elicits expression of WNV E and prM antigens has been used in mice, horses, and birds (44). Vaccination of crows with Kunjin virus, a subtype of WNV, protected against WNV, and a DNA vector, which elicited expression of attenuated Kunjin virus, provided protective immunity against WNV in mice (46).

Future Directions

Since the 1990s, WNV has gained notoriety as a cause of severe neuroinvasive disease in humans. As WNV isolates and genetic sequences accumulate over an increasing geographic and clinical range, the virus shows signs of genetic modifications that likely interact with host factors in causing different patterns of neuroinvasiveness and neurovirulence. Several areas warrant research focus over the next few years. More efficient diagnostic assays will help with both clinical diagnosis and disease surveillance. Improved knowledge about the pathogenesis and natural history of WNV disease is crucial to developing effective treatment, and promising therapies need to be carefully evaluated in controlled clinical trials. Given the focal distribution of WNV outbreaks, and the uncertain distribution of future cases of WNV disease, prospective clinical studies need to be designed with the flexibility to gather information from widely dispersed and changing locations. The development of a safe and effective vaccine for humans is a clear priority for prevention, and the public health strategies and recommendations for vaccination deserve careful thought. Given the relatively low incidence of WNV neuroinvasive disease and the focal occurrence of WNV epidemics thus far, vaccination will likely require targeting to higher risk groups to approach the cost-effectiveness of many recommended public health prevention strategies.

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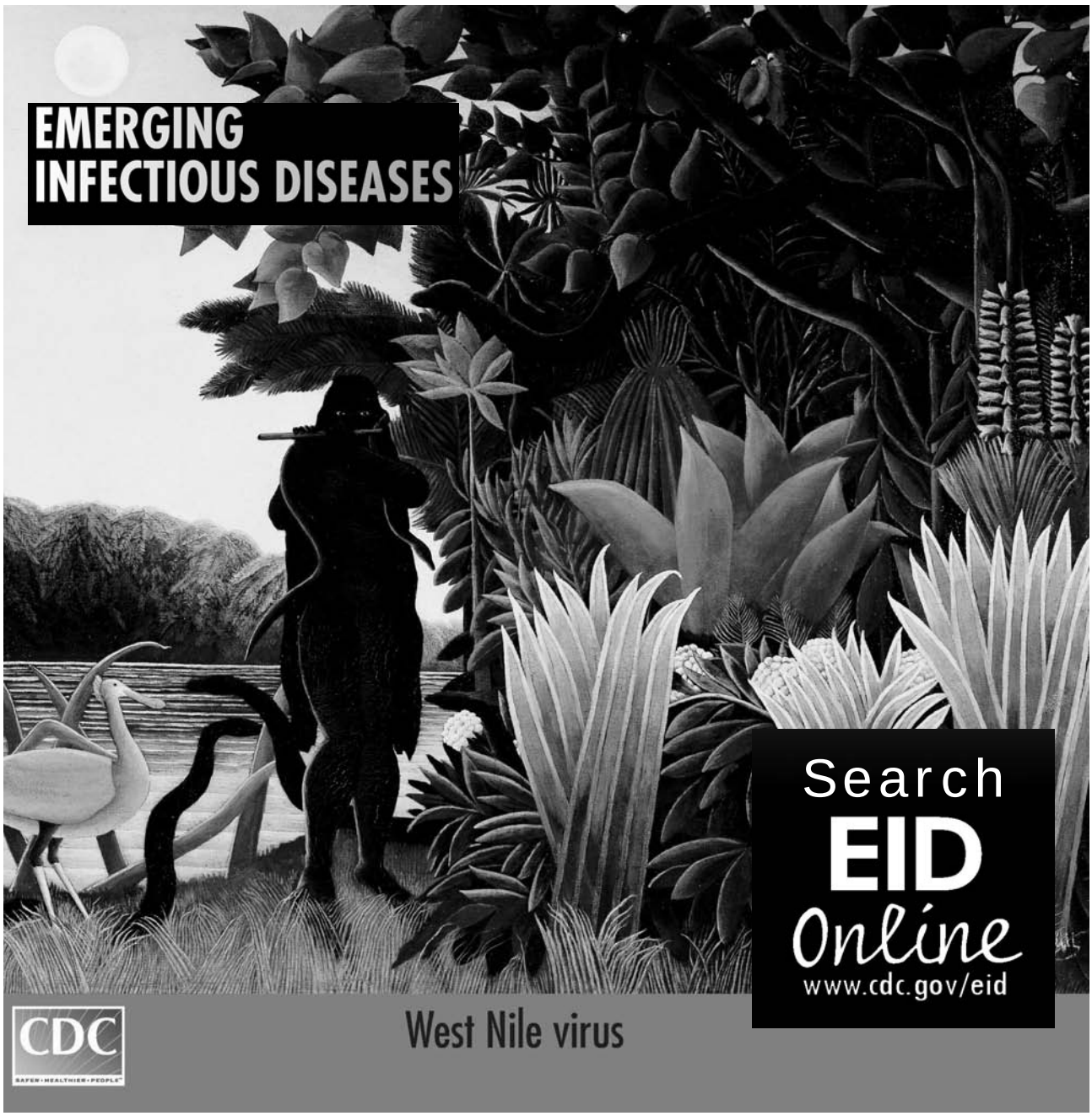

Laboratory Exposures to Brucellae and Implications for Bioterrorism

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Brucellae are class 3 organisms and potential agents of bioterrorism. Because of effective public health measures, brucellosis has become a rare disease in industrialized countries, and clinical microbiology laboratories are frequently unfamiliar with the genus. A low index of suspicion by physicians or failure to notify the laboratory, equivocal Gram-stain results, misidentification of the organism by commercial systems, unsafe laboratory practices, and laboratory accidents have been responsible for numerous cases of exposure to the organism and laboratory-acquired disease in recent years. Discovery of a laboratory exposure to brucellae should prompt an exhaustive investigation of the event and its circumstances, definition of the population at risk, enforcement of safe laboratory practices, and antimicrobial drug prophylaxis for exposed persons. Inadvertent exposures to brucellae in the clinical laboratory indicate a widespread lack of preparedness to cope with eventual biologic threats involving use of the organism.

Brucellosis is a zoonosis usually transmitted to humans by contact with infected animals and consumption of contaminated animal products (1,2). Because of compulsory pasteurization of milk products and strict control of the disease in dairy cattle, the incidence of brucellosis has steadily declined in most industrialized countries during the last 50 years. However, the disease remains among the most commonly recognized causes of laboratory-transmitted infections; 2% of all brucellosis cases are laboratory-acquired (1,3–9).

The Organism

Several biologic characteristics make brucellae easily transmissible within the close confinement of the clinical microbiology laboratory, including the facts that the infecting dose for humans is low, and the organism may enter the body in many ways relevant to laboratory practices (e.g., through the respiratory mucosa, conjunctivae, gastrointestinal tract, or abraded skin) (1,2). Rare cases of acquisition of the organism through organ transplantation, sexual contact, breastfeeding, or the transplacental route have also been reported (1). Because person-to-person transmission does not occur, infected persons do not pose a threat to their surroundings.

Soon after entry into the body through the skin or mucous membranes, brucellae are ingested by polymorphonuclear and mononuclear phagocytes. The organism is able to escape phagocytic killing by inhibiting the phagosome-lysosome fusion and reproducing inside macrophages (1,10). After a variable incubation period ranging from <1 week to several months (usually 2–4 weeks), nonspecific systemic symptoms such as fever, headache, malaise, night sweats, and arthralgia follow, resembling a flulike disease (1,2). During the early stages of the disease, patients are frequently bacteremic. This bacteremia has a continuous pattern, making circulating brucellae easily detectable by blood culture. Once in the bloodstream, the organism is seeded to multiple organ systems and especially to those rich in reticuloendothelial tissue, such as the liver, spleen, and the skeletal and hematopoietic systems, where it may cause localized disease such as hepatitis or arthritis (1,2).

Because of the variable manifestations of human brucellosis, a wide array of different clinical specimens may contain viable brucellae, including pus, blood, bone marrow, synovial fluid and tissues, and more rarely, cerebrospinal fluid, urine, and genital exudates. The concentration of *Brucella* organisms in the blood (11,12) and synovial fluid (13) of patients with brucellosis is usually low, and therefore, these clinical specimens probably pose a low risk for contagion for laboratory personnel. However, the danger of clinically relevant exposure increases exponentially after incubation of both solid and liquid media. Seeded media harbor considerable amounts of viable *Brucella* organisms, and routine bacteriologic procedures such as preparing, centrifuging, and vigorous agitation (vortexing) of bacterial suspensions, performing subcultures and biochemical testing, and particularly the

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catalase test, may create dangerous aerosols and the potential for accidental spillage (14).

Most cases of laboratory-acquired brucellosis have been caused by the more virulent *Brucella melitensis* species (3,4,6,8,14–29). *B. suis* (4,6), *B. abortus* (3,6,8), and *B. canis* (30) have also been implicated, and transmission of the attenuated *B. abortus* 19 and *B. melitensis* Rev-1 vaccine strains has also been reported (31,32).

Clinical Manifestations of Laboratory-acquired Infections

Patients involved in laboratory outbreaks of brucellosis have shown almost the entire range of clinical manifestations of the disease, ranging from the common prolonged febrile syndrome (undulant fever) (14) and a flulike disease (25,27), to focal signs and symptoms, such as hepatitis (17,33), lymphadenopathy (17,25), uveitis (14), breast abscess (28) epididymitis (29), arthritis (17), discitis (29,34), pneumonitis (17), deep vein thrombosis (29), and meningitis (31). Deaths are rare (<5%) even among untreated patients with brucellosis, but 5 fatal cases occurred among the 426 persons with laboratory-acquired disease summarized by Pike in 1978 (5).

Laboratory Exposure to *Brucella* Organisms

Because of effective control measures in animals and animal products, brucellosis has been almost eradicated from most industrialized countries, where the disease is usually limited to persons who have traveled to developing countries or ingested imported contaminated food (14). Human brucellosis has become so rare in the United States that <300 cases have been reported annually in the last 4 decades (9,15) and ≈100 per year in the last 10 years (2). Because of the rare occurrence of brucellosis, technicians working in industrialized countries have become unfamiliar with the staining and other phenotypic characteristics of the organism. In addition, physicians frequently do not consider the diagnosis of brucellosis or fail to communicate their suspicion to the clinical microbiology laboratory, which results in inadvertent handling of cultures on an open bench.

Although the identification of the genus *Brucella* is straightforward (small gram-negative coccobacilli; positive oxidase, catalase, and urease test results; no sugar fermentation; and requirement of aerobic conditions and added CO_2 for its growth), laboratory-acquired disease has frequently resulted from misidentification of the organism (35). In 2 published outbreaks, the *Brucella* isolate resisted decoloration and appeared as a gram-positive or gram-variable coccobacillus and thus was misidentified as micrococcus or a coryneform bacillus (14,29). In other cases, *B. melitensis* organisms tested with the API20NE (bioMérieux, Marcy l'Etoile, France) identification kit produced the 1200004 or 1000004 biochemical profile leading to misidentification of the isolate as *Moraxella phenylpyruvica* at a good identification level (90.5%) (8,18–21). The frequent failure of clinical laboratories to correctly identify isolates as brucellae is particularly worrisome because these organisms are regarded as potential agents for bioterrorism. Brucellae are inexpensive to produce and disperse, and transmission to humans may result in prolonged illness and long-term sequelae. These organisms are considered category B select agents (2). The occurrence in the last few years of inadvertent exposures to brucellae in laboratories in industrialized countries (7,8,14,17–26) indicates a lack of preparedness to deal with a real biologic threat. Lack of recognition of an isolate as a *Brucella* sp. by a laboratory may enable a bioterrorism-related attack to go undetected, whereas a false-positive identification may cause unnecessary alarm.

In laboratories serving countries in which human brucellosis is still endemic, the degree of potential exposure to the organism may be extremely high. In a microbiology laboratory in Ankara, Turkey, the average annual number of cultures positive for *Brucella* spp. reaches 400 (34). A laboratory serving a brucellosis-endemic area in southern Israel processes ≈150 per year; at the peak of the *Brucella* season (April–June), 10% of all positive blood cultures grow *B. melitensis* (27). The risk for exposure in developing countries is frequently aggravated by lack of safety equipment and inadequate laboratory facilities (34). In the aforementioned Turkish laboratory, the disease affected 10 (18%) of 55 laboratory workers, and the calculated hazard was 8% per employee-year (34).

Mechanism of Transmission

The probable source of the infection is usually apparent when disease occurs in laboratories where isolation of brucellae is rare (21). In laboratories serving brucellosis-endemic areas, the time and circumstances of contamination are more difficult to trace because of the existence of multiple potential sources (22,27). Although the exact form of transmission often remains speculative, aerosols have been implicated in most cases (24,27,29,32). This assumption has been strengthened by spread of the disease to distant areas through common ventilation (27,32).

Laboratory accidents such as breakage of centrifuge tubes (7) or blood-culture vials (16) play a minor role in laboratory-acquired disease and are responsible for only 20% of cases (6). More commonly, exposures are the result of unsafe laboratory practices, such as sniffing plates (8,24,26,29,34); working on an open bench with viable organisms (14,16,17,20,22); not using protective equipment such as gloves, masks, and goggles (34); or ingesting suspensions of living brucellae during mouth pipetting (3).

However, even when no apparent breach in safety procedures is apparent, transmission may occur (17). Rare events—such as self-inoculation of brucellae by syringes loaded with a suspension of the organism or with synovial fluid from an infected patient (3,16), injury to the conjunctiva with a broken tube that had contained a living culture (3), or participation in a laboratory exercise in which still-viable organisms were inadvertently used (15)—are also responsible for a small number of laboratory exposures and outbreaks of disease.

Most cases of laboratory-acquired brucellosis have occurred in clinical laboratories, but transmission of the disease in research facilities (4,25) and laboratories that manufactured *Brucella* vaccines has also been documented (21,22). A laboratory technician at the Centers for Disease Control and Prevention (CDC) became infected while working in a safety cabinet with a *B. melitensis* isolate that had originally caused an outbreak of laboratory-acquired disease in a community hospital (17). In a separate episode, the organism isolated from a microbiology technologist with laboratory-acquired disease infected a laboratory worker at the hospital to which the technologist had been admitted (14).

The attack rate of laboratory-associated infections has ranged from 30% to 100% depending, among other factors, on the location of workers, whether aerosol-generating procedures have been performed, and the concentration of microorganisms in the contaminated media (7,8). However, the hazard of transmission is not limited to persons who worked with the isolate. Among the 74 reported cases of laboratory-acquired brucellosis from 1897 to 1939 reviewed by Meyer and Eddie, the disease also affected janitors and occasional visitors (3). In a large outbreak of laboratory-acquired brucellosis, 3 of 7 cases of disease occurred among persons who briefly visited the facility but did not enter the room where *Brucella* cultures were processed (27).

Investigating Outbreaks of Brucellosis

Recognizing sporadic cases and even outbreaks of laboratory-acquired brucellosis is not always easy because the disease lacks distinctive clinical features (14,17) and has a widely variable incubation period (7,17,19,32). In a cluster of disease that originated in a single exposure, the onset of symptoms of the 7 affected laboratory workers spanned 5 months (17). Although a temporal clustering of cases usually suggests a large exposure from a pinpoint source (7,32), this is not always the case. When biotyping was performed in the isolates recovered within a 5-week period from 7 infected hospital workers in southern Israel, 3 distinct strains were recognized, demonstrating that the outbreak was caused by at least 3 separate exposures (27).

Guidelines for Investigating Outbreaks

In-depth investigation of laboratory-acquired cases of brucellosis may lead to the identification of unsafe bacteriologic practices, suitable for correction by educational and technical measures. To guide the investigation, the following recommendations are made. 1) Send isolates to a reference laboratory for confirmation. Organisms should be shipped as "dangerous goods," according to the guidelines established by the World Health Organization and the Office of Biosafety at CDC (36). In the United States, organisms presumptively identified as brucellae should be sent to CDC or another public health laboratory following the specific guidelines for transferring "select agents." 2) Inform infection control services and public health authorities, who may choose to involve CDC. 3) Conduct a meticulous epidemiologic investigation. 4) Determine the date and circumstances of the exposure (17). 5) Exclude other potential sources of transmission, such as previous laboratory exposures, travels to brucellosis-endemic areas, consumption of unpasteurized dairy products, and handling of farm or laboratory animals (14,15,17,27,29). 6) Keep all relevant data and records. 7) Define the exposed population. 8) Determine the level of risk on the basis of type of laboratory procedures performed, proximity to the source, duration of the exposure, and the like, to define persons to whom postexposure prophylaxis should be offered (8,32). 9) Check biologic safety cabinets (27,29). 10) Check the ventilation system (27). 11) Collect baseline serum samples from all known potentially exposed persons (17,27). 12) Freeze isolates for future typing, especially in brucellosis-endemic areas (23,27).

Prevention of Laboratory-acquired Cases

Brucellae are considered class 3 organisms. CDC has strongly recommended that live *Brucella* cultures and suspicious organisms be manipulated in a class II biologic safety cabinet (37). This recommendation, however, is clearly insufficient for preventing laboratory-acquired disease because by the time the organism is suspected or confirmed as *Brucella*, exposure of laboratory personnel may have occurred (27). On the other hand, converting a large and busy clinical microbiology laboratory into a biosafety level III facility, where all specimens are handled in biologic safety cabinets (32), is both impractical and unnecessary, especially in laboratories in areas where the disease is not endemic. However, in regions where brucellosis is highly prevalent, an enhanced safety policy should be adopted. All blood culture vials detected as positive by the automated blood culture system, as well as all bone marrow and synovial fluid specimens, should be manipulated in biologic safety hoods until the isolated microorganisms are definitively determined to be other than *Brucella* sp. (27).

For laboratories serving areas where brucellosis is uncommon, the following recommendations are made. 1) Because of the low incidence of brucellosis, physicians in areas not endemic for the disease are unfamiliar with the clinical and epidemiologic features of human brucellosis, and the possibility of brucellosis is rarely considered in the differential diagnosis. Periodic education of physicians on this subject is indicated. 2) Communication between attending physicians and the laboratory should be improved. The clinical microbiology laboratory should be informed in advance when clinical specimens had been obtained from patients with risk factors for brucellosis, such as recent history of travel to brucellosis-endemic areas, consumption of local or imported unpasteurized dairy products, or professional exposures in veterinarians, shepherds, slaughterhouse employees, and laboratory workers. 3) The use of automated, continuous monitoring blood culture systems for patients with suspected brucellosis should be preferred over the lysis-centrifugation method because the latter involves centrifuging clinical specimens and visually inspecting plates to detect the organism and probably increases the risk for transmission (11,27). Although the use of blood lysis-based methods has been advocated in the past for improving detection of *Brucella* bacteremia, modern automated blood culture systems are faster and more sensitive (11,12). 4) The familiarity of laboratory technicians with the characteristics of the organism, as well as with the safe handling of cultures, should be improved and maintained through periodic education. The reader is referred to the excellent review by Gilligan and York for handling and identifying presumptive *Brucella* organisms (35). 5) Standard precautions and strict adherence to good laboratory practices must be completely adopted, reinforced, and regularly monitored. 6) All work with gram-negative or gram-variable small rods or coccobacilli isolated from tissues, blood, bone marrow, bone, or synovial fluid exudates should be carried out in a biosafety cabinet until *Brucella* has been ruled out. 7) Plates should be sealed for safety when not in use and appropriately disposed and sterilized as soon as they are no longer being actively used (17). 8) Because brucellae are relatively slow-growing bacteria, cultures for the organism have been traditionally kept for several weeks. However, modern blood culture systems enable brucellae to be detected within the routine 5-day incubation period instituted in most clinical laboratories (12). Therefore, safety precautions should not be limited to organisms that tend to grow slowly (8,18,20,26). 9) Antimicrobial drug–susceptibility testing of *Brucella* organisms is not indicated because the therapeutic regimen for brucellosis is standard, and the organism does not usually acquire antimicrobial resistance. Performance of this and other unnecessary tests, and especially of laboratory procedures known to produce aerosols, should be strongly discouraged (27). 10) If a suspension of living brucellae is spilled and the organism is recognized, the entire laboratory should be immediately evacuated, doors should be shut, and an effective germicide such as 3% phenol or 10% bleach should be applied by a trained person wearing a safety mask, goggles, an impermeable laboratory gown, and gloves (7).

Postexposure Prophylaxis

Because of ethical considerations, the heterogeneous nature of the events leading to *Brucella* exposure, difficulties in determining the actual risk for individual workers, late recognition of outbreaks, and the small number of persons involved in each outbreak, no controlled studies have been performed to assess the value of administering postexposure prophylaxis to persons at risk. However, anecdotal evidence suggests that administering prophylactic antimicrobial drug therapy may reduce the risk of developing clinical disease (7,8,26).

In a recently reported event, an isolate from a chest wall exudate culture from an Indian patient, which was originally identified as *M. phenylpyruvica*, was correctly recognized as *B. melitensis* 22 days after the specimen was obtained (8). By that time, 26 laboratory workers had been potentially exposed, and 6 had actually manipulated the organism. These 6 workers were considered to be at high risk and offered a 3-week prophylactic course of combined doxycycline-rifampin or trimethoprim-sulfamethoxazole therapy. None of the 5 laboratory technologists who received postexposure antimicrobial drugs became ill or developed an antibody response. However, *Brucella* bacteremia developed in the only laboratory worker who refused therapy, and she seroconverted 10 weeks after the specimen was received (8). Neither clinical disease nor seroconversion developed in the remaining 19 laboratory workers who were only present in the laboratory but were not in direct contact with the organism (8).

In another event, 3 laboratory technologists who had inadvertently worked with a *B. melitensis* isolate on an open bench and sniffed the plates were given 1 week of prophylactic doxycycline within 24 hours of the exposure; none of them became ill or seroconverted (26). In a large exposure caused by breakage of a tube containing *B. abortus*, patients who started therapy immediately after seroconversion was detected and before symptoms developed (10 weeks after the laboratory accident) had a benign clinical course, suggesting that even if the disease was not prevented, a certain degree of attenuation probably occurred (7).

Because of the high attack rate of brucellosis among exposed workers, the unpredictable and often chronic course of the disease, and the difficulties in eradicating the organism once a symptomatic infection has been established, postexposure prophylaxis is probably indicated for

all persons after an obvious exposure to living brucellae (26). Antimicrobial drug prophylaxis with a combination of oral doxycycline 100 mg twice a day plus rifampin 600 mg 4 × per day for 3 weeks should be started as soon as the exposure to confirmed *Brucella* organisms is recognized (8). For pregnant women, administration of trimethoprim-sulfamethoxazole 160/800 mg 2 × per day for 3 weeks has been advocated (8).

Postexposure Follow-up

Whether exposed persons have received prophylactic therapy or not, increased surveillance for clinical signs of disease should be conducted for at least 6 months. In addition, exposed laboratory workers should be followed for possible subclinical infections or early signs of disease by periodic serologic testing. Weekly or semiweekly serologic surveillance is recommended for the first 3 months and once a month thereafter for 3 to 9 additional months to detect late infections resulting from prolonged incubation (7,8).

Human brucellosis has variable clinical manifestations and may mimic other infections, particularly influenza, as well as noninfectious conditions. Therefore, the diagnosis of the disease requires a high index of suspicion. Education of exposed personnel on the symptoms of the disease and the need for periodic and timely serologic follow-up are particularly important. Increased vigilance during the flu season employing a broad case definition is clearly needed, and administration of influenza vaccine should be strongly recommended to all *Brucella*-exposed persons. If symptoms develop, blood cultures and cultures of other normally sterile body fluids, as clinically indicated, should be obtained, and a new antibody titer should be determined. Although use of nucleic acid amplification methods has been shown to enable early detection of infected persons (38), these tests are not yet commercially available.

Treating Infected Persons and Posttreatment Follow-up

Persons in whom culture-confirmed or serologically proven disease develops should receive therapy with oral doxycycline and rifampin for 6 weeks, or a combination of oral doxycycline for 6 weeks and an intramuscular aminoglycoside (gentamicin or streptomycin) for the first 2 weeks (2,7). Use of 3 drugs is usually reserved for complicated cases or life-threatening clinical manifestations, such as endocarditis or meningitis (1). Pregnant women should receive trimethoprim-sulfamethoxazole 160/800 mg 2 × per day for 6 weeks (1).

Even when patients are appropriately treated, the risk for relapse remains high (≈20%) (39,40). Therefore, patients who have completed a full therapeutic antimicrobial course should be followed clinically and serologically for 1 year. Failure to show declining antibody titers may indicate incomplete cure. If symptoms consistent with brucellosis develop, blood cultures and serologic tests should be performed to detect relapses of the disease (39). Use of nucleic acid amplification methods in the future may further improve the detection of patients in whom the organism was not eliminated (40).

Although human brucellosis has been eradicated from most industrialized countries, isolated cases of disease, usually related to travel or import of contaminated food from disease-endemic areas, continue to occur. Because of the low incidence of disease in industrialized countries, clinical laboratory technologists have become unfamiliar with identifying and handling *Brucella* species. Unsafe laboratory practices while manipulating *Brucella* isolates have resulted in inadvertent exposure to the organism and many cases of laboratory-acquired disease, indicating lack of preparedness to cope with bioterrorism threats involving brucellae. Education of laboratory personnel on the identification of *Brucella* species, adherence to and enforcement of standard precautions, thorough investigation of laboratory exposures, administration of prophylactic antimicrobial drug therapy, and close follow-up of directly exposed persons are strongly recommended.

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Influenza A (H3N2) Outbreak, Nepal

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In July 2004, an outbreak of influenza A (H3N2) was detected at 3 Bhutanese refugee camps in southeastern Nepal. Hemagglutination inhibition showed that ≈40% of the viruses from this outbreak were antigenically distinct from the A/Wyoming/3/03 vaccine strain. Four amino acid differences were observed in most of the 26 isolates compared with the A/Wyoming/3/2003 vaccine strain. All 4 substitutions are located within or adjacent to known antibody-binding sites. Several isolates showed a lysine-to-asparagine substitution at position 145 (K145N) in the hemagglutinin molecule, which may be noteworthy since position 145 is located within a glycosylation site and adjacent to an antibody-binding site. H3N2 viruses continue to drift from the vaccine strain and may remain as the dominant strains during the 2005–2006 influenza season. Thus, the 2005–2006 Northern Hemisphere vaccine strain was changed to A/California/7/2004, a virus with all 4 amino acid substitutions observed in these Nepalese isolates.

The 2003–2004 influenza season was severe in terms of its impact on illness because of widespread circulation of antigenically distinct influenza A (H3N2) Fujian-like viruses. These viruses first appeared late during the 2002–2003 influenza season and continued to persist as the dominant circulating strain throughout the subsequent 2003–2004 influenza season, replacing the A/Panama/2007/99-like H3N2 viruses (1). Of the 172 H3N2 viruses genetically characterized by the Department of Defense in 2003–2004, only 1 isolate (from Thailand) belonged to the A/Panama-like lineage. In February 2003, the World Health Organization (WHO) changed the H3N2 component for the 2004–2005 influenza vaccine to afford protection against the widespread emergence of Fujian-like viruses (2). The annually updated trivalent vaccine consists of hemagglutinin (HA) surface glycoprotein components from influenza H3N2, H1N1, and B viruses.

The HA1 segment of the influenza HA protein is the most rapidly evolving gene product (3) and plays a major role in viral attachment and evasion from the adaptive immune response. Previous studies have demonstrated 5 antigenic sites on the HA1 polypeptide where antibody binding can occur (4,5). Additionally, several studies have documented specific immunodominant codons corresponding to specific amino acids of the HA protein that are directly involved in the divergence of antigenically distinct influenza viruses (6–8).

In July 2004, an outbreak of influenza A (H3N2) was detected in patients at 3 Bhutanese refugee camps in southeastern Nepal. To elucidate the molecular mechanism underlying the emergence of this H3N2 outbreak, we conducted a molecular analysis of the HA1 region of the HA protein. In this report, we describe the epidemiologic and molecular aspects of isolates obtained from this off-season influenza A (H3N2) outbreak.

Materials and Methods

Sample Collection and Antigenic Analysis

Sixty-four patients in Nepal that met US Department of Defense enrollment criteria (9) for influenzalike illness were evaluated by using onsite rapid influenza tests (Optical Immunoassay Rapid Diagnostic Tests, Thermo Electron Corp., San Jose, CA, USA) according to the manufacturer's instructions. Throat swab specimens were collected within the first 72 hours of onset of symptoms, routed through the Armed Forces Research Institute for Medical Sciences in Bangkok, Thailand, and shipped on dry ice to Brooks City Base in San Antonio, Texas, for clinical characterization and diagnosis using traditional culturing techniques and monoclonal antibody staining (10). Antigenic analysis of select isolates was performed at

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the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, by using the hemagglutination inhibition (HI) assay and postinfection ferret antisera (11).

Molecular Analysis

RNA was extracted from 48-hour shell vial cultures (10) by using the MagnaPure LX (Roche Molecular, Mannheim, Germany) and RNA Isolation Kit II (Roche Molecular) according to the manufacturer's protocols. For reverse transcription-polymerase chain reaction (RT-PCR) amplification, 5 μL RNA was added to a 50-μL master mixture containing 1× reaction buffer, 1.6 mmol/L $MgSO_4$, 1× enzyme mixture, and 400 nmol/L primers (H3-F7, 5′-ACT-ATC-ATT-GCT-TTG-AGC-3′ and H3R-1184, 5′-ATG-GCT-GCT-TGA-GTG-CTT-3′) by using the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA). PCR thermocycling consisted of an RT step at 50°C for 30 min, hot start activation at 95°C for 3 min, followed by 40 amplification cycles of 95°C for 30 s, 52°C for 15 s, and 68°C for 1 min, with a final extension cycle at 68°C for 7 min. All PCR products were visualized after electrophoresis in 2% precast gels stained with ethidium bromide (Invitrogen) under UV illumination. PCR products were purified by using the PCR Purification Kit (Qiagen Inc., Valencia, CA, USA). The HA1 amplicon (1177 bp) was sequenced by using the H3-F7 and H3R-1184 PCR primers (described above) and 2 additional internal oligonucleotides, H3R-466 (5′-GGT-GCA-ACC-AAT-TCA-ATC-3′) and H3F-282 (5′-CAG-CAA-CTG-TTA-CCC-3′). Unincorporated fluorescent nucleotides were removed by using a Dye Ex 96-well plate kit (Qiagen) according to the manufacturer's recommendations. Nucleotide sequencing was performed by using the Big Dye Terminator v3.1 Kit and analyzed by using an ABI 3100 Genetic Analyzer (both from Applied Biosystems, Foster City, CA, USA) according to the manufacturer's specifications. Multiple sequence alignments, protein translation, and phylogenetic analysis were performed with the DNAStar (DNAStar Inc., Madison, WI, USA) software package. Three-dimensional HA protein structures were generated by using MOLMOL (12) and the Swiss-PDB Viewer programs (13). HA nucleotide sequences for all 26 Nepal isolates depicted in the phylogenetic analysis are available from GenBank under accession nos. AY945263–AY945288.

Results

Epidemiologic and Laboratory Assessment

Clinical evaluations and throat specimens were obtained from 64 patients from 3 refugee camps in southeastern Nepal (Figure 1). Of the 64 patients, 61 were refugees from Bhutan, 1 was a foreign aid worker from Japan, and 2 were Nepalese nationals. Most of the patients were <10 years of age; 36 were male and 28 were female. None had previously been vaccinated against influenza and of the 64 specimens collected, 42 (66%) tested positive for influenza A by culture.

Antigenic Analysis

HI was performed by using postinfection ferret antisera with reference antigens that included the 2004–2005 H3N2 vaccine seed strain (A/Wyoming/03/2003) and the 2005–2006 Southern Hemisphere H3N2 vaccine strain (A/Wellington/1/2004). When compared with A/Wyoming/03/2003, 4 of 9 Nepal isolates showed 4-fold lower titers (1:320 versus 1:1,280 HI units) than that allowed for homologous titer of the reference antisera. This indicated that these 4 isolates were antigenically distinct. Six of 9 Nepal isolates were antigenically distinct

Figure 1. Early outbreak of influenza A (H3N2) in southeastern Nepal. The green circle shows the location of 3 Bhutan refugee camps where the outbreak occurred in early July 2004. (Map courtesy of www.maps.com)

when compared with the A/Wellington/1/2004 strain and showed a 4-fold (1:160 versus 1:640) reduction in titer to ferret antisera (Table 1).

Molecular Analysis

RT-PCR-based molecular subtyping showed that all 42 specimens were the H3N2 influenza subtype. Twenty-six of the 42 influenza A–positive samples were randomly selected for molecular characterization using direct nucleotide sequencing of the HA gene. The 26 Nepal isolates exhibited 99.8% nucleotide sequence identity and contained the Fujian-like amino acid substitutions at positions 155 (H155T) and 156 (Q156H) in the HA protein (Table 2). Alignment of the 329–amino acid HA protein from 26 isolates obtained from this outbreak with the 2004/05 A/Wyoming/3/03 vaccine strain and previous H3N2 vaccine strains indicated 4 evident amino acid changes present in most of the isolates (Table 2). All 4 amino acid changes observed within most of these outbreak isolates are present within A/California/7/04, a variant strain selected as the H3N2 vaccine strain for the 2005–2006 influenza season.

Of the 26 Nepal strains examined, 24 exhibited a novel lysine-to-asparagine substitution at position 145 in the HA protein (K145N). This substitution is noteworthy because most strains characterized in 2003–2004, including the Fujian/411/2002 vaccine strain, contained a lysine (K) at this position. Prior to this outbreak, the US Department of Defense had only observed K145N substitutions in 6 strains obtained from Ramstein, Germany, (data not shown) in June 2004. Additionally, all 26 Nepal sequences exhibited a serine-to-asparagine substitution at position 189 (S189N) that had also been observed in the 6 isolates from Germany, as well as in a few isolates from Asia characterized at the end of the 2003–2004 influenza season.

Two other substitution mutations in the HA1 hemagglutinin, i.e., valine to isoleucine at position 226 (V226I) and serine to proline at position 227 (S227P), were also observed in 24 (92%) and 26 of 26 of the Nepal isolates, respectively. Both substitutions differ from most influenza A H3N2 field isolates collected in 2003–2004, including the Fujian and Wyoming vaccine strain for 2004–2005 (Table 2).

The phylogeny of H3N2 HA proteins indicates a drifting of the Nepal isolates from the A/Fujian/411/03 and A/Wyoming/03/03 vaccine strains and shows that these outbreak isolates have a higher genetic homology to A/Wellington/1/04, a prototype strain selected as the 2005–2006 Southern Hemisphere H3 vaccine strain (Figure 2). The A/Wellington/1/04 strain contains 2 of the 4 amino acid changes (S227P and S189N) observed in the Nepal isolates, but does not contain the K145N and V226I substitutions.

Three-dimensional views of influenza HA proteins highlighting amino acid changes in a representative Nepal isolate and the A/Wyoming/3/03 vaccine strains are shown in Figure 3A and B, respectively. The mutation at position 145 (shown in yellow), which is located adjacent to antibody-binding site A and within a known glycosylation site, introduces an asparagine-for-lysine substitution. This substitution results in a more accessible receptor-binding cleft located directly above residue 145 (comparing panels A and B). Located above the receptor-binding pocket is a serine-to-asparagine change (shown in green) that possibly alters the regional surface topography at position 189 within antibody-binding site B. A serine-to-proline mutation at

Table 1. Hemagglutination inhibition (HI) reciprocal titers of influenza A (H3N2) viruses with ferret antisera*

Strain designation	Reference ferret antisera						Date collected
	PAN/2007	KO/770	WY/03	TX/40	OK/8	WEL/01	
Reference antigens							
A/Panama/2007/99	2,560	160	640	320	640	160	7/12/99
A/Korea/770/2002†	80	640	1,280	640	640	320	12/2/02
A/WyomingG/03/2003	640	320	1,280	1,280	1,280	320	2/13/03
A/Texas/40/2003	640	1,280	1,280	2,560	1,280	640	10/2/03
A/Oklahoma/8/2004	160	1,280	1,280	2,560	1,280	640	12/8/03
A/Wellington/1/2004	160	320	320	640	320	640	1/26/04
Test antigens							
A/Nepal/1679/04	40	160	1,280	640	2,560	320	7/2/04
A/Nepal/1685/04	40	320	1,280	640	2,560	320	7/2/04
A/Nepal/1670/04	10	160	1,280	320	1,280	320	7/2/04
A/Nepal/1659/04	10	320	640	320	1,280	160	7/2/04
A/Nepal/1660/04	10	160	640	320	2,560	160	7/2/04
A/Nepal/1680/04	20	320	320	320	1,280	160	7/2/04
A/Nepal/1672/04	10	160	320	160	1,280	160	7/2/04
A/Nepal/1678/04	40	160	320	160	1,280	160	7/2/04
A/Nepal/1694/04	10	160	320	160	2,560	160	7/3/04

*Test antigens are considered antigenically different from the reference strain if HI titers show a 4-fold difference.
†A/Korea/770/2002 is antigenically equivalent to the A/Fujian/411/02 vaccine strain.

Table 2. Unique hemagglutinin amino acid substitutions from influenza virus isolates obtained during July 2004 influenza outbreak in southeast Nepal compared with 5 vaccine strains*

	Amino acid position					
Virus strain	145 Glycosylation site adjacent to antibody site A	155 Fujian-like lineage amino acid substitution	156 Fujian-like lineage amino acid substitution	189 Antibody site B	226 Antibody site D	227 Antibody site D
A/Nepal Consensus/04†	N	T	H	N	I	P
A/Fujian/411/02	K	T	H	S	V	S
A/Wyoming/3/03	K	T	H	S	I	S
A/Wellington/1/04	K	T	H	N	V	P
A/California/7/04	N	T	H	N	I	P
A/Panama/2007/99	K	H	Q	S	V	S

*N, asparagine; T, threonine; H, histidine; I, isoleucine; P, proline; K, lysine; S, serine; V, valine; Q, glutamine.
†Consensus sequence derived from a multiple sequence protein alignment of 26 HA1 hemagglutinin sequences from Nepal.

position 227 (shown in magenta) appears to marginally affect the HA surface features. This substitution resides within antibody-binding site D, which corresponds to residues 225–228, which make up the left side of the receptor-binding pocket (14). Interestingly, this proline residue is located within a β barrel (a protein motif consisting of an antiparallel β sheet domain) and does not appreciably alter the predicted protein structure, as shown by the absence of any substantial changes in the computer-modeled, 3-dimensional structure compared with the HA1 of A/Wyoming/3/2003.

Discussion

The 4 substitutions described represent a growing lineage of influenza A (H3N2) viruses characterized since July 2004. Three amino acid changes are confined within known antibody-binding sites, i.e., the S189N change within antibody-binding site B (4,5) and the V226I and S227P changes residing in antibody-binding site D (4,5). Because of rotational restrictions, a proline substitution at position 227 (S227P) would typically give rise to considerable conformation change; however, this particular substitution is located within a β barrel motif and therefore has little effect on regional protein conformation. Cumulatively, field isolates characterized subsequent to this outbreak continue to exhibit these 4 changes, and they appear to constitute a distinct branch in the phylogeny of HA sequences when compared with H3N2 isolates from the 2003–2004 season.

The K145N mutation represents a change from a charged to uncharged amino acid R group. This change may affect protein-protein interactions since it is immediately adjacent to antibody-binding site A, where neutralizing antibodies have been shown to bind (4,5). Furthermore, since the K145N substitution is located within a glycosylation site, the charge alteration may affect glycosyl transferase activity, which results in altered glycosylation. Differences in glycosylation have been shown to contribute to antigenic variation by preventing antibody binding to antigenic sites (15). Additionally, 3-

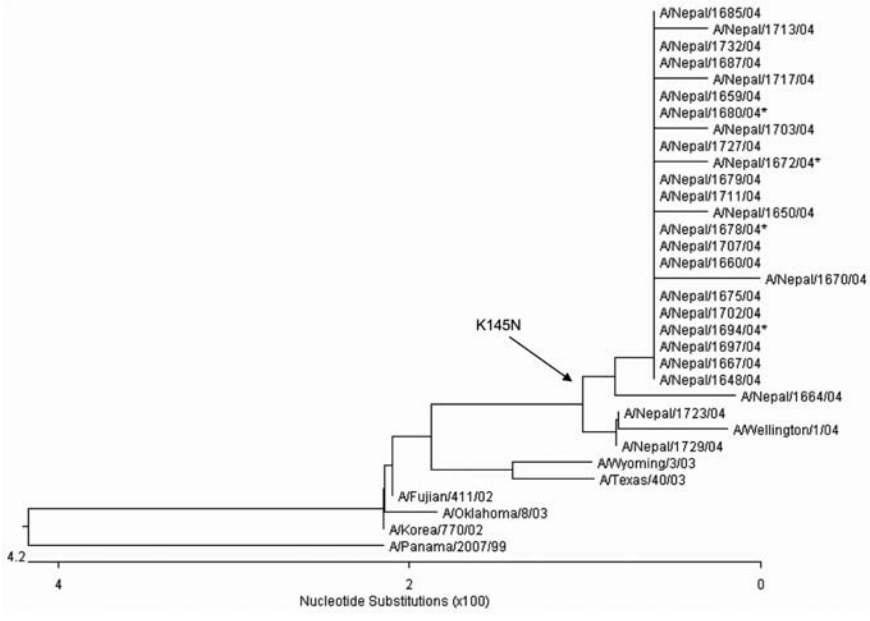

Figure 2. Unrooted phylogenetic analysis of HA1 hemagglutinin nucleotide sequences from 26 Nepal isolates and H3N2 vaccine and reference strains. The Nepal isolates have drifted from the 2004–2005 A/Fujian/411/03 vaccine strain (and A/Wyoming/03/03 vaccine seed strain) and are genetically equivalent to A/California/7/04, the 2005–2006 Northern Hemisphere vaccine strain. A K145N substitution (branch point indicated by the arrow) was observed in 24 of 26 Nepal isolates and represents a genetic marker for the dominant lineage of H3N2 viruses during the 2004–2005 season. Nucleotide and amino acid sequences for all Nepal isolates are available from GenBank under accession no. AY945263–AY945288. The asterisk indicates isolates from Table 2 that were antigenically distinct from A/Wyoming/303.

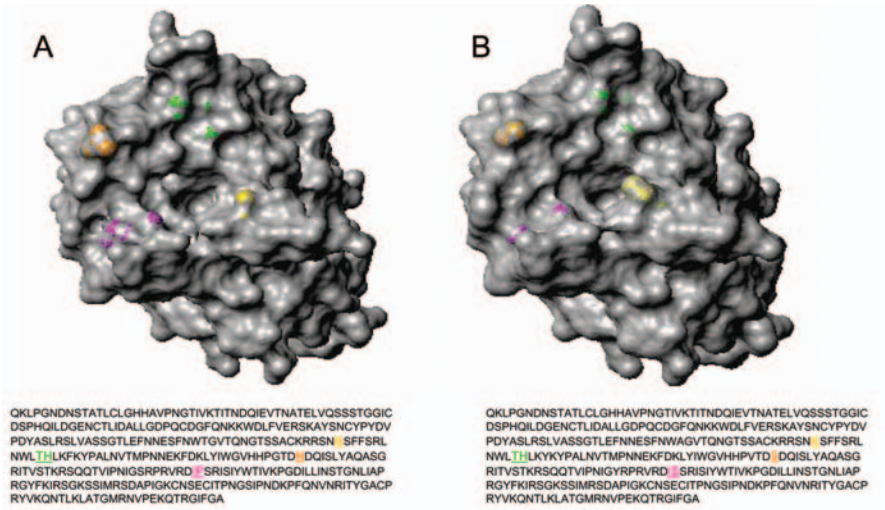

Figure 3. Three-dimensional top view of the HA1 hemagglutinin structures for A) a representative A/Nepal/1648/04 virus and B) vaccine strain A/Wyoming/3/03. Most (24/26) of the Nepal isolates contain a lysine to asparagine substitution (shown in yellow) at position 145 (K145N). Magenta, residues 226 and 227; orange, residue 189; green, residues 155 and 156; yellow, residue 145. Hemagglutinin molecules were generated by using the respective amino acid sequences with MOLMOL (12). A/Nepal/1648/04 is available from GenBank under accession no. AY945264.

dimensional analysis suggests this amino acid substitution may also promote enhanced receptor binding since the asparagine R group is shorter, which may make binding requirements less stringent and the receptor cleft more accessible. The 3-dimensional depiction provides a unique regional residue perspective, demonstrating how the rapidly evolving HA surface antigens in the vaccine strain differ at the molecular level. These changes are consistent with both antigenic and genetic data.

Collectively, the clinical isolates obtained from this outbreak in Nepal cannot be considered antigenically distinct from the A/Wyoming/3/03 vaccine strain because only 4 of 9 isolates evaluated exhibited 4-fold lower titers by HI (Table 1). Furthermore, the varying reactivity noted in several isolates from this outbreak having identical HA1 sequences is suggestive that other viral antigens aside from the HA1 protein may have contributed to the antigenic variability observed in the HI panel.

With the exception of A/Nepal/1670/2004 and A/Nepal/1672/2004, all isolates evaluated by HI (Table 1) exhibited identical HA1 amino acid sequences and varying antigenicity profiles to A/Wyoming/03/2003 reference antisera. One explanation for this observation is that genetic differences in other influenza surface proteins contribute to the observed immunoreactivity. Alternative viral surface protein candidates include the neuraminidase, HA2, and M2 glycoproteins, which have been shown to exhibit antigenic properties (16–19).

In this report, we describe the genetic analysis of the HA proteins from viruses obtained from an early season outbreak and compare them to current vaccine strains. Three amino acids changes (S189N, I226V, and S227P) were noted in known (4,5) antibody-binding sites (Table 2). The fourth change (K145N), which was located within a glycosylation site, may enhance viral binding since the smaller asparagine R group is located close to the HA receptor-binding cleft (Figure 3). Phylogenetic analyses show that the Nepal isolates make up a distinct branch in the evolution of H3N2 viruses when they are compared with vaccine and reference strains (Figure 2). However, antigenic data appear more ambiguous, suggesting a multigenic effect that cannot solely be attributed to properties of the influenza HA (Table 1). Studies are in progress to characterize the neuraminidase, M2, and HA2 proteins to determine the molecular basis responsible for antigenicity differences observed within isolates from this outbreak.

The K145N substitution change has become a marker for an increasingly large subset of the Fujian-like viruses. CDC and the US Department of Defense have recently characterized viruses with the K145N change in Singapore, Taiwan, China, Australia, Canada, and the United States. In February 2005, WHO reported the emergence of a new influenza H3N2 strain in the United States. The A/California/7/2004 strain, which was first identified in the United States in September 2004, contains all 4 changes observed in isolates from this Nepalese outbreak. The A/California/7/2004 strain differs by only 1 amino acid in HA1 (which is of no immunologic importance) from most isolates from the outbreak in Nepal. All viruses characterized (≈150 globally isolated strains) subsequent to the preparation of this report (March 2005) by the US Department of Defense are genetically similar in amino acid sequence to these Nepalese strains (and the A/California strain). Most of the isolates (80%) analyzed by CDC since October 2004 are antigenically related to A/California (20), which indicates that this strain has emerged as the dominant influenza A H3N2 strain. These data indicate that these viruses may persist as the dominant strain at the onset of the 2005–2006 influenza season. In February 2005, WHO recommended inclusion of an A/California/7/2004-like strain in the 2005–2006 trivalent influenza vaccine to afford immunologic protection from

this variant H3N2 virus. Our findings emphasize the importance of continued molecular surveillance for characterizing emerging influenza drift variants.

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cephalosporin

[sef′ə-lo-spor′in]

Any of a class of broad-spectrum, relatively penicillinase-resistant, β-lactam antimicrobial drugs originally derived from species of the fungus *Acremonium* (formerly called *Cephalosporium*). Italian scientist Giuseppe Brotzu first isolated the parent compound cephalosporin C from a sewer in Sardinia in 1948. Cephalosporins available for medical use today are semisynthetic derivatives of this natural antimicrobial compound.

Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003. and Merriam-Webster's collegiate dictionary. 11th ed. Springfield (MA): Merriam-Webster's, Inc; 2003.

Cephalosporin-resistant Pneumococci and Sickle Cell Disease

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Sickle cell anemia patients have 600 times the risk for invasive pneumococcal disease than their healthy peers. High-level cephalosporin resistance was described in the 1990s in healthy children from Tennessee, but its prevalence in sickle cell disease patients is unknown. Pneumococcal isolates from sickle cell disease patients from Tennessee were subjected to multilocus sequence typing to characterize antimicrobial drug–resistant strains. Twenty-one percent of strains were resistant to cefotaxime and penicillin. Of the 14 cephalosporin-resistant strains, 9 were sequence types previously described as highly cephalosporin resistant, while resistance was found for the first time in 3 clones: Maryland^{6B}, ST660, and a novel clone, ST1753. High-level cephalosporin resistance exists in more settings than initially recognized, and its high prevalence in sickle cell disease patients may decrease the efficacy of third-generation cephalosporins in invasive pneumococcal disease.

Streptococcus pneumoniae is a gram-positive bacterium that causes substantial illness and death in children. Children with sickle cell disease have an increased risk for invasive infection from this pathogen. Before the routine use of prophylactic measures, invasive pneumococcal disease was 600 times more likely to develop in patients with sickle cell disease than in their healthy peers (1). Thus, colonization with pneumococci is viewed as a high-risk event for sickle cell disease patients.

The risk for fatal infection increases if the patient is colonized with antimicrobial drug–resistant pneumococci. The prevalence of colonization with pneumococci is generally the same in healthy persons (12%) and sickle cell disease patients (7%) (2). However, penicillin-resistant pneumococci are consistently more common in children with sickle cell disease (62% versus 41% in healthy children) (2). A similarly high incidence of penicillin resistance (55%) in pneumococci infecting sickle cell disease patients was reported by Daw et al. (3) and has been sustained throughout the 1990s (4).

In the early 1990s, the Centers for Disease Control and Prevention described a series of community-acquired invasive infections in healthy children from Memphis, Tennessee, caused by pneumococci with unusually high resistance to extended-spectrum cephalosporins (5). These strains displayed MICs of cefotaxime and ceftriaxone from 4 to 32 μg/mL, exceeding the MICs of penicillin by as much as 5-fold (6). This finding is of clinical importance since it precludes using cephalosporins as a treatment option (6,7). Richter et al. extended the analysis of this resistant Tennessee cluster and identified a novel clone termed TN^{23F}-4 with MIC values of third-generation cephalosporins as high as 32 μg/mL (8,9).

The epidemiology of the cephalosporin-resistant TN^{23F}-4 clone in sickle cell disease patients is unknown. Colonization with this clone would have implications in terms of drug therapy, since this patient population routinely receives standard doses of extended-spectrum cephalosporins to treat invasive pneumococcal disease. Infection with a strain exhibiting high-level cephalosporin-resistance could result in treatment failure. In this study, we reexamined pneumococci collected from sickle cell disease patients in Memphis, Tennessee, from 1994 to 1995, the time of the original description of the TN^{23F}-4 clone, to determine the prevalence of this clone and any other highly cephalosporin-resistant clones circulating in the sickle cell disease population.

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Materials and Methods

Pneumococcal Strains

Sixty-four nasopharyngeal isolates were collected from 42 patients between July 1994 and December 1995 at the Mid-South Sickle Cell Center (3). Frozen strains were recovered by overnight growth on blood agar plates at 37°C, followed by resuspension in 15% glycerol solution. Strains were refrozen at –80°C for further use. Since this study was retrospective and used clinical pneumococcal strains, institutional review board permission was granted to review characteristics specific to the isolates themselves. Limited patient demographics were obtained, including patient diagnosis and penicillin prophylaxis. Antimicrobial susceptibility testing was conducted as described (3). Susceptibility breakpoints were defined according to the Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines for the years 1994 and 1995 (10,11). Pneumococcal isolates were serotyped by the slide agglutination method (12) with the Pneumotest-Latex Kit (Statens Serum Institut, Copenhagen, Denmark).

Genomic DNA Preparation

Genomic DNA was prepared by sodium dodecyl sulfate (SDS) lysis (13) and standard phenol:chloroform extraction (14). Each strain was grown in 20 mL casein/yeast broth (15) at 37°C in 5% CO_2 until turbid. Bacteria were harvested by centrifugation; resuspended in 500 μL (1:20 volume) iced buffer containing Tris-HCl, glucose, and EDTA (13); and treated with 15 μL 10% deoxycholate and 1.25 μL 10% SDS. After incubation at 37°C for 30 min, 30 μL 10% SDS was added and gently mixed by inversion. The mixture was incubated with 200 μg/mL proteinase K (Invitrogen, Carlsbad, CA, USA) overnight. An equal volume of phenol:chloroform:isoamyl (Invitrogen) was added to each sample and centrifuged for 5 min at 12,000 rpm. The upper phase was treated 2 more times. The extract was treated with 10% volume of 3 mol/L sodium acetate. DNA was then precipitated by adding 2 volumes of cold 95%–100% ethanol. The DNA pellet was treated twice with cold 70% alcohol. The resultant sample was air dried and resuspended in 20 μL distilled water.

Multilocus Sequence Typing

To assign the strains to a sequence type (ST), 7 housekeeping genes were subjected to polymerase chain reaction (PCR) amplification and DNA nucleotide sequencing: *aroE* (shikimate dehydrogenase), *gdh* (glucose 6-phosphate dehydrogenase), *gki* (glucose kinase), *recP* (transketolase), *spi* (signal peptidase I), *xpt* (xanthine phosphoribosyltransferase), and *ddl* (D-alanine-D-alanine ligase). The primer sets were obtained from the multilocus sequence typing (MLST) Web site (www.mlst.net). Fifty-microliter reaction mixtures were prepared with 1.25 U Taq polymerase (Applied Biosystems, Foster City, CA, USA), 1× Taq polymerase buffer (10 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KCl), 1.5 mmol/L MgCl, 0.2 mmol/L each deoxynucleoside triphosphate, and 0.2 mmol/L each primer. One microliter of genomic DNA was added to each reaction. The following parameters were used for amplification: denaturation at 95°C for 5 min, 30 subsequent cycles of amplification, each consisting of 1 min at 95°C, 1 min at 50°C, and 30 s at 72°C, with a final extension at 72°C for 7 min. PCR products were analyzed by electrophoresis on a 1.0% wt/vol agarose gel, and the amplicon size was evaluated by comparing it with 1-kb ladder (Invitrogen). PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Sequencing, with forward and reverse primers, was performed on the ABI 377 DNA sequencer with Big Dye chemistry (Applied Biosystems), according to ABI protocols, by the St. Jude Hartwell Center for Bioinformatics and Biotechnology.

Phylogenetic Analysis

Sequencing results were assembled by using SeqAssem version 09/2004 (http://www.gwdg.de/~dhepper/). Sequences were subsequently queried against the NCBI nonredundant database by using both nucleotide-BLAST [blastn] and protein-BLAST [blastp] programs and compared by alignment with the ClustalW algorithm in BioEdit (16). Concatenated DNA alignments of the 7 housekeeping genes were used for phylogenetic analysis. The phylogenetic relationship among the 64 pneumococcal strains was inferred by using the Bayesian approach (17), a variant of the maximum likelihood algorithm. Although eBURST (18,19) defines clonality based on 6/7 shared alleles, the Bayesian approach allows resolution of clonality based on 7/7 alleles. Thus, the Bayesian approach allows branch placement, due to differing alleles, into a paraphyletic clade versus clustering as a monophyletic clade. Clade credibility for the consensus tree topology was calculated by using MrBayes version 3.0b4 (20) with the following parameters: 1 million generations, 4 simultaneous Monte Carlo chains, and exclusion of the first 1,000 trees. The tree was rooted by using data from the *S. pneumonia*e TIGR4 strain (21) as an outgroup. An evolutionary model of nucleotide substitution was selected by using the MrModeltest program version 2.1 (22).

Results

We analyzed 64 nasopharyngeal strains from patients in whom homozygous sickle cell disease (HgbSS), hemoglobin SC sickle cell disease (HgbSC), or hemoglobin Sβ^+ thalassemia (HgbSβ^+) was diagnosed. Antimicrobial drug–susceptibility results are represented in Tables 1 and 2.

Table 1. Distribution of β-lactam resistance in nasopharyngeal pneumococcal isolates from sickle cell disease patients

Penicillin susceptibility*	No. isolates	Cefotaxime susceptibility*	No. isolates
Sensitive	31	Sensitive	31
Intermediate	24	Sensitive	19
		Intermediate	3
		Resistant	2
Resistant	9	Intermediate	3
		Resistant	6

*Definition of susceptibility per Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines from 1994 and 1995 (10,11). Sensitive, penicillin V MIC<0.06 μg/mL, cefotaxime MIC<0.05 μg/mL; intermediate, penicillin V MIC 0.12–1 μg/mL, cefotaxime MIC 1 μg/mL; resistant, penicillin or cefotaxime MIC>2 μg/mL.

Fifty-one percent (33/64) of the strains were penicillin-resistant (intermediate strains included). Of these strains, 14 (42%) of 33 were resistant to cefotaxime. No serotypes consistently correlated with antimicrobial drug resistance (Table 2).

All strains were subjected to MLST and phylogenetic analysis (Table 2, Figure). Isolates were designated as novel if they possessed ≥1 unrecognized alleles based upon known sequences listed in the MLST database. Isolates were designated as nontypeable (NT) if the allele profile was not listed in the MLST database. The 31 penicillin-sensitive strains were distributed broadly through 19 known STs (13, 43, 62, 124, 146, 176, 180, 205, 208, 425, 433, 439, 447, 547, 647, 690, 876, 899, and 1499), 2 putative novel strains (1752 and pending ST), and 3 NT strains (1755 and 1757). Intermediate penicillin resistance (n = 24) was also broadly represented by 8 known STs (37, 199, 236, 344, 384, 460, 660, and 690), 4 novel STs (1754 and 3 distinct STs with pending designations), and 2 NT sequence types (1756 and pending ST referred to as NT3) (Table 2).

In contrast to the broad ST distribution of sensitive and intermediate penicillin-resistant strains, high-level

Table 2. Multilocus sequence types of penicillin- and cephalosporin-resistant isolates*†

Strain	Serotype	Allele numbers							Sequence type	Resistance		MIC values	
		aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*		PNV	CTX	PNV	CTX
38	3	8	37	9	29	2	12	NA	Novel	I	S	0.38	0.50
48	6	8	37	9	29	2	12	53	344	I	S	0.38	0.19
1	6	7	6	9	2	6	1	67	384	I	I	1.00	0.75
49	6	5	7	4	10	10	1	27	460	I	NA	0.38	
63	6	5	7	4	10	10	1	27	460	I	S	0.38	0.50
35	6	1	25	1	8	15	20	14	660	I	I	1.00	0.75
81	6	1	25	72	1	15	20	28	690	I	S	0.094	0.094
84	6	2	13	8	6	6	3	NA	1754	I	NA	0.25	
75	6	7	25	4	4	15	20	NA	Novel	I	S	0.094	0.094
13	6	1	5	4	5	5	3	101	NT3	I	S	0.38	0.19
29	6	1	5	4	5	5	3	101	NT3	I	S	0.94	0.25
19	6	7	6	9	2	6	1	67	384	R	R	3.00	3.00
22	14	2	8	7	4	6	1	1	67	R	I	4.00	1.00
27	14	2	8	7	4	6	1	1	67	R	I	4.00	1.00
57	14	2	8	7	4	6	1	1	67	R	I	2.00	1.00
59	14	2	8	7	4	6	1	1	67	R	I	3.00	1.50
60	19	8	13	14	4	17	4	14	199	I	S	0.125	0.064
70	19	8	13	14	4	17	4	14	199	I	S	0.190	0.094
79	19	8	13	14	4	17	4	14	199	I	S	0.125	0.064
80	19	8	13	14	4	17	4	14	199	I	S	0.094	0.064
24	19	15	16	19	15	6	20	26	236	I	S	0.25	0.25
34	19	15	16	19	15	6	20	26	236	I	S	0.25	0.25
65	19	15	16	19	15	6	20	26	236	I	I	1.00	0.75
42	19	8	20	14	4	17	4	14	1756	I	S	0.190	0.094
77	19	8	20	14	4	17	4	14	1756	I	S	0.125	0.094
78	19	8	20	14	4	17	4	14	1756	I	S	0.190	0.064
41	19	15	NA	NA	NA	6	NA	NA	Novel	I	S	0.25	0.19
2	23	1	8	6	2	6	4	6	37	I	R	1.00	1.50
53	23	1	8	6	2	6	4	6	37	I	R	0.125	1.50
26	23	1	8	6	2	6	4	6	37	R	R	4.00	6.00
47	23	1	8	6	2	6	4	6	37	R	R	8.00	8.00
62	23	1	8	6	2	6	4	6	37	R	R	3.00	8.00
58	23	1	8	6	2	6	4	NA	1753	R	R	3.00	8.00

*PNV, penicillin V; CTX, cefotaxime; S, susceptible; I, intermediate; R, resistant; NT, nontypeable; NA, not available; ND, not done.
†An expanded version of this table, including isolates that were susceptible to penicillin, is available online at http://www.cdc.gov/ncidod/EID/vol11no08/05-0152.htm#table2

Figure. Bayesian analysis of the phylogenetic relationship among pneumococcal isolates from sickle-cell disease patients as determined by multilocus sequence type (MLST). ST, sequence type, as defined by the MLST database. Strains in **boldface** had recognized allele numbers but not recognized profiles according to the current database (listed as NT). Putative novel sequence types (those with unrecognized alleles) are in **boldface** and *italics*. Dots indicate resistance to penicillin. Boxes indicate sequence types determined to be resistant to cephalosporins. Red box, intermediate resistance, previously described; black box, highly resistant, previously described; yellow box, intermediate resistance, not previously described; white box, highly resistant, not previously described. Clade credibility scores >80% are listed on the tree.

penicillin and cephalosporin resistance was restricted to 4 STs (37, 67, 384, and the novel 1753). Two of these STs have been reported previously to be highly cephalosporin resistant: TN^{23F}-4 (ST37) and TN^{14}-18 (ST67) (23). Five (8%) of 64 strains were classified as the TN^{23F}-4 clone, all of which were highly resistant, and 4 (6%) were designated as TN^{14}-18; 1 of these was highly resistant and 3 were intermediate. Two STs, Maryland^{6B}-17 (ST384) and Taiwan^{19F}-14 (ST236), have been reported to be intermediately cephalosporin resistant (24,25). In this study cohort, 1 Maryland^{6B}-17 was highly resistant and 1 intermediate. One of the 3 Taiwan^{19F}-14 strains was intermediately resistant, while the other 2 were sensitive. Seven isolates (11%) of the study cohort represented distinct putative novel clones (ST1752, 1753, and 1754; additional designations from MLST database are still pending); 1 of these isolates (ST1753) was highly cephalosporin resistant. ST1753 is closely related to TN^{23F}-4 (ST37). Two of the newly recognized cephalosporin-resistant clones (ST384 and 660) were not closely related to each other or the TN clones (Figure).

Discussion

Although the incidence of carriage of penicillin-nonsusceptible pneumococcus is highly variable, depending on area (from <5% to <50%) (7,26), the incidence is generally increasing worldwide. Children with sickle cell disease routinely receive penicillin prophylaxis as well as empiric therapy with third-generation cephalosporins and have a higher rate of carriage of penicillin-resistant strains (2–4).

We analyzed cephalosporin resistance in pneumococcal nasopharyngeal isolates collected from 1994 to 1995 from patients with sickle cell disease in Memphis, a time and place corresponding to the initial description of the highly cephalosporin-resistant TN^{23F}-4 clone. Fifty-one percent of strains were penicillin resistant, a percentage consistent with that seen in many previous sickle cell disease studies (2–4). Strikingly, 14 (21%) of 64 isolates were resistant to cefotaxime, 12.5% at high level, and all of the cephalosporin-resistant strains were also resistant to penicillin. Although this sample is small and precludes the ability for direct comparison, this percentage is much greater than the 4.6% reported for clone TN^{23F}-4 (8). Nine of the 14 cephalosporin-resistant strains were either the TN^{23F}-4 clone or TN^{14}-18, the 2 sequence types reported previously to be highly cephalosporin resistant (5,6,23). Of the remaining 5 cephalosporin-resistant strains, 4 STs, Maryland^{6B}-17 (25), Taiwan^{19F}-14 (24), ST660, and a novel clone ST1753, had increased levels of cephalosporin resistance not previously described. Of these newly described resistant strains, only the novel clone was closely related to either TN clones, suggesting that high-level third-generation cephalosporin resistance exists in a wider array of backgrounds than previously recognized.

Fifty-three (80%) of the 66 strains had serotypes contained in the Prevnar vaccine (Wyeth Pharmaceuticals, Philadelphia, PA, USA), including all cefotaxime-resistant strains. Routine administration of Prevnar since 2000 (5 years after these strains were collected) is likely to have protected most of the sickle cell disease population from risk for cephalosporin-resistant disease. However, the

possibility of colonization with nonvaccine serotypes or the extension of cefotaxime resistance into previously unrecognized backgrounds should not be underestimated.

Aggressive management is warranted to prevent death from invasive pneumococcal infections in children with sickle cell disease. With the increasing prevalence of penicillin and extended-spectrum cephalosporin resistance in the sickle cell disease population, alternative antimicrobial drug therapies may be needed for prophylaxis. If the high prevalence of third-generation cephalosporin resistance is documented in the sickle cell disease population in other geographic areas, extended-spectrum cephalosporins should be reconsidered as empiric therapy when invasive pneumococcal disease is suspected. As antimicrobial drug alternatives are eliminated, vaccination becomes more important as the mainstay of prophylactic management of disease.

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Pseudomonas aeruginosa, *Staphylococcus aureus*, and Fluoroquinolone Use

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Few long-term multicenter investigations have evaluated the relationships between aggregate antimicrobial drug use in hospitals and bacterial resistance. We measured fluoroquinolone use from 1999 through 2003 in a network of US hospitals. The percentages of fluoroquinolone-resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) were obtained from yearly antibiograms at each hospital. Univariate linear regression showed significant associations between a hospital's volume of fluoroquinolone use and percent resistance in most individual study years (1999–2001 for *P. aeruginosa*, 1999–2002 for *S. aureus*). When the method of generalized estimating equations was used, a population-averaged longitudinal model incorporating total fluoroquinolone use and the previous year's resistance (to account for autocorrelation) did not show a significant effect of fluoroquinolone use on percent resistance for most drug-organism combinations, except for the relationship between levofloxacin use and percent MRSA. The ecologic relationship between fluoroquinolone use and resistance is complex and requires further study.

Antimicrobial drug resistance in bacterial pathogens is of national and international concern (1,2). Although use of antimicrobial agents is accepted as a major driving force behind the spread of resistance, the nature of this relationship is complex (3). Two problematic nosocomial pathogens are *Pseudomonas aeruginosa* and *Staphylococcus aureus*; both often express multidrug resistance. A number of case-control studies at individual hospitals have identified fluoroquinolone use as a risk factor for acquisition of fluoroquinolone-resistant *P. aeruginosa* and methicillin-resistant *S. aureus* (MRSA) (4–8). While outcomes for individual patients are most important from a clinical point of view, an ecologic perspective is also useful to assess the relationship of aggregate antimicrobial drug use to aggregate measures of bacterial resistance. Ecologic investigations across multiple hospitals have reported significant correlations between fluoroquinolone use and percent resistance for MRSA (9) and *P. aeruginosa* (10,11). However, these studies have primarily focused on teaching institutions, used drug expenditure data rather than hospital billing records as a measure of use, or were conducted over a limited time span. We measured fluoroquinolone use as well as the percentages of MRSA and fluoroquinolone-resistant *P. aeruginosa* across 24 US hospitals during a 5-year period. The purpose of this observational study was to determine if volume of aggregate fluoroquinolone use in individual hospitals and bacterial resistance in individual years and during the entire study period are associated.

Methods

Participating Hospitals

Hospitals included in this study were participants in the Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE)–MediMedia Information Technology (MMIT) Antimicrobial Surveillance Network. MMIT (http://www.mminfotech.com) is a healthcare informatics corporation that extracts drug-use data from hospital billing records. Data collection for this project began in 1999 with 19 participating hospitals; that number increased to 48 hospitals in 2003. Of these, 15 hospitals in 1999, 23 hospitals in 2000 and 2001, and 24 hospitals in 2002 and 2003 provided adequate drug-use and microbiology data

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for inclusion in this study. Demographic data for the hospitals in the year 2002 were obtained from the MMIT database and the American Hospital Directory (http://www.ahd.com). Members of the Council of Teaching Hospitals and Health Systems (http://www.aamc.org/members/coth/start.htm) were designated as teaching hospitals.

Measurement of Hospital Fluoroquinolone Use

Total grams for each fluoroquinolone used during each year were electronically extracted from individual patient billing records and aggregated to reflect hospitalwide usage. The total number of patient days (PD) for the corresponding time period at each hospital was determined from the sum of individual patient lengths of stay. These data were used to express normalized antimicrobial drug use in defined daily doses per 1,000 patient days (DDD/1,000PD) as recommended by the World Health Organization (WHO) (http://www.whocc.no/atcddd/). The DDDs used were levofloxacin 500 mg, moxifloxacin 400 mg, gatifloxacin 400 mg, and ciprofloxacin 1,000 mg. (Because the MMIT database does not indicate the proportion of intravenous versus oral ciprofloxacin used, we used the oral DDD for ciprofloxacin of 1,000 mg for all ciprofloxacin use).

Measurement of Hospital Susceptibility for *P. aeruginosa* and *S. aureus*

Hospital antibiograms were requested from each participating hospital for each study year. To be included in the analysis, antibiograms must have reported data on organisms from all clinical sites (i.e., systemic and urinary isolates) and all units (including intensive care units). Ciprofloxacin or levofloxacin susceptibility was used to determine the percentage of fluoroquinolone-resistant *P. aeruginosa*. Oxacillin or nafcillin susceptibility was used to determine the percentage of MRSA.

Statistical Analysis

Mixed-effects repeated-measures analysis of variance was used to analyze changes in fluoroquinolone use and resistance over the study period, and the Tukey HSD (honestly significantly difference) test was used to compare differences between individual years. Relationships between total and individual fluoroquinolone usage and resistance in target pathogens for each year were determined by univariate linear regression. To analyze the relationship between fluoroquinolone use and percent resistance over the course of the study period, the method of generalized estimating equations (GEE) was used to construct a population-averaged longitudinal model (12). Unlike traditional least squares regression, GEE models do not assume that each observation is statistically independent. Instead, only observations across units are assumed to be independent (i.e., the group of observations from the first hospital are independent from the observations from the second hospital). Other methods, such as generalized least squares, can also account for this correlation of observations within a given unit; however, these methods are based on the assumption that the correlation structure among observations from the same unit is correctly specified. The GEE method provides some protection against this misspecification. This protection can be improved by using a modified sandwich variance estimator to calculate robust standard errors. The model constructed in the current study assumed a first-order autoregressive correlation structure since a 1-year lagged resistance term was used in the model to control for the prior year's resistance. GEE methods also allow data across time points to be analyzed simultaneously, rather than in a year-by-year fashion. Therefore, this GEE model represents the longitudinal change in the outcome in relation to the longitudinal change in the set of predictor variables using all of the available data. A p value <0.05 was considered significant, and all tests were 2-tailed. Because of the exploratory nature of this analysis, p values from univariate linear regressions were not adjusted for multiple testing.

Results

Characteristics of Study Hospitals

Table 1 shows the demographic characteristics of the study hospitals in the year 2002. Ten hospitals were designated as teaching hospitals. The location of the hospitals was predominantly from the East (12 hospitals), with 7 hospitals from the South, 3 from the Midwest, and 2 from the West. No significant relationships were seen between resistance in *P. aeruginosa* or percent MRSA in the year 2002 and any of the demographic characteristics by univariate linear regression ($p>0.05$ for all comparisons).

Fluoroquinolone Use

Figure 1 shows changes in the mean of total and individual fluoroquinolone use from 1999 through 2003. For that period, the mean of total fluoroquinolone use increased from 119.6 ± 45.6 DDD/1,000 PD in 1999 to 150.4 ± 44.4 in 2003 ($p = 0.011$). These changes were driven by the early increase in fluoroquinolone use, as mean values were not significantly different in later (2000–2003) study years ($p>0.05$, Tukey HSD). Changes in levofloxacin use during the entire study were also significant ($p = 0.016$), but changes in ciprofloxacin use were not ($p = 0.186$). Fluoroquinolone use in individual hospitals was significantly correlated with the previous year's use ($r>0.75$ for all years).

The diversity of use of individual fluoroquinolones in hospitals changed during the study period. Figure 1 shows

Table 1. Demographic characteristics of study hospitals, year 2002*

Characteristic	Mean ± SD	Median (range)
No. admissions	19,122 ± 12,208	14,720 (5,206–40,676)
No. patient-days	96,488 ± 64,719	76,408 (19,244–219,634)
Case mix index	1.51 ± 0.245	1.52 (1.13–2.01)
Length of hospital stay, d	5 ± 0.67	5 (3.6–6.6)
No. staffed beds	358 ± 203	310 (105–778)
No. intensive care unit beds	22 ± 16	18 (3–80)
No. surgical procedures/1,000 admissions	346 ± 184	281 (163–779)

*SD, standard deviation.

the mean use of individual fluoroquinolones across all hospitals. The number of hospitals in which a particular fluoroquinolone represented most total fluoroquinolone use also changed. In 1999, levofloxacin use represented >90% of total fluoroquinolone use in 4 (27%) of 15 hospitals; 1 hospital used >90% ciprofloxacin. In 2003, 8 (33%) of 24 hospitals used >90% levofloxacin while in 7 hospitals (30%) moxifloxacin and ciprofloxacin combined to account for >90% of total fluoroquinolone use. The percentage of MRSA and fluoroquinolone-resistant *P. aeruginosa* in 2003 was compared between the 8 hospitals that predominantly used levofloxacin, the 7 that predominantly used moxifloxacin and ciprofloxacin, and the remaining 9 hospitals that used a mixture of fluoroquinolones. No significant differences in mean percent resistance were found between the groups (predominant levofloxacin, predominant moxifloxacin/ciprofloxacin, or neither) for either pathogen in 2003.

Antimicrobial Drug Resistance

Figure 1 shows the changes in mean percentage resistance for the studied pathogens from 1999 to 2003. Most hospitals reported the number of isolates tested; at least 100 isolates of *S. aureus* and 25 isolates of *P. aeruginosa* were tested per year. From 1999 to 2003, mean percent fluoroquinolone resistance in *P. aeruginosa* increased from 33.6% to 40.5% ($p = 0.001$). Mean percent MRSA increased from 42.1% in 1999 to 50.9% in 2003 ($p<0.0001$). Correlations between percent resistance in a given year and percent resistance in the previous year were high for both pathogens ($r>0.8$).

Associations between Fluoroquinolone Use and Percent Resistance in Individual Years

The results of the univariate regressions between fluoroquinolone use and resistance are summarized in Table 2. For *P. aeruginosa*, significant relationships occurred between total fluoroquinolone use and resistance in the same year for 1999, 2000, and 2001; the relationship had borderline significance in 2002 ($p = 0.0562$) and was not significant in 2003. Total fluoroquinolone use was associated with percent MRSA in every year except 2003. Table 2 also shows the results of univariate regression with the individual fluoroquinolones levofloxacin and ciprofloxacin. Levofloxacin use was a significant predictor of fluoroquinolone-resistant *P. aeruginosa* in 1999, 2000, and 2001. Increasing levofloxacin use was significantly associated with increased percent MRSA for all study years except 2003. For ciprofloxacin, a negative slope (increasing ciprofloxacin use associated with decreased percent resistance) was observed for most associations with percent resistant *P. aeruginosa* and MRSA, but none of these associations were significant.

Modeling of Relationship between Fluoroquinolone Use and Resistance

Table 3 shows the results of population-averaged GEE models using data over the study period from 2000 to 2003. The data from 1999 were not used as an outcome variable because of the lagged resistance term used in the model. After adjusting for the previous year's percent resistance, fluoroquinolone use was generally associated with a small and nonsignificant effect on percent resistance in a given year for either pathogen. Levofloxacin did display a significant contribution to percent resistance in MRSA; the coefficient of 0.012 suggests that an additional 100 DDD/1,000PD would lead to a 1.2% increase in percent MRSA over the previous year ($p = 0.033$).

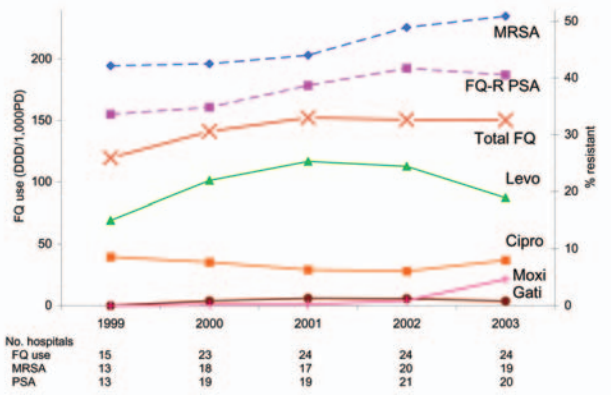

Figure 1. Fluoroquinolone use and resistance over study period. FQ, fluoroquinolone; Levo, levofloxacin; Cipro, ciprofloxacin; Moxi, moxifloxacin; Gati, gatifloxacin; DDD/1,000PD, defined daily doses/1,000 patient-days; FQ-R PSA, fluoroquinolone-resistant *Pseudomonas aeruginosa*; MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 2. Associations between fluoroquinolone use and resistance in individual years*

		FQ-R *P. aeruginosa*			MRSA		
FQ use		Total FQ	Levofloxacin	Ciprofloxacin	Total FQ	Levofloxacin	Ciprofloxacin
1999	R^2	**0.525**	**0.593**	0.233	**0.392**	**0.644**	0.255
	p	**0.008**	**12**	0.116	**0.029**	**0.0017**	0.0937
	n	**12**	**0.003**	12	**12**	**12**	12
2000	R^2	**0.397**	**0.285**	0.519	**0.617**	**0.617**	0.112
	p	**0.004**	**0.019**	0.348	**0.0001**	**0.0001**	0.175
	n	**19**	**19**	19	**18**	**18**	18
2001	R^2	0.481	0.335	0.067	**0.318**	**0.32**	0.159
	p	0.001	0.009	0.2821	**0.018**	**0.018**	0.1134
	n	19	19	19	**17**	**17**	17
2002	R^2	0.178	0.112	0.09	**0.267**	**0.28**	0.145
	p	0.056	0.137	0.184	**0.019**	**0.017**	0.097
	n	21	21	21	**20**	**20**	20
2003	R^2	0.104	0.012	0.01	0.157	0.02	0.001
	p	0.16	0.641	0.66	0.092	0.538	0.9924
	n	20	20	20	19	19	19

*Linear regression of fluoroquinolone (FQ) use versus percent resistance for hospitals. R^2, coefficient of determination; n, number of hospitals. **Bold** indicates significant relationships ($p<0.05$). FQ-R *P. aeruginosa*, fluoroquinolone-resistant *Pseudomonas aeruginosa*; MRSA, methicillin-resistant *Staphylococcus aureus*.

Figure 2 shows the baseline-to-endpoint changes in fluoroquinolone use and resistance in *P. aeruginosa* and MRSA for those 9 hospitals with complete data from 1999 to 2003. Fluoroquinolone use increased in 7 hospitals (arrows pointing to the right), and the percentage of fluoroquinolone-resistant *P. aeruginosa* increased in 7 and decreased in 1 (Figure 2A). For the 2 hospitals that reduced total fluoroquinolone use, percent resistance increased slightly in both. The percentage of MRSA increased in 7 hospitals; 5 of these increased fluoroquinolone use and 2 decreased use. The percentage of MRSA decreased in 2 hospitals; both increased quinolone use during the same period (Figure 2B).

Discussion

The results of this longitudinal, multicenter study of fluoroquinolone use and bacterial resistance suggest a complex relationship between fluoroquinolone use in hospitals and percentage of methicillin-resistant *S. aureus* and fluoroquinolone-resistant *P. aeruginosa* when viewed from an ecologic level. Fluoroquinolone use was linked to greater percent resistance in study years by univariate linear regression. This observation is consistent with the results of other studies linking fluoroquinolone use to resistance in these pathogens (4–11), although the pathways for selection of resistance differ between the 2 pathogens. Fluoroquinolone resistance in *P. aeruginosa* is believed to arise largely from the selection of organisms with point mutations in the topoisomerase enzymes that are targets for the fluoroquinolones (13). This hypothesis is supported by studies demonstrating the emergence of resistance during therapy with fluoroquinolones (4). For methicillin-resistant *S. aureus*, de novo emergence of resistance as seen with *P. aeruginosa* is not a common event (14); rather, patients are generally believed to acquire methicillin-resistant strains of *S. aureus* from the environment (e.g., through cross-transmission); antimicrobial drug use may increase the likelihood of colonization or amplify the resistant population after colonization (15). Fluoroquinolones are not active against most methicillin-resistant isolates (16), providing a selective pressure for MRSA. While any antimicrobial agent that is not active against MRSA should increase a patient's risk for infection, fluoroquinolones may be particularly likely to do so, since they have appear to have unique effects on the expression of MRSA resistance determinants (17) and fibronectin-binding proteins (18).

Using a model incorporating the previous year's percent resistance as well as fluoroquinolone use and time over the entire study period, we did not find an additional effect of total fluoroquinolone use on percent resistance. Many factors beyond the volume of use of an antimicrobial agent affect the emergence and spread of antimicrobial resistance to that drug in the hospital setting. Cross-transmission between patients, acquisition of organisms from the hospital environment, and the use of different antimicrobial agents with linked resistance to the agent under study are all factors that also affect the number of resistant isolates in a given hospital (19–21). These factors are difficult to control for, since quantitative measures of infection control are lacking, and examining the effects of multiple antimicrobial agents complicates analysis. We also did not account for antimicrobial drug use in the community, which we have previously reported to be associated with hospital resistance rates (22). Because of the large number of variables that may influence resistance in the hospital setting, we would expect hospital fluoroquinolone use to have at best a modest effect on resistance. The

Table 3. Longitudinal GEE models*

	FQ-R *P. aeruginosa*		MRSA	
Drug	Coefficient	p value	Coefficient	p value
Total FQ				
Previous year's resistance	0.875	<0.001	0.804	<0.001
Total FQ use	0.002	0.883	0.025	0.155
Time	-0.312	0.554	1.04	0.040
Constant	6.75	0.001	4.61	0.058
Levofloxacin				
Previous year's resistance	0.868	<0.001	0.818	<0.001
Levofloxacin use	0.005	0.548	0.012	0.033
Time	-0.317	0.579	1.04	0.041
Constant	6.78	0.001	6.38	0.001
Ciprofloxacin				
Previous year's resistance	0.866	<0.001	0.845	<0.001
Ciprofloxacin use	-0.018	0.226	-0.004	0.848
Time	-0.393	0.475	0.991	0.079
Constant	8.185	<0.001	6.55	0.033

*Association of fluoroquinolone and pathogen resistance over time controlling for prior year resistance. GEE, generalized estimating equations; FQ-R *P. aeruginosa*, fluoroquinolone-resistant *Pseudomonas aeruginosa*; MRSA, methicillin-resistant *Staphylococcus aureus*.

results of our model suggest that the ecologic effect size of fluoroquinolone use is such that substantial changes in fluoroquinolone use may be required to affect percent resistance. Thus, a larger sample size, which incorporates more hospitals over a greater period, may be required to demonstrate significance, if such effects exist.

Because percent antimicrobial resistance in a given period is highly correlated to previous percent resistance (i.e., the observations are not independent), we incorporated the previous year's percent resistance into our longitudinal model. Failing to account for this autocorrelation may result in spurious associations (23). However, our use of a 1-year lag period was imposed by the nature of our data (which was aggregated on a yearly basis) rather than biologic considerations and may not be the most accurate method of modeling this relationship. We did not time-lag the effect of fluoroquinolone use. Monnet et al. used time-series analysis with autoregressive integrative moving average (ARIMA) functions to model the changes in percent MRSA in a hospital in Scotland during a 3-year period (24). Their transfer function model for predicting percent MRSA used the previous month's percent MRSA; the effect of fluoroquinolone use had a 4-month lag in the effect of changes in fluoroquinolone use on percent MRSA. No study has specifically examined lag effects with fluoroquinolone use and fluoroquinolone-resistant *P. aeruginosa*, although Lopez-Lozano et al. used a lag period of 3 and 5 months in percent resistance and a 1-month lag in ceftazidime use to model the relationship between ceftazidime use and ceftazidime-resistant gram-negative bacilli (25). Thus, use of yearly antibiograms may not be adequate to properly model the relationships between percent resistance in a given year, previous resistance, and fluoroquinolone use. Future studies should attempt to attain more detailed data to allow for more flexibility in modeling the relationship between antimicrobial use and resistance.

The results of this study raise a number of questions for further investigation. Why did the association between fluoroquinolone use and resistance become weaker in the later study years? Does this finding represent random fluctuation or an underlying trend? Mean fluoroquinolone use, after increasing through the first 3 study years, reached a plateau in the last 2 years (Figure 1). Perhaps associations with resistance are reflected most strongly when antimicrobial drug use is increasing, as was seen in the first 3 study years. The study by Zervos et al. (11) found a significant association between changes in fluoroquinolone use and changes in resistance in *P. aeruginosa* in 10 teaching hospitals from 1991 to 2000. During this period, fluoroquinolone use increased by a mean of 97% in the participating hospitals; in the present study, the mean increase during the study period was 23%. Also, the percentage of resistance among MRSA and *P. aeruginosa* was relatively high in all hospitals from the beginning of this study. Monnet et al., in their study of the impact of antimicrobial drug use on an outbreak of MRSA in Scotland, observed that "…antimicrobial drug use was a more important ecologic risk factor at the start of the outbreak than once MRSA had become endemic in the hospital" (24). After antimicrobial use "drives" resistance to a certain point, other effects such as cross-transmission may become the dominant mode of spread. From a scientific standpoint, this finding suggests that ecologic studies may be more likely to detect a significant effect when a particular form of resistance is in its "infancy" (current examples might be linezolid-resistant *S. aureus* or fluoroquinolone-resistant *Streptococcus pneumoniae*). From a clinical point of view,

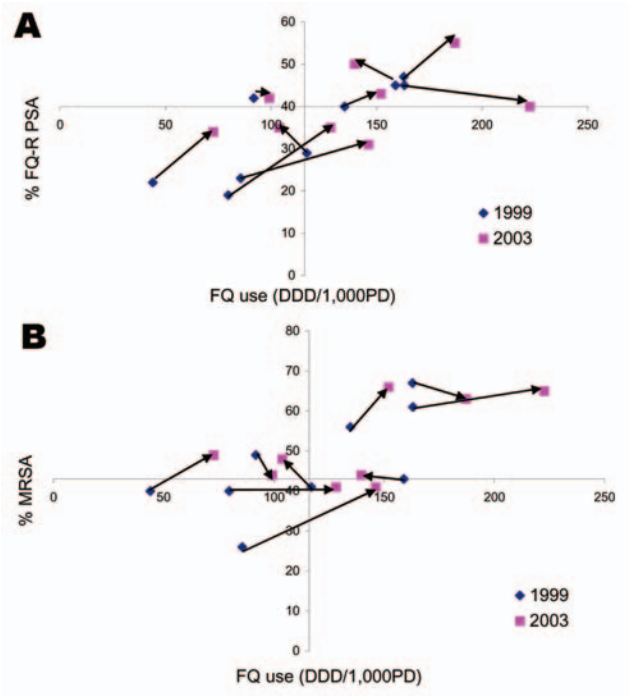

Figure 2. A) Changes in fluoroquinolone use (x axis) and resistance in *Pseudomonas aeruginosa* (y axis) for 9 hospitals with complete data, 1999–2003. Origin is median values of fluoroquinolone use and resistance in 1999. DDD/1,000 PD, defined daily doses/1,000 patient-days. FQ-R PSA, fluoroquinolone-resistant *P. aeruginosa*; MRSA, methicillin-resistant *Staphylococcus aureus*. B) Changes in fluoroquinolone use (x axis) and resistance (y axis) in *S. aureus* for 9 hospitals with complete data, 1999–2003.

this finding suggests that measures to ensure proper antimicrobial drug use would have the most effect before resistance becomes widespread.

Also of interest is the effect of individual fluoroquinolones on resistance. In our univariate analyses, levofloxacin use had a much stronger association with resistance in both pathogens than did use of ciprofloxacin. Levofloxacin also showed the only significant relationship (with percent MRSA) among the longitudinal models. However, the univariate models did not control for prior resistance levels. Also, the population-averaged modeling approach results in a pooling of effects across all hospitals, resulting in some degree of effect attenuation. Indeed, the estimates from a GEE analysis can sometimes be smaller than those estimates produced from a corresponding mixed-effect model (12). This apparent effect attenuation can be viewed as a trade-off for the reduced vulnerability to model misspecification, as previously discussed. Relationships between individual fluoroquinolone use and resistance should be interpreted with caution, as it is possible that our study is biased (an unknown confounder that is causally associated with MRSA or resistant *P. aeruginosa* occurs more frequently in hospitals that use more levofloxacin). However, other ecologic studies have found similar results. At a single teaching institution in an 8-year period, Mohr et al. observed an association between increasing levofloxacin use and increasing percent resistance in *P. aeruginosa*; this effect was not observed for total fluoroquinolone use or use of other fluoroquinolones (26). An ecologic study across 174 hospitals across 6 years by Bhavnani et al. showed that increasing use (measured as drug expenditures) of levofloxacin and ofloxacin, but not ciprofloxacin, was associated with increased percent resistance to ciprofloxacin in *P. aeruginosa* (10). This ecologic effect might arise from a greater in vitro potential for levofloxacin to select for resistant mutants of *P. aeruginosa*, as Gilbert and colleagues have observed (27). For MRSA, a different relationship between fluoroquinolone use and resistance is relevant, since the emergence of methicillin resistance during therapy is not of concern. When viewed from the perspective of selecting for preexisting methicillin-resistant, fluoroquinolone-resistant *Staphylococcus aureus* from a population that contains both resistant and susceptible isolates, levofloxacin might be more likely to select for the preexisting resistant clones because of its greater activity against the susceptible organisms. In such a scenario, the proportion of resistant isolates (percent resistance) may increase, although the incidence of infections due to resistant bacteria may be relatively stable, as argued by Schwaber et al. (28). Thus, analysis of incidence rates of isolation of resistant organisms may tell a different story from analysis of percentage of resistant organisms. We did not determine incidence rates because some of our study hospitals did not include the number of organisms isolated on their antibiograms. Although percent resistance is most likely of concern to a clinician, the incidence rates determine the overall effect on impact of resistance from an ecologic perspective. Future studies should incorporate resistance rates alongside changes in percent resistance to give the complete picture of the effect of antimicrobial drug use on resistance. The differential effects of individual fluoroquinolones on antimicrobial drug resistance are an important area for future study, as hospitals manipulate their formularies with regard to use of individual fluoroquinolones, often for economic reasons.

This study has a number of limitations. The study hospitals do not represent a random sample of US hospitals; further studies are required to determine whether the results are broadly applicable. We were unable to control for differences between hospitals in their methods of antibiogram construction, including methods and reporting of duplicate isolates, which can affect reported resistance (29), as well as hospital culturing practices (such as MRSA

screening). Standardization of methods to report antibiograms has been advocated by the Clinical and Laboratory Standards Institute (formerly NCCLS), but adherence is poor (30). We also were not able to control for differences in infection control measures between hospitals, which are a likely source of variability in the prevalence of resistant organisms. Finally, the associations between antimicrobial drug use and resistance found in ecologic studies such as this may not always coincide with those observed on an individual patient level (31). Case-control studies are more appropriate to quantify the risk associated with antimicrobial drug exposure in individual patients.

Our results suggest that the ecologic relationship between the hospital use of fluoroquinolones and antimicrobial resistance in *P. aeruginosa* and *S. aureus* is complex. Future studies to better define this relationship would be worthwhile, in that they would help hospitals determine where to best invest their resources to reduce the overall impact of resistance in their institutions, whether through enhanced infection control measures or more active antimicrobial stewardship, although both measures are likely important. Meanwhile, judicious use of fluoroquinolones is advocated to prevent the loss of this valuable therapeutic class.

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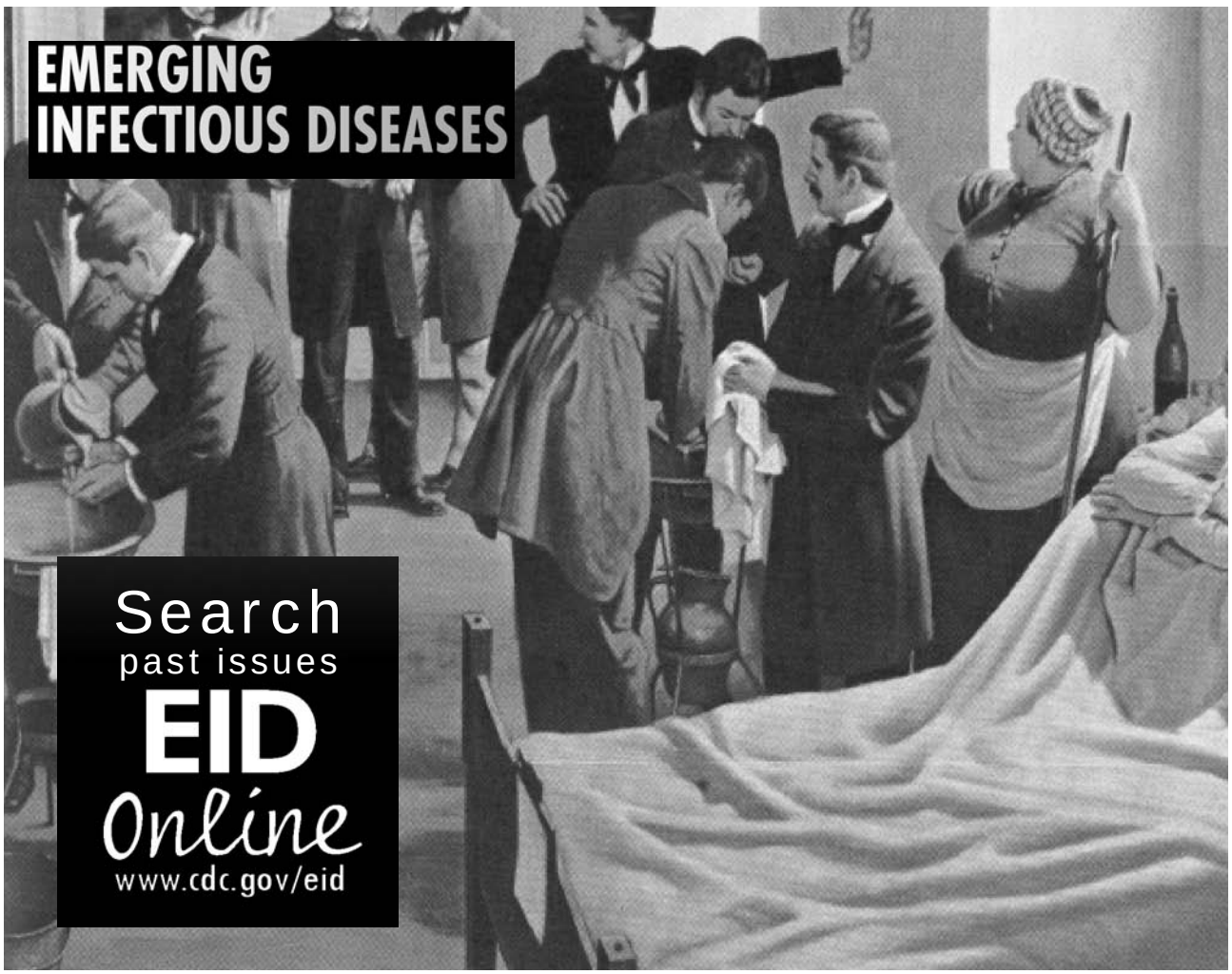

Methicillin-resistant *Staphylococcus aureus*, Hawaii, 2000–2002

Fenfang Li,* Sarah Y. Park,† Tracy L. Ayers,† F. DeWolfe Miller,* Ralph MacFadden,† Michele Nakata,† Myra Ching Lee,† and Paul V. Effler†

The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) has generated considerable concern among medical and public health professionals. We used a statewide, population-based antimicrobial resistance surveillance system to examine epidemiologic trends for MRSA from outpatients and inpatients in Hawaii. Pediatric and adult patient populations were compared to assess characteristics of MRSA isolates specific for each group. From 2000 to 2002, 8,206 (26%) of 31,482 total *S. aureus* isolates were MRSA. During this period, the proportion of MRSA isolates increased in both outpatient and inpatient clinical settings ($p<0.01$). When stratified by age, annual trends showed a significant increase in the proportion of MRSA in adult patients (from 24% to 30%, $p<0.01$) but not in pediatric patients (from 25% to 27%, $p>0.05$). Although MRSA isolates from adults demonstrated high resistance to most non–β-lactams, most MRSA isolates from pediatric outpatients remained susceptible to most non–β-lactams.

First detected in the 1960s, methicillin-resistant *Staphylococcus aureus* (MRSA) has become the leading cause of nosocomial infections during the last 2 decades (1). MRSA isolates are increasingly resistant to multiple non–β-lactam antimicrobial drugs. Recent reports of vancomycin-resistant *S. aureus* foreshadow an era of chemotherapy in which effective bactericidal drugs to treat infection with this organism may not be readily available (2,3).

Established risk factors for MRSA infection include a history of recent hospitalization or surgery, dialysis, residing in long-term care facilities, presence of an indwelling catheter, and use of injectable drugs (4–6). More recently, however, outbreaks of MRSA infections have been reported in healthy persons without these previously recognized risk factors in a variety of community settings (7–12).

Preliminary work suggests that community isolates of MRSA differ from their hospital counterparts in their demographic, clinical, and molecular characteristics (13–18). However, the epidemiology of MRSA in outpatient settings has not been fully described. In particular, knowledge is limited regarding the epidemiology of MRSA in persons visiting hospital outpatient settings, public or community health centers, and private physicians' offices, i.e., settings where most MRSA infections are treated and the greatest percentage of total antimicrobial use occurs (19). The objective of this study was to better characterize the epidemiology of MRSA from both inpatient and outpatient settings in Hawaii by using a population-based surveillance system.

Materials and Methods

Data Collection

Antimicrobial susceptibility test data, collected retrospectively through the State of Hawaii Antimicrobial Resistance Project (SHARP) from 2000 to 2002, were used for this analysis. The SHARP system captures electronic laboratory data from 2 large private clinical laboratories, which serve most of Hawaii's total population (N = 1,211,537) (20). These 2 commercial laboratories provide susceptibility testing services for >85% of all nonhospital outpatient settings in Hawaii (21). They also perform susceptibility testing for 18 of 24 acute care hospitals in the state, including the outpatient services associated with these hospital facilities (22). The remaining 6 acute care hospitals perform susceptibility testing in their own laboratories. Data from 2 of these hospitals are incorporated into the SHARP database, which provides a final dataset

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that encompasses 20 (83%) of Hawaii's 24 acute care hospitals.

Isolate-level data, including the specimen collection date, source (e.g., blood, urine, and cerebrospinal fluid), susceptibility testing methods (e.g., Kirby-Bauer), and susceptibility test results, are provided by the laboratories participating in SHARP. Limited demographic patient information is also included in the record, e.g., date of birth and sex. However, detailed clinical histories and patient names are not available. In lieu of names, patients were assigned personal identifiers created by concatenating values from the birth date, sex, reporting laboratory, and hospital/clinic location of specimen collection.

All cultures that yielded *S. aureus* isolates from 2000 through 2002 were identified, and any isolates from patients in nonacute care beds (i.e., long-term care homes) and correctional facilities were excluded. Duplicate isolates were then removed according to Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines (23). Only the first isolate per patient, irrespective of body site, antimicrobial susceptibility profile, or other phenotypic characteristics (e.g., biotype), in a defined period (for our purposes, 90 days) was included. Therefore, after the initial isolate, successive isolates for the same patient during the next 90 days were excluded.

The breakpoint for MRSA was an MIC ≥4 μg/mL or a zone diameter ≤10 mm. The breakpoint for oxacillin (methicillin)–intermediate isolates was an MIC 2–4 μg/mL or a zone diameter from 11 to 12 mm. The breakpoint for oxacillin (methicillin)–susceptible isolates was an MIC ≤2 μg/mL or a zone diameter ≥13 mm. Susceptibility interpretations for other antimicrobial drugs were based also on breakpoints established by NCCLS (24).

Patient Classification

Patients ≤18 years of age were defined as pediatric patients and those ≥19 years of age as adult patients. Classification of inpatient or outpatient status was based on patients' location at the time of specimen collection. Inpatient isolates were defined as those collected from patients in both regular hospital wards and intensive care units. Outpatient isolates were defined as those collected from patients in 1) an outpatient department, emergency department, ambulatory clinic, or same-day-surgery clinic associated with a hospital, or 2) a private physician's office, community public health center, or university health center.

Data Analysis

The proportion of MRSA was calculated as the number of MRSA isolates divided by the total number of *S. aureus* isolates during a defined period (e.g., per year) for a given setting (e.g., inpatient) and population (e.g., pediatric). The proportion of MRSA isolates resistant to a specific antimicrobial agent was calculated as the proportion of resistant isolates divided by the total MRSA isolates tested against the particular antimicrobial agent of interest during a defined period. Categorical data analysis was performed using EpiInfo version 6.04c statistical software (Centers for Disease Control and Prevention, Atlanta, GA, USA). Chi-square and Fisher exact tests were used to compare proportions, and significance was defined as $p<0.05$. The Kruskal-Wallis test was used to compare median age of patients, and Pearson correlation coefficient was used to determine the correlation of age and MRSA proportions in different clinical settings.

Results

A total of 41,250 *S. aureus* isolates were identified from combined inpatient and outpatient settings from 2000 to 2002. After removal of duplicate data, 31,482 isolates remained in the analysis; 23,550 were from outpatients, and 7,932 were from hospital inpatients. A total of 8,206 (26%) of all isolates included in the analyses were MRSA. The overall proportion of MRSA from 2000 to 2002 in outpatients was 22% (5,135/23,550) versus 39% (3,071/7,932) for inpatients ($p<0.01$). Although the proportion of MRSA remained significantly higher in inpatients compared with outpatients each year of the study, an overall significant increase in the proportion of MRSA infections was observed in both clinical settings during the 3-year study period ($p<0.01$, Table 1). The proportion of MRSA isolates during the 3-year period was significantly higher in pediatric outpatients (24%, 1,092/4,571) than that in adult outpatients (21%, 4,043/18,979; $p<0.01$). However, adult inpatients had a significantly higher proportion of MRSA isolates (40%, 2,868/7,217) when compared with pediatric inpatients (28%, 203/715; $p<0.01$).

A significant increase in the proportion of MRSA isolates was observed in all adult patients (from 24% to 30%, $p<0.01$) during the 3-year study period, and an increasing, although not statistically significant, trend was observed in pediatric patients (Figure 1). When the total MRSA isolates for all study years were examined by 10-year age groups, the proportion of MRSA isolates increased with age in inpatients, but not in outpatients (Figure 2). Correspondingly, inpatients with MRSA infections were significantly older (median age 67 years) than outpatients with MRSA infections (median age 44 years; $p<0.01$).

Of the 31,482 *S. aureus* isolates in the analysis, 31,240 (99%) had identifiable specimen sources. Table 2 summarizes the frequency of MRSA anatomic specimen sources for pediatric and adult patients in outpatient and inpatient settings. Wounds were the most common specimen sources for MRSA isolates in both pediatric and adult populations in all clinical settings.

Table 1. Distribution of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates by patient group and clinical setting in Hawaii

		Outpatients			Inpatients		
Year	Patient group	No. *S. aureus*	No. (%) MRSA	Contributing to total MRSA (%)	No. *S. aureus*	No. (%) MRSA	Contributing to total MRSA (%)
2000	Total	7,633	1,557 (20)*	–	2,330	842 (36)*	–
	Pediatric	1,593	384 (24)	25	216	65 (30)	8
	Adult	6,040	1,173 (19)	75	2,114	777 (37)	92
2001	Total	7,543	1,495 (20)*	–	2,661	997 (37)*	–
	Pediatric	1,401	302 (22)	20	249	53 (21)	5
	Adult	6,142	1,193 (19)	80	2,412	944 (39)	95
2002	Total	8,374	2,083 (25)*	–	2,941	1,232 (42)*	–
	Pediatric	1,577	406 (26)	19	250	85 (34)	7
	Adult	6,797	1,677 (25)	81	2,691	1,147 (43)	93

*p<0.001, by chi-square test for trend for outpatient and inpatient settings.

For both adult and pediatric patients, MRSA resistance to non–β-lactam antimicrobial agents was significantly higher in inpatient isolates than in outpatient isolates for nearly all of the antimicrobial agents tested (p<0.01, Table 3). For all years combined, a significantly higher level of resistance to non–β-lactam antimicrobials was observed in all adult MRSA isolates compared with all pediatric MRSA isolates (p<0.01). Most MRSA isolates from pediatric outpatients remained susceptible to most non–β-lactams, with the exception of erythromycin, to which 24% of the isolates were resistant.

Discussion

Our analyses identified an increase in the proportion of MRSA for both outpatient and inpatient settings in Hawaii from 2000 through 2002. In the final year of the study, the proportion of MRSA isolates in outpatients and inpatients was notable, i.e., 25% and 42%, respectively.

Whereas high rates of MRSA in inpatient settings have been described previously (1,14), our data highlight an issue of more recent concern, i.e., increasing MRSA infections occurring in the outpatient setting. In the absence of detailed clinical histories, isolates obtained in the outpatient setting cannot be equated to community-associated MRSA. However, isolates from a large population of pediatric outpatients may be a reasonable surrogate for community-associated *S. aureus* infections because they should reflect infections occurring in a predominantly healthy, nonhospitalized population. Furthermore, previous studies have established that skin and soft tissue infections are the most common sources of community-associated MRSA (16,25,26). In our setting, ≈25% of all *S. aureus* infections from pediatric outpatients were MRSA, with 95% of these isolated from wounds. These data suggest that MRSA is an important cause of wound infections in children in Hawaii, a finding consistent with a growing number of reports of MRSA in pediatric outpatients elsewhere (27,28).

Although the proportion of MRSA isolates was significantly lower for adult outpatients compared with pediatric outpatients (21% versus 24%, p<0.01), the total number of MRSA infections in adult outpatients outnumbered those in pediatric outpatients nearly 4:1. In addition, we observed a positive correlation between the proportion of MRSA isolates and advancing age for inpatients, but not for outpatients (R = 0.92, p<0.01). This finding is not unexpected if one considers that older adults are more likely to have chronic illnesses that require more frequent hospitalizations and, thus, potentially encounter increased exposure to resistant microorganisms (1,4,5,14).

MRSA isolates obtained from outpatient settings tended to be less resistant to nonoxacillin antimicrobial drugs than their inpatient counterparts, as observed elsewhere (12,14,16,25–28). Proportionally more pediatric isolates remained susceptible to multiple non–β-lactams compared with adult isolates, most notably when compared with adult inpatient isolates. The dissimilar antimicrobial resistance profiles observed between clinical settings may reflect differences in cohorts of circulating MRSA isolates, especially between outpatients and inpatients, as has been demonstrated in other investigations (16,27,28).

Of clinical importance is that pediatric outpatient MRSA isolates remained largely susceptible to most non–β-lactams. However, ≈25% of pediatric outpatient

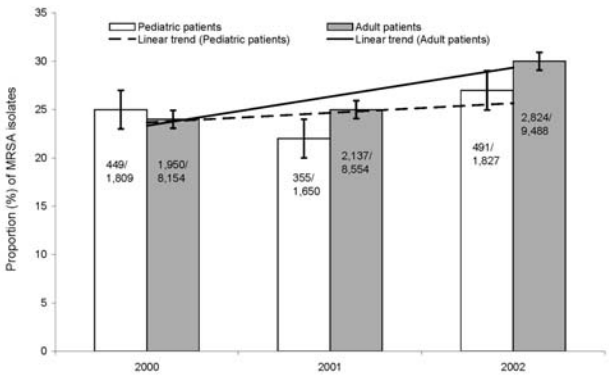

Figure 1. Proportion of methicillin-resistant *Staphylococcus aureus* (MRSA) in pediatric and adult patients, Hawaii, 2000-2002. Error bars show 95% confidence intervals.

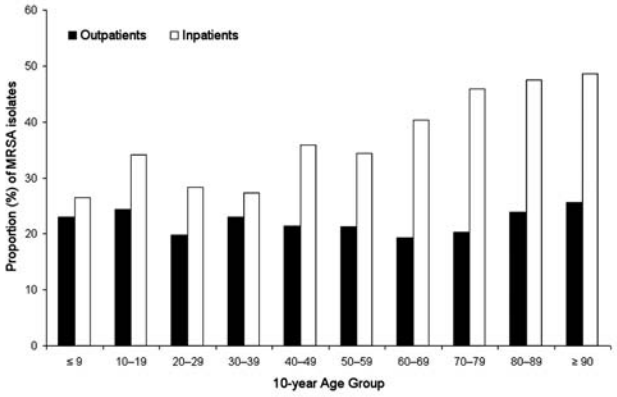

Figure 2. Proportion of methicillin-resistant *Staphylococcus aureus* (MRSA) by 10-year age group and clinical setting, Hawaii 2000–2002.

MRSA isolates in Hawaii were resistant to erythromycin, which is consistent with findings in other studies of MRSA macrolide resistance in pediatric outpatients (25,27,28). In contrast, only 7% of the pediatric outpatient isolates were resistant to clindamycin. This antimicrobial drug has been used to successfully treat MRSA infections in children in community settings. However, clinicians should be aware of inducible resistance to clindamycin due to methylation of ribosomal RNA encoded by the genes *ermA* or *ermC* in initially clindamycin-susceptible but erythromycin-resistant *S. aureus* isolates (25,29). Therefore, clindamycin monotherapy should be avoided in critically ill children with MRSA infections until inducible clindamycin resistance has been excluded by double-disk testing (25,29,30).

A major limitation of this study is the potential misclassification of the clinical setting (i.e., outpatient versus inpatient). Because of the lack of named personal identifiers, patients could not be traced from isolates to be interviewed, and medical records could not be reviewed. Therefore, we could not directly correlate our findings for outpatients and inpatients with the more conventional definitions of community-associated and nosocomial-associated MRSA, respectively. We assigned an isolate to a clinical setting based on the location where the patient's specimen was collected, so misclassification was possible. For example, a patient whose specimen was obtained in an outpatient setting (e.g., an emergency department) may have actually acquired *S. aureus* during a recent hospitalization. If misclassification of this type occurred, the difference in MRSA rates between outpatient and inpatient isolates would be underestimated. However, this misclassification type might be expected to remain consistent over a 3-year period, so the observed temporal trends would likely remain valid. Moreover, the significant differences in resistance to nonoxacillin antimicrobial drugs observed between outpatients and inpatients suggest that the MRSA isolates from outpatients were not, on average, nosocomial-associated isolates cultured in the outpatient setting (13,14,16).

Another study limitation is the inability to fully assess the effect of potential changes in clinical practice during the 3-year study period. If physicians increased or decreased the tendency to obtain cultures, these changes might affect the number or proportion of MRSA isolates identified each year. However, retrospective examination of the total number of cultures requested by physicians each year from 6 acute care hospitals demonstrated that 102,537, 99,772, and 99,461 total cultures were ordered at these facilities in 2000, 2001, and 2002, respectively. These data do not suggest any major shift in practice patterns during the relatively short duration of this study. Therefore, the observed temporal trends cannot likely be attributed to changing physician practices.

A third possible limitation is that we were unable to include susceptibility testing data from 4 of Hawaii's 24 acute care medical centers and their associated outpatient locations. However, a separate review of antibiogram data available from all laboratory facilities in the state in 2001 found that in aggregate, the 4 excluded hospital laboratories accounted for only 1,632 (11%) of the 14,539 total *S. aureus* isolates identified that year. Therefore, the SHARP data in this study captured ≈90% of all *S. aureus* susceptibility testing data in Hawaii from 2000 to 2002.

To our knowledge, this is the first study of MRSA using susceptibility testing data from inpatient and outpatient settings representative of an entire state population. Our major finding, substantive and increasing rates of MRSA in the outpatient setting, is particularly important as reports of MRSA acquired in the community increase across the

Table 2. Specimen sources of methicillin-resistant *Staphylococcus aureus* isolates from pediatric and adult patients by clinical setting, Hawaii, 2000–2002

Specimen sources	Pediatric outpatients (%) n = 1,086	Pediatric inpatients (%) n = 199	Adult outpatients (%) n = 4,012	Adult inpatients (%)* n = 2,829
Wound	95	72	80	46
Sputum	0	1	4	29
Urine	2	2	7	8
Blood	0	4	4	7
Other†	3	21	5	9

*Total percentage may not equal 100% because of rounding.
†Includes medical devices, respiratory swabs, gynecologic specimens, stool, synovial fluid, and gallbladder specimens.

Table 3. Resistance patterns of MRSA isolates from pediatric and adult patients by clinical setting, Hawaii, 2000–2002*

	Pediatric patients					Adult patients				
	Outpatients		Inpatients			Outpatients		Inpatients		
Antimicrobial drug	No. isolates tested*	No. (%) resistant	No. isolates tested*	No. (%) resistant	p value†	No. isolates tested*	No. (%) resistant	No. isolates tested*	No. (%) resistant	p value†
Ciprofloxacin	604	11 (2)	110	10 (9)	<0.01	1,502	545 (36)	1,305	1,071 (85)	<0.01
Clindamycin	1,083	71 (7)	202	39 (19)	<0.01	3,936	1,462 (37)	2,830	2,128 (75)	<0.01
Erythromycin	1,092	261 (24)	203	78 (38)	<0.01	4,018	2,186 (54)	2,859	2,412 (84)	<0.01
Gentamicin	997	9 (1)	199	12 (6)	<0.01	3,289	418 (13)	2,609	906 (35)	<0.01
Levofloxacin	215	5 (2)	59	9 (16)	<0.01	629	245 (39)	549	433 (79)	<0.01
Rifampin	1,028	4 (0)	199	5 (3)	<0.01	3,773	134 (4)	2,795	355 (13)	<0.01
Tetracycline	812	27 (3)	178	16 (9)	<0.01	3,414	667 (20)	2,622	976 (37)	<0.01
Trimethoprim/ sulfamethoxazole	1,092	1 (0)	203	2 (1)	NS	4,035	199 (5)	2,862	278 (10)	<0.01
Vancomycin	1,092	0 (0)	203	0 (0)	NA	4,036	0 (0)	2,865	0 (0)	NA

*The number of MRSA isolates tested against each antimicrobial drug varies in each laboratory because the drug panel used differed for each MRSA.
MRSA, methicillin-resistant *Staphylococcus aureus*; NS, not significant; NA, not applicable.
†By chi-square test comparing outpatients to inpatients within each patient group. Fisher exact test was used for rifampin within the pediatric population.

country, and optimal strategies for the management of MRSA infections in the outpatient setting have yet to be established (14,16,25,27–30). More studies are needed to improve the understanding of MRSA epidemiology in the community and to identify appropriate strategies to prevent *S. aureus* infections, including MRSA.

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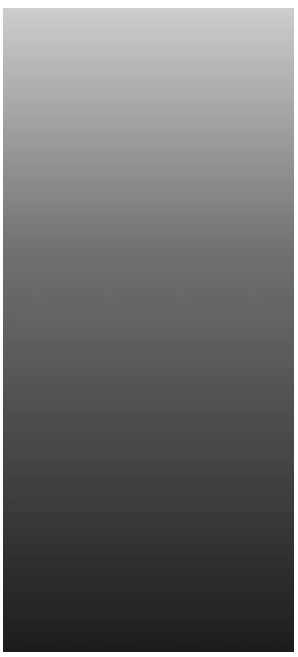

Coxiella burnetii Genotyping

Olga Glazunova,*[1] Véronique Roux,*[1] Olga Freylikman,*† Zuzana Sekeyova,*‡ Ghislain Fournous,* Judith Tyczka,§ Nikolai Tokarevich,† Elena Kovacava,‡ Thomas J. Marrie,¶ and Didier Raoult*

Coxiella burnetii is a strict intracellular bacterium with potential as a bioterrorism agent. To characterize different isolates of *C. burnetii* at the molecular level, we performed multispacer sequence typing (MST). MST is based on intergenic region sequencing. These regions are potentially variable since they are subject to lower selection pressure than the adjacent genes. We screened 68 spacers in 14 isolates and selected the 10 that exhibited the most variation. These spacers were then tested in 159 additional isolates obtained from different geographic areas or different hosts or were implicated in different manifestations of human disease caused by *C. burnetii*. The sequence analysis yielded 30 different allelic combinations. Phylogenic analysis showed 3 major clusters. MST allows easy comparison and exchange of results obtained in different laboratories and could be a useful tool for identifying bacterial strains.

Coxiella burnetii is a strict intracellular microorganism, included in the γ subdivision of the Proteobacteria phylum (1). It is found in close association with arthropod and vertebrate hosts, and it causes Q fever in humans and animals. Cattle, goats, and sheep are the primary reservoirs of human infection. In humans, the disease may appear in 2 forms, acute and chronic (2). Acute Q fever may be asymptomatic or appear as atypical pneumonia, granulomatous hepatitis, or self-limited febrile illness. In some persons, the immune system is unable to control the infection and chronic Q fever occurs. The manifestations of chronic Q fever are endocarditis, hepatitis, osteomyelitis, or infected aortic aneurysms. *C. burnetii* is highly infectious by the aerosol route and can survive for long periods in the environment.

Previous studies have shown that *C. burnetii* isolates differed respect to their plasmid type (QpH1, QpRS, QpDG, and QpDV) (3–6), lipopolysaccharide profiles (7), and analysis of endonuclease-digested DNA separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8) or pulsed-field gel electrophoresis (PFGE) (9–11). Differentiation was also obtained by sequence determination of the isocitrate dehydrogenase gene (12), *com1* gene, and *mucZ* gene, which was renamed *djlA* when the whole genome of *C. burnetii* was sequenced (13,14).

Several other methods have been used to type different isolates of the same species, in particular, multilocus enzyme electrophoresis (15) and multilocus sequence typing (MLST) (16). Many bacterial species have been studied by using these approaches (17–19).

Recently, the whole genome of the *C. burnetii* Nine Mile strain was sequenced (14). We decided to investigate parts of the genome located between 2 open reading frames (ORFs) because they are considered potentially variable since they are subject to lower selection pressure than the adjacent genes. The 16S/23S ribosomal spacer region has been widely used to genotype bacteria (20–23). We investigated the utility of multispacer sequence typing (MST) with 173 *C. burnetii* isolates. After screening, we selected 10 variable spacers and showed that the combination of the different sequences allowed us to characterize 30 different genotypes. Phylogenetic analysis inferred from compiled sequences characterized 3 monophyletic groups, which could be subdivided into different clusters.

Methods

Bacterial Strains

The *C. burnetii* strains included in this study are listed in online Appendix Table 1 (available at http://www.cdc.gov/ncidod/EID/vol11no08/04-1354_app.htm#table1). All the strains were propagated on Vero cell monolayers (ATCC CRL 1587). Minimal essential medium (MEM) (Invitrogen, Cergy-Pontoise, France) supplemented with 4% fetal bovine serum (Invitrogen) and 1% L-glutamine (Invitrogen) was used for cultivation. Infected cells

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[1]Dr. Glazunova and Dr. Roux contributed equally to this work.

Table 1. Primers used for PCR amplification and sequencing of *Coxiella burnetii* gene spacers

Spacer name	ORF	Nucleotide sequence (5′–3′)*	Amplified fragment length (bp)
Cox2	Hypothetical protein	Cox20766 CAACCCTGAATACCCAAGGA	397
	Hypothetical protein	Cox21004 GAAGCTTCTGATAGGCGGGA	
Cox5	Sulfatase domain protein	Cox77554 CAGGAGCAAGCTTGAATGCG	395
	Entericidin, putative	Cox77808 TGGTATGACAACCCGTCATG	
Cox18	Ribonuclease H	Cox283060 CGCAGACGAATTAGCCAATC	557
	DNA polymerase III, epsilon subunit	Cox283490 TTCGATGATCCGATGGCCTT	
Cox20	Hypothetical protein	Cox365301 GATATTTATCAGCGTCAAAGCAA	631
	Hypothetical protein	Cox365803 TCTATTATTGCAATGCAAGTGG	
Cox22	Hypothetical protein	Cox378718 GGGAATAAGAGAGTTAGCTCA	383
	Amino acid permease family protein	Cox378965 CGCAAATTTCGGCACAGACC	
Cox37	Hypothetical protein	Cox657471 GGCTTGTCTGGTGTAACTGT	463
	Hypothetical protein	Cox657794 ATTCCGGGACCTTCGTTAAC	
Cox51	Replicative DNA helicase, intein-containing	Cox824598 TAACGCCCGAGAGCTCAGAA	674
	Conserved hypothetical protein – Uridine kinase	Cox825124 GCGAGAACCGAATTGCTATC	
Cox56	OmpA-like transmembrane domain protein	Cox886418 CCAAGCTCTCTGTGCCCAAT	479
	Conserved hypothetical protein	Cox886784 ATGCGCCAGAAACGCATAGG	
Cox57	Rhodanese-like domain protein	Cox892828 TGGAAATGGAAGGCGGATTC	617
	Hypothetical protein	Cox893316 GGTGGAAGGCGTAAGCCTTT	
Cox61	Dioxygenase, putative	Cox956825 GAAGATAGAGCGGCAAGGAT	611
	Hypothetical protein	Cox957249 GGGATTTCAACTTCCGATAGA	

*The numbers are beginning or end locations of the genes where the primers were chosen.

were maintained in a 5% CO_2 atmosphere at 35°C. *C. burnetii* cells were harvested, pelleted, resuspended in 200 µL MEM, and mixed with 500 µL Chelex 100 20% (Bio-Rad, Ivry sur Seine, France). The preparation was boiled for 30 min, centrifuged at 10,000 x *g* for 30 min (24), and the supernatant containing DNA was transferred to a clean Eppendorf tube and stored at 4°C or –20°C.

Multispacer Sequence Typing

The whole genome of *C. burnetii* was accessible in the NCBI server (GenBank NC 002971). We kept spacers that were 300–700 bp in length. Primers were chosen in neighboring genes to allow polymerase chain reaction (PCR) amplification at 57°C and are listed in Table 1. Each PCR was carried out in a T3 Thermocycler Biometra (Biolabo, Archamps, France). Two microliters of the DNA preparation was amplified in a 50-µL reaction mixture containing 200 µmol/L of each primer, 200 µmol/L (each) dATP, dCTP, dGTP, and dTTP (Invitrogen), 1.5 U Taq DNA polymerase (Roche, Meylan, France) in 1x Taq buffer. Amplifications were carried under the following conditions: initial denaturation of 10 min at 95°C, followed by 37 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 57°C, and extension for 1 min at 72°C. PCR products were purified and sequenced as previously described (25).

PCR products were cloned in PGEM-T Easy Vector (Promega, Charbonnières, France) according to the manufacturer's instructions. Ten clones were cultivated in LB medium (USB, Cleveland, OH, USA) overnight, and PCR and sequencing were performed as described previously.

Plasmid Sequence Type, *com1* Type, and *djlA* Type Determination

PCR for QpH1 and QpRS sequence plasmids were performed with the primers previously described QpH11/12 and QpRS01/02 (5). PCR was carried out as described for MST, except that annealing temperature was 55°C and cycle number was 35. PCR primers for QpDV and QpRS sequence plasmid amplification were chosen after comparison of the entire sequence of the 2 plasmids. The primers were QpDV1f and QpDV1r. PCR amplification was carried out at 63°C for 30 cycles. PCR was performed as previously described for *com1* and *djlA* (13) (Appendix Table 2, available at http://www.cdc.gov/ncidod/EID/vol11no08/04-1354_app.htm#table2).

Data Analysis

Statistical analyses were performed by using the chi-square test in the program EpiInfo 6 (26). The spacer sequences were compiled and aligned by using the multisequence alignment program ClustalX (1.8). The phylogenetic relationships between the *C. burnetii* isolates were determined by using Mega version 2.0 (27). A matrix of pairwise differences in allele profiles was constructed, and the similarities between the allelic profiles of the isolates were assessed by cluster analysis using the unweighted pair-group method with arithmetic mean (UPGMA). Another analysis of the results was performed by using the BURST algorithm (http://www.mlst.net), which defines clonal complexes in which every isolate shares at least 5 identical alleles with at least 1 other isolate (Cox2, Cox5,

Cox18, Cox20, Cox37, Cox56, and Cox57 were kept for the analysis) and characterizes ancestral genotypes. *C. burnetii* MST database was entered at the following website: http://ifr48.timone.univ-mrs.fr, and ST determination by sequence comparison is possible at this site.

Results

Choice of Spacers for Typing and Analysis by MST

Initially 14 isolates were chosen to test the genetic diversity of the spacers: Nine Mile, Priscilla, Q212, Heizberg, Brasov, Dog ut Ad, CB15, CB20, CB26, CB28, CB33, CB35, CB114, and CB115. We chose 68 spacers, but we retained only 51 spacers for which PCR amplification was obtained for all the isolates. We kept 10 spacers (Cox2, Cox5, Cox18, Cox20, Cox22, Cox37, Cox51, Cox56, Cox57, and Cox61) (Table 1) because they were representative of the results found when we analyzed the entire test set of 51 spacers. For each spacer, the number of variable sites in the sequences was determined, and the percentage of variability was calculated. They were, respectively, 1.1, 1.4, 1.9, 0.7, 2.3, 1.2, 1.4, 2.5, 1.7, and 2.1. We kept Cox18, Cox22, Cox51, Cox56, Cox57, and Cox61 because the percentage of variability in these spacers was high compared with the other spacers. We kept Cox2, Cox5, Cox20, and Cox37 because they allowed the characterization of CB35, CB15, CB26 and CB28, and Nine Mile respectively. To test the reliability of the spacers we kept, chi-square value was determined by using the value of 1% as the threshold value. The Fisher value was found to be statistically significant (9×10^{-4}). We then added 159 other isolates. Sequences were obtained for all the isolates with spacers Cox2, Cox18, Cox20, Cox22, Cox37, Cox51, and Cox57. Mixed sequences were obtained with the isolate Poker Cat with spacers Cox5, Cox56, and Cox61. We cloned the PCR products and showed that several sequences were present after PCR amplification, including insertions or deletions. Allele distribution of the different gene spacers are described in Table 2. Each of the different sequences in a locus defined a distinct genotype, even if it differed from the others by only a single nucleotide. Thirty different sequence types (STs) were identified by using MST.

The nucleotide sequence accession numbers are noted in online Appendix Table 3 (available at http://www.cdc.gov/ncidod/EID/vol11no08/04-1354.htm#table3).

Table 2. Alleles of 10 spacers which allow the definition of the different *Coxiella burnetii* sequence types

COX ST	2	5	18	20	22	37	51	56	57	61
1	5	6	3	4	6	5	8	1	5	6
2	5	6	3	5	6	5	8	1	5	6
3	5	6	3	4	6	7	8	1	5	6
4	5	6	3	2	6	5	8	1	5	6
5	4	6	3	5	6	2	8	2	5	6
6	4	3	3	5	6	5	8	2	5	6
7	4	6	3	5	6	5	8	2	5	6
8	5	4	2	5	1	5	3	3	4	4
9	1	4	2	5	1	5	2	3	4	6
10	5	4	2	5	1	5	2	3	2	6
11	6	5	1	6	5	4	5	4	3	2
12	3	5	1	6	5	4	5	4	3	2
13	3	5	1	6	5	4	5	5	3	2
14	7	5	1	6	5	6	9	4	3	2
15	7	5	1	6	5	6	9	6	3	2
16	3	7	5	3	4	1	6	7	6	5
17	3	7	5	7	4	1	10	8	6	7
18	3	7	1	6	3	4	7	9	6	3
19	3	2	7	8	5	4	11	9	6	5
20	3	2	6	1	5	4	4	10	6	5
21	2	1	4	6	2	3	1	11	1	1
22	3	7	1	6	3	8	7	9	6	8
23	3	7	1	6	3	8	7	9	6	3
24	3	5	1	6	5	4	5	12	3	9
25	3	7	1	6	3	4	7	9	7	3
26	9	4	8	5	8	5	2	3	4	6
27	3	5	1	6	5	4	5	12	3	2
28	8	4	8	5	7	5	2	3	4	6
29	3	7	1	9	3	4	7	9	6	3
30	5	6	9	5	6	5	8	13	8	6

Accession numbers for Poker Cat isolate clones are, respectively, AY619726, AY619728, and AY619729, and AY619721 for Cox5, Cox56, and Cox61.

Computer Analysis of MST Data

The dendrogram in the Figure was constructed from a matrix of pairwise allelic differences between the compiled sequences of the 30 STs. We identified 3 monophyletic groups within the tree. The first group, representing 13 different STs, included isolates from France, Spain, Russia, Kyrgyzstan, Namibia, Kazakhstan, Ukraine, Uzbekistan, and the United States. It was divided in 2 subgroups. The first one included 36 isolates representing 8 different STs (ST1 to ST7 and ST30). Nineteen were represented by ST1. The second subgroup included 39 isolates which represented 5 different STs (ST8, ST9, ST10, ST26, and ST28). Twenty-eight were represented by ST8.

The second group included isolates from Europe (France, Germany, Switzerland, Romania, Italy, Greece, Austria, Slovakia), the United States, Russia, Africa (Central Africa and Senegal), and Asia (Kazakhstan, Uzbekistan, Mongolia, and Japan). It was divided into 4 subgroups. The first one included 26 isolates, which represented 7 different STs (ST11, ST12, ST13, ST14, ST15, ST24, and ST27). The second subgroup included 34 isolates that were included in ST18, ST22, ST23, ST25, and ST29 groups. The third subgroup included 18 isolates (ST16 and ST17), and the fourth subgroup included 10 isolates (ST19 and ST20).

The third group consisted of only 1 ST, ST21, and included the 7 Canadian isolates, 2 isolates from France (CB4 and CB7), and 1 isolate from the United States (Scurry). The clusters determined by the BURST algorithm were consistent with those determined by the phylogenetic analysis. Five groups were defined. The first one included ST1 to ST7; the putative ancestral genotype in this group was ST1. ST8 (putative ancestral genotype), ST9, ST10, ST26, and ST28 were included in the second group; ST11, ST12 (putative ancestral genotype), ST13, ST14, ST15, and ST24 in the third group, ST16 and ST17 in the fourth group; and ST18 (putative ancestral genotype), ST22, ST23, ST25, and ST29 in the fifth group. ST19, ST20, ST21, and ST30 were considered as singletons.

Sequence Type Determination and Correlation with Pathology

In the monophyletic group 1, the sequence of plasmid QpRS was found for isolates included in ST4, ST5, ST6, ST7, ST8, ST9, ST10, ST26, ST28, and ST30. The QpDV plasmid sequence was amplified for isolates included in ST1, ST2, ST3, and ST4. In the monophyletic group 2, the QpH1 plasmid sequence was found in all the isolates. In the monophyletic group 3, the QpH1 plasmid sequence or none of the searched plasmid sequences was detected. Sequence comparison of *djlA* generated 4 different groups. Group I included all STs included in the monophyletic group 2 defined by MST analysis. Group II included ST1, ST2, ST3, and ST4. Group III included ST5, ST6, ST7, ST30, ST26, ST28, ST8, ST9, and ST10. Group IV corresponded to ST21. *Com1* sequence comparison generated 6 different groups. Group I included all the STs included in the monophyletic group 2 defined by MST analysis except ST14 (group V) and ST20 (groupVI). Group II included ST1, ST2, ST3, and ST4. Group III included ST5, ST6, ST7, ST30, ST26, ST28, ST8, ST9, and ST10. Group IV corresponded to ST21. When *com1* typing was used, only 1 strain was not in accordance with MST typing results.

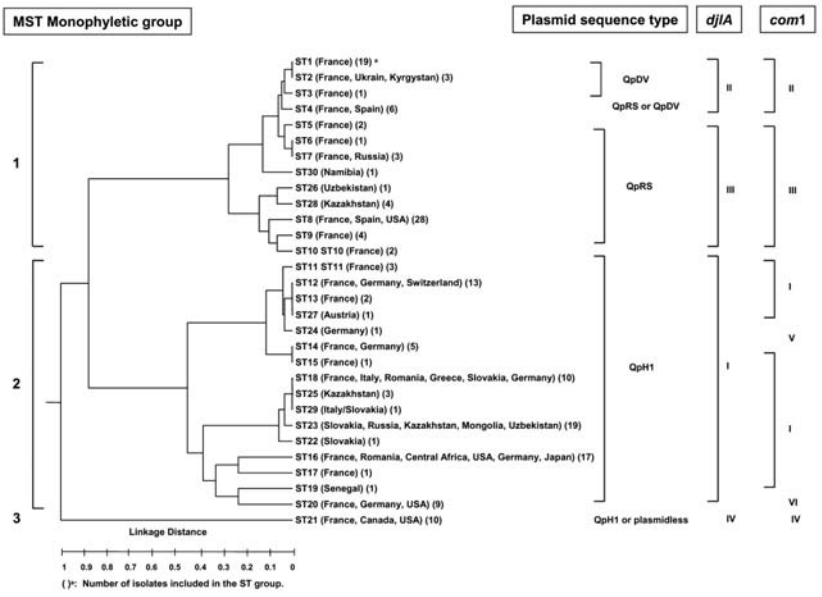

Figure. Dendrogram of the genetic relatedness among the 30 different sequence types defined by multispacer sequence type (MST) analysis. The dendrogram was constructed by unweighted pair-group method with arithmetic mean. Plasmid sequence type, *com1* group, and *djlA* group corresponding to each ST are indicated on the right of the figure. The 3 monophyletic groups defined by MST analysis are indicated on the left.

This strain, CB95, was included in ST8 but exhibited a group II *com1* sequence.

QpDV plasmid presence in human isolates was correlated with the acute form of the disease ($p = 2 \times 10^{-7}$), and QpRS plasmid presence was correlated with the chronic form of the disease ($p = 2 \times 10^{-4}$). The acute form of the disease was correlated with ST1 ($p = 10^{-3}$), ST4 ($p = 7 \times 10^{-4}$) ST16 ($p = 3 \times 10^{-3}$), ST18 ($p = 10^{-2}$), and the chronic form of the disease was correlated with ST8 ($p = 2 \times 10^{-3}$).

Modifications in ORFs Surrounding Studied Spacers

As primers were chosen in ORFs surrounding the studied spacers, mutations, deletions, or insertions were noted in the protein sequences. Mutations were noted in the hypothetical protein (gi29653385) for ST11; in the hypothetical protein (gi29653385) for ST9 and ST26; in entericin (gi29653446) for ST20, in ribonuclease H (gi29653667) in ST1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 21, 26, 28, and 30; in amino acid permease family protein (gi29653908) in ST28; in hypothetical protein (gi29654047) in ST1, 2, 4, 5, 6, 7, 8, 9, 10, 26, 28, and 30. In CB118 (ST3), a stop codon appeared which shortened the length of the ORF. Mutations were noted in uridine kinase (gi29654198) in ST18, ST22, ST23, ST25, and ST29; in ompA-like transmembrane domain protein (gi29654257), in ST20; in rhodanese-like domain protein (gi29654263) in ST20 (the protein was longer by 2 amino acids); in dioxygenase (gi29654325) in ST21 and ST22; in hypothetical protein (gi29732244), in ST17.

Insertions or deletions were noted in hypothetical protein (gi29653386) in ST5, 6, and 7; in hypothetical protein (gi29653755) in ST1 and ST3 (insertion of a base G in the DNA sequence made the protein sequence longer of 22 amino acids); in the amino acid permease family protein (gi29653772) in ST8, 9, and 10 (deletion of a base A in the DNA sequence made the protein sequence longer of 24 amino acids); in ompA-like transmembrane domain protein (gi29654257) in ST11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25, 27, and 29.

Discussion

Q fever in humans and animals, caused by *C. burnetii*, is found worldwide. In humans, it causes a variety of diseases such as acute flulike illness, pneumonia, hepatitis, and chronic endocarditis. In animals, *C. burnetii* is found in the reproductive system, both uterus and mammary glands and may cause abortion or infertility.

Molecular methods are now almost universally used to characterize strains and to determine the relatedness between isolates causing diseases in different contexts. The most discriminative approach used for *C. burnetii* isolates until this study was PFGE. Twenty different restriction patterns were distinguished after *Not*I restriction of total *C. burnetii* DNA and PFGE (11). Comparison of PFGE profiles is sometimes difficult because good separation of the different fragments is required. For example, the isolate Heizberg was classified in group 1 by Thiele et al. (10) and in group 2 by Jäger et al. (11). This fact highlights the difficulty of comparing results obtained by this technique. Moreover, in some species, rapid genomic rearrangements occur because of repeats or insertion sequences, so even if isolates descended from a common ancestor that arose several decades ago, they may not readily be seen to be minor variants of the same clone. In these cases, PFGE does not contribute to tracing of isolates. The great advantage of MST over PFGE as a typing method is the lack of ambiguity and the portability of sequence data, which allow results from different laboratories to be compared without exchanging strains. This work is the first to include so many isolates in a rigorous examination of molecular epidemiology. The study of this bank of sequences will contribute to understanding the propagation mode of the bacteria as variations accumulate relatively slowly, thus making it an ideal tool for global epidemiology. For example, in ST16 we characterized isolates that were obtained from 1935 (Nine Mile) to 1991 (CB25).

Most of the French isolates were included in monophyletic group 1. Nineteen were included in ST1, and 24 were included in ST8. Thus, an isolate has a geographic distribution even if genetic modifications appear (insertions, deletions or mutations) over time, giving rise to a new ST that is related to the ancestor isolate. This fact was highlighted when the analysis of the STs was performed by using the BURST algorithm. ST1 and ST8 were described as the ancestral genotypes and for example, ST9 and ST10 corresponded to SLVs of ST8 (isolates that differ at only 1 of the 7 loci) and ST26 and ST28 corresponded to DLVs of ST8 (double locus variants). But some types were not delineated on the basis of geographic origin because they were isolated from different parts of the world. This distribution in distant countries is likely related to movements of infected patients, animals, or ticks. This is particularly true for ST16 isolates that were encountered on 4 different continents, America, Europe, Asia, and Africa. The homology of the Canadian isolates from Nova Scotia should be noted. Q fever is just as endemic in Nova Scotia as in France. This may indicate rapid and recent spreading of a single strain. The association between ST21 and Canada is significant as tested with the chi-square test with a Fisher value $<10^{-8}$. Notably, patient CB115, who had Q fever endocarditis, was living in Edmonton, Alberta (≈3,000 miles from Nova Scotia) when this illness was diagnosed. He grew up in Nova Scotia, and the molecular epidemiologic findings show that he acquired his disease there. Q fever is uncommon in Alberta. Most of the STs are found in Europe. A sample bias could exist as most of the isolates

tested were from this continent, but the results obtained may also indicate that *C. burnetii* originated from the Old World and spread later in the New World, excluding New Zealand.

Concordant results were found when MST was compared with *com1* and *djlA* sequences comparison (Figure). However MST was more discriminant. Plasmid profile investigation of *C. burnetii* detected 4 different plasmids QpH1, QpRS, QpDV, and QpDG and 1 group of plasmidless isolates. QpH1 was first found in the Nine Mile tick isolate (28). QpRS was first found in the goat isolate Priscilla (29). QpDG was described from isolates obtained from feral rodents near Dugway, Utah (8). QpDV was found in French and Russian isolates (5,6). Another not-well-characterized plasmid type was described in China (30). The existence of a plasmidless *C. burnetii* isolate, Scurry Q217 was described (31), but a chromosomally integrated plasmid-homologous DNA fragment was found in this isolate by hybridization (32,33). Plasmid type sequence detection was also correlated with MST. Group 2 included isolates that PCR amplification found to be positive with primers specific for QpH1. Group 3 included 3 isolates, 2 from France (CB4 and CB7) and 1 from Nova Scotia (Poker Cat), in which plasmid sequence type of QpH1 was detected. No such sequence was detected in the other isolates of Nova Scotia origin included in group 3. Group 1 included isolates that were positive by PCR amplification with primers specific for QpRS (47/77). QpDV plasmid was described in isolates from France, Spain, Ukraine, and Kyrgyzstan. In fact, regions shared by QpH1, QpRS, and QpDV were termed "core plasmid sequences" and encompassed 25 kb. QpH1, QpRS, and QpDV are, respectively, 37 kb, 39 kb, and 33 kb in size. Integrated sequences in American isolate represent 18 kb. Differences in plasmid size and sequence can be explained by notable sequence rearrangements, such as deletions, insertions, or duplications, because several repeat sequences have been identified through which such rearrangements might have occurred. For CB13, we were able to characterize sequences for plasmids QpH1 and QpDV, which can be caused by several situations: this isolate may have 1) 2 different plasmids, 2) a QpH1 plasmid and sequences of QpDV integrated in the chromosome, or 3) a new plasmid that arose from combination of QpH1 and QpDV. All these hypotheses are in agreement with the presence of QpH1 plasmid in the ancestor of *C. burnetii* isolates. This plasmid was lost by some of them (monophyletic group 3) but genetic information of crucial importance for the organism was integrated in the chromosome. For other isolates, QpH1 plasmid evolved to QpRS plasmid, in some isolates QpRS plasmid evolved to QpDV plasmid.

This study showed a correlation between QpDV and acute infections, between QpRS and chronic infections, and an association between some genotypes and disease type. A bias in sampling exists since acute disease is 20 times more frequent than chronic disease, but in this study, most of the human isolates were from chronic disease patients, and the isolates from acute infections were mainly obtained from France. These facts reflect the difficulty in isolating the bacteria. A genomic typing method such as MST could be applied directly to samples to obtain a more precise idea of how *C. burnetii* is spreading in the environment and the pathogenetic implications in acute and chronic forms of Q fever.

Comparison of DNA sequences is the best approach to investigate bacterial evolution. MLST in association with BURST analysis has been used to type isolates of many species. But this method is useful only if housekeeping gene diversity exists in the studied species. For example, in the species *Yersinia pestis* no diversity was found in the housekeeping genes studied (34). With the MST approach, differentiation of the 3 biovars Antiqua, Medievalis, and Orientalis was possible (25), which shows that the discriminatory power of MST is higher than that of MLST and is comparable to that of tandem repeats analysis (35). Low variability was found in *C. burnetii* housekeeping genes such as 16S rRNA (36) and *rpoB* (37). MST is the first method that allows a rapid and reliable typing of *C. burnetii* isolates during investigations of outbreaks by sequencing the PCR product obtained from the 10 spacers described. We did not test isolates from Australia and only 8 from the United States. Two isolates from Africa (Namibia and CB119) were considered as singletons in the BURST analysis denoting lack of closely related isolates. In the future, isolates that were not available in our laboratory during this study must be tested so the missing links in our phylogenetic analysis can be determined. The constitution of a database in a website will allow isolates from all the countries in the world to be compared and increase understanding of the propagation of the isolates of *C. burnetii*.

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Multidrug-Resistant *Acinetobacter* Extremity Infections in Soldiers

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War wound infection and osteomyelitis caused by multidrug-resistant (MDR) *Acinetobacter* species have been prevalent during the 2003–2005 military operations in Iraq. Twenty-three soldiers wounded in Iraq and subsequently admitted to our facility from March 2003 to May 2004 had wound cultures positive for *Acinetobacter calcoaceticus-baumannii* complex. Eighteen had osteomyelitis, 2 burn infection, and 3 deep wound infection. Primary therapy for these infections was directed antimicrobial agents for an average of 6 weeks. All soldiers initially improved, regardless of the specific type of therapy. Patients were followed up to 23 months after completing therapy, and none had recurrent infection with *Acinetobacter* species. Despite the drug resistance that infecting organisms demonstrated in this series, a regimen of carefully selected extended antimicrobial-drug therapy appears effective for osteomyelitis caused by MDR *Acinetobacter* spp.

Casualty statistics from the 2003–2005 military operations in Iraq show an increase in the ratio of wounded to fatal casualties compared to previous operations in the Persian Gulf, Vietnam, and Korea (1). This relative increase of wounded casualties has led to an increased incidence of war wound infection and osteomyelitis, especially caused by multidrug-resistant (MDR) *Acinetobacter* species. The incidence of bacteremia at military medical facilities caused by *Acinetobacter baumannii* has also increased (2). The current incidence of infection with *Acinetobacter* should not be surprising. These organisms were the most frequently recovered gram-negative isolate from war wounds and the second most frequent bacterium causing bloodstream infection in US Marines with extremity wounds during the Vietnam War (3). In nonconflict environments, *Acinetobacter* species are rarely responsible for community-acquired infections. In the hospital setting, *Acinetobacter* species are an important cause of nosocomial infection, yet these infections were rarely encountered in our facility until we began observing them in soldiers with infected wounds. Nosocomial infections caused by *Acinetobacter* species include pneumonia, meningitis, bloodstream, urinary tract, surgical wound, and soft tissue infections (4). Such infections are challenging to treat because of extensive antimicrobial drug resistance. Osteomyelitis caused by *Acinetobacter* occurs, but it is less frequently reported and had not been identified in our facility during the 14 months before March 2003. Optimal therapy for osteomyelitis caused by these organisms is not well defined because of limited available data. This case series reviews 1 military medical center's experience with these infections, including species identified, antimicrobial drug–susceptibility patterns, antimicrobial drug therapy, and clinical outcomes.

Methods

Case reports were compiled from active-duty soldiers admitted to Brooke Army Medical Center (BAMC) in San Antonio, Texas. This tertiary military medical center serves a population of active-duty and retired soldiers and their dependents along with a limited number of civilian trauma patients admitted from the local area. The hospital was operating at an average capacity of 175 beds during the study period. This facility also houses the US Army's Institute of Surgical Research, which treats both active-duty and civilian trauma patients with burn injuries. Data collection for this case series was completed under a study protocol approved by BAMC's Department of Clinical Investigation Institutional Review Board.

Identification of Patients

All wound, sputum, urine, and blood culture results completed at our hospital from March 1, 2003, to May 31, 2004, were reviewed. Those patients who had *Acinetobacter*-positive cultures were then compared to all

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active-duty soldiers admitted to our facility. A soldier was considered for inclusion if he had an *Acinetobacter*-positive culture and had been deployed to Iraq or Afghanistan and had an admission diagnosis of injury (ICD codes 800.0–900.0). Similarly, hospital admission and laboratory data were reviewed for the 14 months before the study period to define the incidence of *Acinetobacter* infection in hospitalized, active-duty soldiers before the onset of military action in Iraq.

Case Definitions

Patients with either *Acinetobacter* contiguous focus osteomyelitis or wound infection are included in this series. Cases were defined as osteomyelitis if bone tissue collected during surgical procedures (primarily open debridements but also including placement of external or internal fixators or bone grafting) was positive for *Acinetobacter* spp. on routine culture (5,6). In addition, patients with open fractures or exposed bone with gross findings of infection (purulence, necrotic tissue, or environmental contamination with exposed bone), clinical evidence of infection (temperature >38°C, leukocyte count >12,000/μL), and *Acinetobacter* spp. identified from culture of deep wound tissue obtained intraoperatively, excluding bone, were also defined as having osteomyelitis (7). Cases were defined as wound infection if similar deep wound cultures were positive for *Acinetobacter* spp. with gross findings and clinical evidence of infection but no exposed bone and no fracture. Colonization with *Acinetobacter* was defined as a positive culture for *Acinetobacter* without gross findings or clinical evidence for infection.

The *Acinetobacter* isolate was defined as MDR if it was resistant to ≥3 classes of antimicrobial agents as tested by automated antimicrobial drug–susceptibility testing (Vitek, bioMérieux, Hazelwood, MO, USA) (8). On occasion, isolates were further evaluated with disk diffusion antimicrobial testing for susceptibilities to alternate antimicrobial drugs, such as colistin, or to confirm automated susceptibility results. Confirmatory disk diffusion susceptibility testing was completed only for those isolates that were resistant to all antimicrobial agents by automated testing or if only 1 antimicrobial drug was listed as susceptible. Disk diffusion testing was performed in accordance with Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines (9). Colistin susceptibility was assumed if the zone of inhibition was ≥14 mm (10).

Patients were evaluated for recurrence of infection. Many patients underwent subsequent reconstructive surgeries, and the bone tissue was sent for culture. Definitions of recurrent infection followed the previously described criteria for the case definitions with the following additions: recurrent infection was defined as having *Acinetobacter* spp. isolated at the original site of infection after completing an antimicrobial drug treatment course for the initial infection; secondary infection was defined as infection with a different organism at the same site as the initial *Acinetobacter* infection.

Data Collection

Both electronic and paper charts of all patients who met case definition criteria were retrospectively reviewed for demographic, diagnostic, and treatment data. Laboratory results were reviewed for *Acinetobacter* species isolated and antimicrobial drug susceptibilities. Patients were also interviewed either in person or by telephone to confirm mechanism of injury, length of antimicrobial drug treatment course, recurrence of infection, subsequent hospital admissions, and clinical outcome of the sustained injury and infection (resolved, continuing convalescence, or amputation). Follow-up was defined as the time from completing the initial antimicrobial treatment course to the date of the study interview.

Results

Case Inclusion Criteria

From March 1, 2003, to May 31, 2004, a total of 24,114 cultures (blood, urine, wound, sputum) were completed in our hospital. Of these, 145 (0.6%) were positive for *Acinetobacter* spp. During the same period, 237 active-duty patients were admitted to our facility with the admission diagnosis of injury (Figure). Of these admitted soldiers, 151 (64%) had been deployed to OIF/OEF. Cultures of blood, wound, sputum, urine, or skin were obtained for 84 of these patients; 48 (32% of admitted deployed soldiers) were positive for *Acinetobacter* spp. Of these, 30 (63%) represented clinical infection; the remaining 18 represented colonization with *Acinetobacter.* Of those patients with cultures that represented clinical infection, 23 met the case definition for *Acinetobacter* osteomyelitis (Table 1) or *Acinetobacter* wound infection (Table 2). During the 14 months before the study period, only 2 active-duty soldiers, of 326 admitted to our facility, had any *Acinetobacter* infection. The incidence of *Acinetobacter* infection during the study period represents a significant increase when compared to the control period ($p<0.01$ by 2-tailed Fisher exact test).

Demographics

All patients included in this series had been transferred to BAMC through the military airmobile medical evacuation system. All, excluding one, were evacuated through, and admitted for at least 1 day to, Landstuhl Army Medical Center in Landstuhl, Germany; 3 patients were admitted to a second US Army medical center before admission to BAMC. The median time from injury to admission at

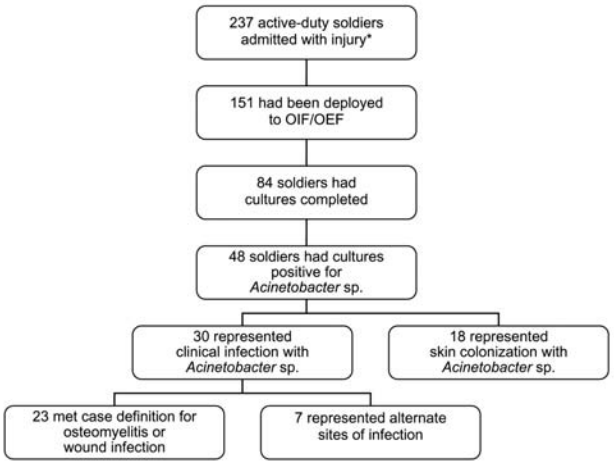

Figure. Flow chart illustrating active-duty soldier admissions to Brooke Army Medical Center from March 1, 2003, to May 31, 2004, and those who met case definitions for *Acinetobacter* osteomyelitis or wound infection. *Soldiers with diagnosis of injury, ICD codes 800.0–900.0. OIF/OEF, Operation Iraqi Freedom/ Operation Enduring Freedom.

BAMC was 6 days (range 2–36 days, Table 3). The median time from injury to identification of infection was also 6 days (range 3–12 days). *Acinetobacter* infection was initially identified at BAMC in 15 of the 23 patients; the remainder were identified at a previous medical center. None were initially diagnosed prior to evacuation from Iraq or Afghanistan. The median age of the patients was 26 years (range 20–48), and all but 2 were men. Patients were generally stable on admission to BAMC and did not require admission to an intensive care unit.

Microbiologic Data

Patients with *Acinetobacter* osteomyelitis primarily had bone tissue collected during surgical procedures that was culture-positive for *A. calcoaceticus-baumannii* complex. This was the only species and organism identified in all initial tissue cultures. Ten patients had deep wound cultures, excluding bone tissue, that were positive for *A. calcoaceticus-baumannii* complex. Five (patient numbers 4, 9, 10, 11, and 15, Table 1) had open fractures with environmental contamination and signs of infection that met the case definition of osteomyelitis. The remaining 5 (Table 2) did not meet criteria for diagnosis of osteomyelitis and were diagnosed with wound infection. Two of these patients had burn injuries. Cultures of debrided soft tissue in these 2 patients were positive for *Acinetobacter* within the first 8 days of hospitalization, and pathologic evaluation of tissue demonstrated invasive infection. Patient no. 22 had a soft tissue wound culture positive on hospital day 5 (postinjury day 9); patient no. 23 had a soft tissue wound culture positive on hospital day 8 (postinjury day 10).

Antimicrobial Drug–susceptibility Data

Thirty-eight cultures from the 23 patients reported in this study were positive for *Acinetobacter* spp. (Table 4). Twenty-nine isolates were MDR, as tested by automated susceptibility testing. All but 4 of the MDR isolates were susceptible to imipenem, and no imipenem resistance developed in the 15 patients who received this drug during therapy. Three of these 4 isolates were susceptible only to amikacin. Of the 25 imipenem-susceptible MDR *Acinetobacter* isolates, 10 demonstrated resistance to all other tested antimicrobial agents. Other isolates were susceptible to only 1 other antimicrobial agent: 7 were also susceptible to amikacin, 3 to ampicillin/sulbactam, 2 to tobramycin, and 1 to trimethoprim/sulfamethoxazole. Nine isolates were not MDR. These isolates were susceptible to ≥3 classes of the tested antimicrobial agents. Three MDR isolates were tested for susceptibility to colistin; all 3 were susceptible by disk diffusion testing. One was susceptible only to imipenem, 1 to amikacin alone, and 1 to both amikacin and ceftazidime.

Therapy

Antimicrobial drug treatment of these infections was based on susceptibility testing, and all patients with osteomyelitis underwent multiple surgical debridements of necrotic bone. Ten of the patients with osteomyelitis were treated with dual antimicrobial agents, 7 with monotherapy, and 1 with surgical debridement alone. Only patients with osteomyelitis received dual antimicrobial drug therapy. Of the 10 treated with dual therapy, 5 had MDR *Acinetobacter* spp. and 5 had non-MDR *Acinetobacter* spp. isolated. The primary combination of antimicrobial agents was imipenem (500 mg every 6 h) in combination with high-dose amikacin (15–20 mg/kg daily). In a few instances, when imipenem was not active against the isolated organism, ampicillin/sulbactam or ceftazidime was used if either was active against the particular isolate (Table 1). Of the 7 treated with monotherapy, 5 had MDR *Acinetobacter* isolated. All patients with wound infection received monotherapy based on antimicrobial drug–susceptibility testing results.

Follow-up

The follow-up period was 1–23 months (mean 9 months). During this time, no *Acinetobacter* infections recurred at any site, including the bloodstream. Seven secondary infections occurred, 6 in those with an initial diagnosis of osteomyelitis and 1 with wound infection. Four occurred in patients with MDR *Acinetobacter* (3 with osteomyelitis and 1 with wound infection). These secondary infections primarily involved other resistant nosocomial pathogens (see expanded online Tables 1 and 2, available at http://www.cdc.gov/ncidod/eid/vol11no08/05-0103.htm).

Table 1. *Acinetobacter* osteomyelitis*

Patient	Osteomyelitis location	Mechanism of injury	MDR† isolate	Bacteremia	Parenteral drug therapy	Follow-up, wk‡
1	Left radius/ulna	Landmine explosion	Yes	No	Imipenem 500 mg every 6 h, amikacin 20 mg/kg/d for 8 wk	12
2	Right humerus/shoulder	IED round	Yes	No	Imipenem 500 mg every 6 h, amikacin 15 mg/kg/d for 6 wk	32
3	Right humerus/shoulder	IED blast	Yes	No	Imipenem 500 mg every 6 h, amikacin 15 mg/kg/d for 7 wk	35
4	Left radius/ulna	RPG blast	Yes	Yes	Imipenem 500 mg every 6 h for 7 wk, with amikacin 20 mg/kg/d for 3 wk changed to amp/sulb 3 g every 6 h for 4 wk	4
5	Left tibia	Mortar blast	Yes	No	Ceftazidime 2 g every 8 h; amikacin 12.5 mg/kg/d for 7 wk	7
6	Right distal humerus/elbow	IED blast	Yes	No	Imipenem 500 mg every 6 h for 7 wk	22
7	Left tibia/fibula	Proximate IED blast	Yes	No	Amp/sulb 12 g continuous 24-h infusion for 6 wk	36
8	Left humerus	Proximate mortar round blast	Yes	Yes	Meropenem 1 g every 8 h for 7 wk	5
9	Left tibia/fibula	Land mine	Yes	No	Gentamicin 5 mg/kg/d for 3 wk	40
10	Left distal humerus/elbow	IED blast	Yes	No	Imipenem 500 mg every 6 h for 6 wk	30
11	Right humerus/shoulder	IED blast	Yes	No	None	39
12	Right humerus/elbow	IED blast	No	No	Imipenem 500 mg every 6 h, amikacin 15 mg/kg/d for 6 wk	48
13	Left tibia	50-caliber gunshot	No	No	Amp/sulb 3 g every 6 h, amikacin 20 mg/kg/d for 6 wk	61
14	Left humerus/elbow	Motor vehicle accident	No	No	Imipenem 500 mg every 6 h, gentamicin 5 mg/kg/d for 4 d	57
15	Right femur	RPG round	No	Yes	Imipenem 500 mg every 6 h, amikacin 20 mg/kg/d for 6 wk	9
16	Left tibia	Gunshot	No	No	Imipenem 500 mg every 6 h, amikacin 15 mg/kg/d for 8 wk	35
17	Right tibia/fibula	IED blast	No	No	Imipenem 500 mg every 6 h for 4 wk, followed by meropenem 1 g every 8 h for 2 more wk	50
18	Left tibia	Motor vehicle accident	No	No	Ceftazidime 2 g every 8 h for 4 wk	56

*Expanded online table available at http://www.cdc.gov/ncidod/eid/vol11no08/05-0103.htm#table1.
†MDR, multidrug resistant; MSSA, methicillin-sensitive *Staphylococcus aureus*; IED, improvised explosive device; RPG, rocket-propelled grenade; MRSA, methicillin-resistant *S. aureus*.; amp/sulb, ampicillin/sulbactam.
‡Length of follow-up after completion of antimicrobial drug therapy.

Control Period

During the 14 months before March 2003, only 2 active-duty soldiers had *Acinetobacter* infection. A soft tissue infection with *Acinetobacter* developed in 1 soldier with a history of bullous pemphigoid. Bacteremia with *Acinetobacter* developed in the other soldier, who had a history of Ewing sarcoma. The latter *Acinetobacter* isolate was not a MDR organism and was treated with imipenem (500 mg parenterally) for 14 days.

Discussion

The 23 cases observed during the study period represent a significant increase in the incidence of clinical infection with *Acinetobacter* in our facility. Similarly, the rate of blood, wound, or urine cultures positive for *Acinetobacter* species increased 3-fold during the study period as compared to the control time period (data not shown). This increase and the influx of severe extremity infection due to MDR *Acinetobacter* species posed considerable challenges. The foremost was determining appropriate therapy for osteomyelitis caused by MDR *Acinetobacter* species without institutional or historical experience to guide us. In addition, increasing prevalence of this MDR gram-negative organism in our facility mandated new infection control procedures to limit nosocomial spread. Finally, the occurrence of *Acinetobacter* wound infection was somewhat unexpected, and initially the reservoir for infection was unclear and generated much debate. Recent investigation by the military medical and research community suggests that these are nosocomial infections; however, their exact source remains unclear.

Most *Acinetobacter* infections reported in the literature reflect nosocomial *Acinetobacter*, as hospitalized patients

Table 2. *Acinetobacter* wound infection*

Patient	Wound infection location	Mechanism of injury	MDR† isolate	Bacteremia	Parenteral drug therapy	Follow-up, wk‡
19	Right achilles tendon wound	RPG blast	Yes	No	Imipenem 500 mg every 6 h for 5 wk	36
20	Left thigh wound	Proximate car-bomb blast	Yes	No	Imipenem 500 mg every 6 h for 2 wk	11
21	Right elbow wound	RPG fire	Yes	No	Cefazolin 1 g every 8 h for 10 d	92
22	Scalp wound	Land mine	Yes	No	Imipenem 1 g every 8 h for 16 d	89
23	Hand wound	RPG blast	Yes	No	Imipenem 500 mg every 6 h for 14 d	30

*Expanded online table available at http://www.cdc.gov/ncidod/eid/vol11no08/05-0103.htm#table2.
†MDR, multidrug-resistant; RPG, rocket-propelled grenade.
‡Length of follow up after completion of antimicrobial drug therapy.

are at increased risk because of severe illness or disability, extremes of age, and relative states of immunocompromise (4). *Acinetobacter* species can cause infection in any organ system, including bacteremia, pneumonia, endocarditis, meningitis, urinary tract infection, intraabdominal abscess, osteomyelitis, soft tissue infection, and surgical site infections (11). Data collected from a review of sentinel hospitals in the United States demonstrated that 1.5% of all nosocomial bloodstream infections were due to *Acinetobacter* species (12). Crude death rates associated with nosocomial *Acinetobacter* infection are 19%–54% (12–15). The difficulty in treating these infections is not due to any excessive virulence of the organism per se but rather to its antimicrobial drug resistance. Many nosocomial isolates are resistant to ≥3 classes of antimicrobial agents, which classifies them as MDR organisms (8,12). A common susceptibility pattern in this case series was resistance to all antimicrobial agents except imipenem and amikacin.

When these patients were first evaluated, data to guide therapeutic decisions were limited. Previous reported experience with osteomyelitis caused by *Acinetobacter* species is scant. It has been described after a hamster bite in an 8-year-old boy (16) and in a patient who previously had an artillery fragment injury that caused an open fracture of the right femur (17). Other reviews have described osteomyelitis as a sequela of infection with *Acinetobacter* species but did not report details of therapy or follow up (4,11). Patients in our case series primarily received extended dual antimicrobial–drug therapy based on

Table 3. Patient demographics*

Patient	Age, y	Time (d) from injury to		No. MC admissions before BAMC admission	Infection initially diagnosed at BAMC
		BAMC admission	Diagnosis of infection		
1	20	13	4	1	N
2	26	10	10	1	Y
3	31	11	12	1	Y
4	21	9	7	2	N
5	21	4	5	1	Y
6	37	13	NA	2	N
7	33	5	6	1	Y
8	48	5	5	1	Y
9	21	4	NA	1	N
10	34	13	NA	1	N
11	21	6	7	1	Y
12	37	6	7	1	Y
13	22	3	3	0	Y
14	23	5	9	1	Y
15	33	3	4	1	Y
16	22	5	6	1	Y
17	27	13	NA	1	N
18	26	36	6	1	N
19	26	16	9	2	N
20	21	6	6	1	Y
21	26	5	5	1	Y
22	20	4	9	1	Y
23	24	2	10	1	Y

*BAMC, Brooke Army Medical Center; MC, medical center; N, no; Y, yes; NA, not available.

Table 4. *Acinetobacter calcoaceticus-baumannii* complex antimicrobial drug susceptibilities for 38 isolates recovered from wound or blood cultures

Antimicrobial drug	Susceptible (%)
Amikacin	48
Amoxicillin/clavulanate	9
Ampicillin/sulbactam	50
Cefepime	14
Cefotetan	3
Ceftazidime	12
Ceftriaxone	6
Ciprofloxacin	11
Colistin*	100
Gentamicin	8
Imipenem	89
Tobramycin	14
Trimethoprim/sulfamethoxazole	29

*Colistin susceptibility evaluated in 3 multidrug-resistant isolates.

susceptibility patterns of the recovered organisms. Combination therapy has been shown to decrease the risk for development of more highly resistant organisms, which has been reported when single agents are used alone (18). While on this antimicrobial regimen, patients demonstrated clinical improvement with marked reduction of inflammatory markers. Many of these patients had internal stabilizing hardware placed into the infected area at the time of diagnosis of infection. This hardware remained in place at the completion of parenteral therapy. In these situations, when the causative organism was susceptible to oral antimicrobial agents, oral suppressive therapy was continued as long as the stabilizing hardware remained in place. In most cases, however, because of extended antimicrobial drug resistance, no oral agents maintained activity against the *Acinetobacter* isolate. Once those infected with MDR isolates demonstrated clinical improvement and normalization of inflammatory markers, antimicrobial drug therapy was discontinued without continuing long-term suppressive therapy (see expanded online Tables 1 and 2, available at http://www.cdc.gov/ncidod/eid/vol11no08/05-0103.htm).

During the follow-up period, no recurrent episodes of *Acinetobacter* osteomyelitis have occurred. The relative brevity of follow-up is a limitation of this study. The ultimate outcome for these patients will not be known for many years, as they have increased risk for recurrent infection throughout their lifetime. In addition, *Acinetobacter* organisms do not possess substantial inherent virulence. None of the patients in this series failed therapy, and none died because of *Acinetobacter* infection. Such is not the case in outbreaks among immunocompromised or intensive care patients, in whom *Acinetobacter* infection leads to increased mortality (12–15). The successful outcomes in this case series may be a reflection of the youth and general good health of the soldiers infected.

MDR *Acinetobacter* is an important nosocomial pathogen with multiple recent outbreaks reported (18–22). It has the capacity to survive in dry environments (23,24), which increases the risk for nosocomial transmission. The increasing prevalence of MDR *Acinetobacter* in our facility led to new infection control procedures. Currently, all injured soldiers admitted to our facility returning from OIF/OEF are placed in contact isolation. Screening cultures of the axilla, groin, and any open wound are completed to assess for colonization with MDR *Acinetobacter*, which was identified in 18 of 151 admitted soldiers during the study period (Figure). If all cultures taken on admission are negative, the soldier is then removed from contact isolation. Soldiers with wound infection or osteomyelitis caused by MDR *Acinetobacter* are kept in contact isolation for the duration of hospitalization. Implementation of these types of infection control procedures has limited nosocomial spread in previously reported outbreaks (18,20,22), which is the goal of our current policy, in addition to controlling the continuing reservoir of this organism.

As previously noted, we initially suspected that colonized soldiers themselves were the reservoir for MDR *Acinetobacter*, and that this colonization was obtained from the environment. This hypothesis was based on 2 facts. First, these organisms are ubiquitous in the environment (4,25), and inoculation of these organisms into war wounds during traumatic blast, shrapnel, or projectile injuries seemed to be plausible. Second, *Acinetobacter* spp. had previously been described as common pathogens in war wounds (3), supporting the initial hypothesis. However, these infections are apparently similar to recently reported nosocomial MDR *Acinetobacter* infections. Investigation into the cause of these infections is ongoing, but the source is unlikely to be environmental. Multiple follow-up soil samples have not yielded *Acinetobacter*, yet it has been recovered from environmental cultures within field medical facilities. The final outcome of this investigation is pending further analysis.

Data from this case series demonstrate that highly resistant *Acinetobacter* infection, including osteomyelitis, can be successfully treated with appropriate surgical debridement, directed antimicrobial drug therapy, and careful follow-up. Our patients responded to this multifaceted approach, although their final outcome will not be determined for several years. These patients continue to be followed for recurrence of MDR *Acinetobacter* infection. Clearly, guided therapy based on antimicrobial drug susceptibility leads to suppression of recurrent infection up to 23 months. Most of the patients in this series did not receive extended continuation therapy with oral antimicrobial agents; whether such therapy would provide added benefit is unclear. However, few antimicrobial drug options are currently available, with none soon to be

released, to treat infections caused by resistant gram-negative organisms. Increasing prevalence of these types of infections highlights the necessity for newer antimicrobial agents with activity against these organisms.

Dr. Davis is a fellow in infectious disease at BAMC, Ft. Sam Houston, Texas. His primary research interest is nosocomial methicillin-resistant *Staphylococcus aureus* infections.

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Human Coronavirus NL63, France

Astrid Vabret,* Thomas Mourez,* Julia Dina,* Lia van der Hoek,† Stéphanie Gouarin,* Joëlle Petitjean,* Jacques Brouard,* and François Freymuth*

The human coronavirus NL63 (HCoV-NL63) was first identified in the Netherlands, and its circulation in France has not been investigated. We studied HCoV-NL63 infection in hospitalized children diagnosed with respiratory tract infections. From November 2002 to April 2003, we evaluated 300 respiratory specimens for HCoV-NL63. Of the 300 samples, 28 (9.3%) were positive for HCoV-NL63. The highest prevalence was found in February (18%). The main symptoms were fever (61%), rhinitis (39%), bronchiolitis (39%), digestive problems (33%), otitis (28%), pharyngitis (22%), and conjunctivitis (17%). A fragment of the spike protein gene was sequenced to determine the variety of circulating HCoV-NL63. Phylogenetic analysis indicated that strains with different genetic markers cocirculate in France.

Human coronaviruses (HCoVs) were first recorded in the late 1960s; they are associated mainly with respiratory tract illness but are also involved in enteric and central nervous system diseases. They are represented by 2 prototype strains, HCoV-229E and HCoV-OC43, which belong to antigenic groups 1 and 2, respectively. In 2003, human coronaviruses received worldwide attention with the emergence of severe acute respiratory syndrome (SARS) caused by a novel coronavirus (SARS-CoV). In 2004, the increase in research on theses viruses soon led to the discovery of 2 other human coronaviruses, HCoV-NL63 in the Netherlands and, more recently, CoV-HKU1 in China (1–3). In March 2004, van der Hoek et al. isolated HCoV-NL63 from a nasopharyngeal aspirate taken from a 7-month-old child hospitalized with bronchiolitis, conjunctivitis, and fever (1). One month later, Fouchier et al. characterized the same virus isolated from a nasal swab that had been collected from a child with pneumonia in April 1988 (2). Phylogenetic analysis showed that HCoV-NL63 is a new group 1 coronavirus, most closely related to HCoV-229E. Partial HCoV-NL63 sequences from Australia, Japan, and Canada have been submitted to the GenBank database, which indicates that this virus is distributed worldwide. Two retrospective studies were conducted in the Netherlands, and 11 additional HCoV-NL63–positive samples were detected from November 2000 to February 2003.

We tested for HCoV-NL63 in children with acute respiratory tract infection hospitalized in Caen from November 2002 to April 2003, described symptoms associated with this infection, and examined local strains for the genetic variability. We also evaluated a multiplex reverse transcription–polymerase chain reaction (RT-PCR) assay for classical coronaviruses as a tool to test human coronaviruses (except SARS-CoV).

Materials and Methods

From November 2002 to April 2003, the virology laboratory (University Hospital, Caen, France) received 1,427 respiratory samples (nasal aspirates and swabs) from patients <20 years of age. All specimens were tested for influenza virus A and B; respiratory syncytial virus (RSV); parainfluenza virus 1, 2, 3, and 4; and adenovirus by direct or indirect immunofluorescence or virus isolation. Samples were also tested for human metapneumovirus (HMPV), rhinovirus, enterovirus, and HCoV 229E and OC43 by virus isolation and RT-PCR. A total of 556 samples (39%) were positive for any of these viruses. Symptoms indicated viral infection, as judged by the clinical department; therefore, samples were not tested for bacterial pathogens. Of the 556 positive samples, the following respiratory viruses were detected: RSV (37%, n = 205), rhinovirus (18%, n = 101), influenza virus A and B (15%, n = 86), HMPV (9.7%, n = 54), and HCoV-OC43 (1.2%, n = 7); no HCoV-229E were detected.

Of the 871 negative samples, 300 (50 per month) were tested for HCoV-NL63. These 300 samples represented 191 patients <2 years of age (64%), 46 patients 2–5 years of age (15%), and 63 patients 6–20 years of age (21%). All patients were hospitalized with acute respiratory tract illness. Data for 18 patients with recorded HCoV-NL63 infection were available and were examined retrospectively for specific respiratory symptoms. All patients consented to having their samples tested for respiratory viruses, including coronaviruses.

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Two RT-PCR assays were used to detect HCoV-NL63 in respiratory samples. RNA was extracted by using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The first RT-PCR assay was a 1-step simple RT-PCR that amplified a 255-bp fragment of the nucleocapsid (N) gene of HCoV-NL63 by using the following primers: N5-PCR2 (5′-GATAACCAGTCGAAGTCACCTAGTTC-3′) and N3-PCR2 (5′-ATTAGGAATCAATTCAGCAAGCTGTG-3′). The second assay was a 1-step multiplex RT-PCR that amplified the same 255-bp fragment of the N gene of HCoV-NL63, a 574-bp fragment of the membrane (M) gene of HCoV-229E, and a 334-bp fragment of the M gene of HCoV-OC43 by using previously described primers (4,5). These assays (OneStep RT-PCR kit, Qiagen) were undertaken in 25-μL reaction volume containing 2.5 μL RNA extract, 5 μL 5× Qiagen OneStep RT-PCR buffer, 1 μL 10 mmol/L deoxynucleoside triphosphate (dNTP), 1 μL Qiagen OneStep RT-PCR Enzyme Mix, 1.2 μL of 10 μmol/L each primer, 3 μL Qiagen OneStep RT-PCR kit Q solution, and RNase-free water to 25 μL. The reaction was carried out in a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with an initial reverse transcription step at 50°C for 30 min, followed by PCR activation at 95°C for 15 min, 40 cycles of amplification (30 s at 95°C, 30 s at 58°C, 1 min at 72°C), and a final extension step at 72°C for 10 min. Each RT-PCR test included water controls that were treated identically to the virus samples throughout and was performed with usual precautions to avoid contamination.

RT-PCR products were subject to electrophoresis on an agarose gel, stained with ethidium bromide, and visualized under UV light. The comparative analytical sensitivities of these simple and multiplex RT-PCR assays were previously studied on prototype strains by analyzing serial 10-fold dilutions of positive control for HCoVs NL63, OC43, and 229E. The analytical sensitivity was equivalent to detect HCoV-OC43, the multiplex assay was more sensitive (by one 10-fold dilution) to detect HCoV-229E, and less sensitive (by one 10-fold dilution) to detect HCoV-NL63 (data not shown). No cross-reaction of these tests was observed between these coronaviruses. Samples that were positive for HCoVs NL63, 229E, and OC43 were confirmed by using a DNA enzyme immunoassay (GEN-ETI-K DEIA, Sorin, Saluggia, Italy) carried out as recommended by the manufacturer with original probes previously described for HCoVs 229E and OC43 and the following probe defined in the N gene for HCoV-NL63: 5′-(Biotin)CCTCTTTCT-CAACCCAGGGCTGATA-3′ (4). A third RT-PCR assay was carried out on 12 HCoV-NL63–positive samples amplifying a 523-bp fragment with spike (S) gene–specific primers NL63-S-sens (position 22557–22582: 5′-ACCGCTGTTAATGAGTCTAGATATG-3′) and NL63-S-antisens (position 23043–23063: 5′-GTCCTGCTATACG-GCTTGAA-3′). This assay was performed essentially as described above. The RT-PCR products were purified by using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and sequenced with the primers by using the CEQ Dye Terminator Cycle Sequencing Quick Start Kit on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). The nucleotide sequences of the partial S gene (GenBank accession nos. AY994243–AY994254) were compared with the 2 prototype HCoV-NL63 sequences available in GenBank (NL63-Amsterdam1: NC005831 and NL: AY518894). Both nucleotide and predicted amino acid sequence alignments were prepared by using ClustalX version 1.83. The phylogenetic trees were constructed by using HCoV-229E as an outgroup.

Results

HCoV-NL63 was detected in 28 (9.3%) of the 300 samples evaluated from November 2002 to April 2003. Twenty-two samples were positive for HCoV-NL63 by both simple and multiplex RT-PCR (Figure 1). Discordant results were found for the remaining 6 samples: 3 were positive only in simple RT-PCR, and 3 were positive only in multiplex RT-PCR. These discordant samples were controlled by using the same methods from the RNA extraction product; the results obtained were identical. The specificity of the RT-PCR products under UV was confirmed by hybridization. Multiplex RT-PCR identified 3 samples with HCoV-OC43 and 1 sample with both HCoV-NL63 and HCoV-OC43. The 28 HCoV-NL63–positive samples were obtained from 18 patients <2 years of age (65%), 4 patients 2–5 years of age (14%), and 6 patients 6–15 years of age (21%). The age distribution of the patients infected by HCoV-NL63 was identical to the age

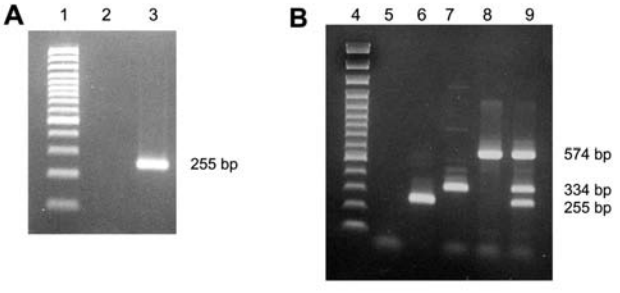

Figure 1. Ethidium bromide stain of 2% agarose gel showing reverse transcription-polymerase chain reaction (RT-PCR) products of human coronaviruses (HCoVs). A) Simple 1-step RT-PCR (HCoV-NL63, gene N): lane 1, size markers (100 bp); lane 2, negative control RT-PCR mix; lane 3, positive control HCoV-NL63. B) Multiplex 1-step RT-PCR (HCoVs NL63, OC43, 229E): lane 4, size markers (100 bp); lane 5, negative control RT-PCR mix; lane 6, positive control HCoV-NL63; lane 7, positive control HCoV-OC43; lane 8, positive control HCoV-229E; lane 9, mix of 3 positive control HCoVs (NL63, OC43, and 229E).

distribution of the sample. Positive specimens were collected throughout the study period; no epidemic was observed. The temporal distribution of HCoV-NL63 infection is shown in Figure 2.

All patients included in this study had a respiratory tract illness. The medical reports of 18 patients with HCoV-NL63–positive samples were retrospectively examined, and the following symptoms were noted: fever (61%, n = 11), rhinitis (39%, n = 7), lower respiratory tract illness (bronchiolitis, pneumonia [39%, n = 7]), digestive problems (diarrhea and abdominal pain [33%, n = 6]), otitis (28%, n = 5), pharyngitis (22%, n = 4), and conjunctivitis (17%, n = 3). One patient had severe underlying disease (congenital immunodeficiency) and had upper respiratory tract illness with fever, another had a family history of atopic allergy, and pneumonia was diagnosed. Overall, more than one third of the patients infected by HCoV-NL63 had severe lower respiratory tract infection (6 bronchiolitis and 1 pneumonia). All of them recovered completely.

To determine if the isolates from France contain different genetic markers, we sequenced a part of the S protein gene of 12 isolates for molecular analysis. The phylogenetic analysis shows that the isolates from France are a divergent group containing sequences with different markers. One isolate (23034101) had characteristics of an outlier (Figure 3A). The phylogenetic analysis of the predicted amino acid sequence also shows that this isolate is an outlier (Figure 3B). However, the branches of this tree are based on only 1 amino acid difference. Care should be taken because of the limited informative sites in the sequence.

Discussion

A number of viruses cause respiratory infections, and many infections cannot be attributed to any known pathogen (6). This fact may be because some detection methods lack sensitivity, because some respiratory viruses are not systematically tested for, or because some pathogens are not yet identified. Of the 4 novel agents, HMPV, SARS-CoV, HCoV-NL63, and CoV-HKU1, identified recently, 3 were coronaviruses (1,3,7–9). Coronaviruses infect many species of mammals and birds, they possess the largest genome of all RNA viruses (≈30 kb), and they have a high frequency of recombination. In addition, the potential to infect other species has been described for bovine coronavirus and is suspected to have caused the SARS outbreak (10,11). Therefore, coronaviruses represent a potential major infectious agent in humans. Based on genotypic and serologic characteristics, coronaviruses were divided into 3 distinct groups: with HCoVs 229E and NL63 in group 1 and HCoVs OC43 and HKU1 in group 2. SARS-CoV is not definitively assigned

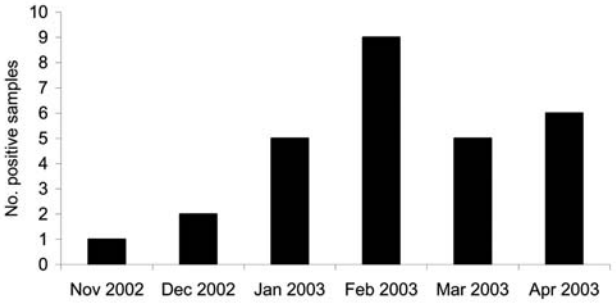

Figure 2. Number of human coronavirus NL63-positive samples per month. Fifty samples from patients hospitalized for acute respiratory symptoms were tested each month.

to any of these groups. However, an early split-off of SARS-CoV from the group 2 lineage was suggested (12). Until recently, only the HCoVs 229E and OC43 and SARS-CoV have been thoroughly studied. As suggested by epidemiologic surveys conducted in the 1970s, human coronaviruses are distributed worldwide and circulate during seasonal outbreaks (13–15). In this study, we determined whether this is also the case for HCoV-NL63. Furthermore, we looked at the symptoms of a HCoV-NL63

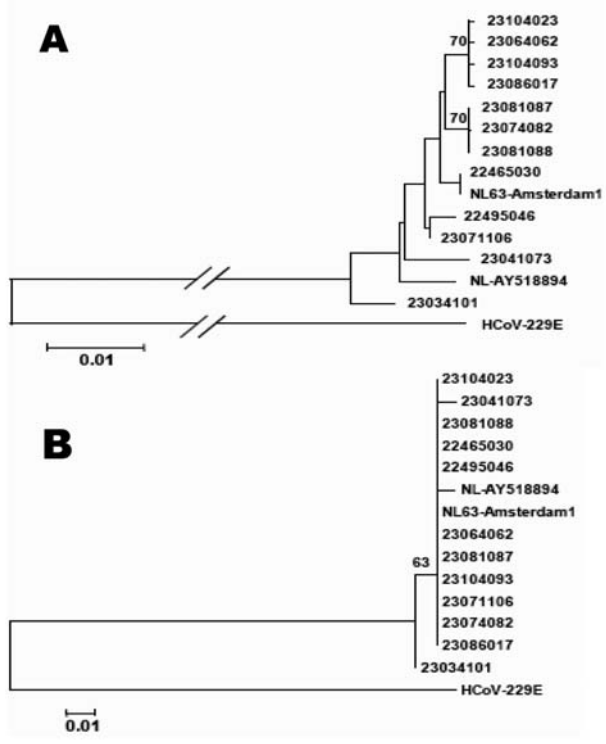

Figure 3. A) Phylogenetic analysis of a 523-bp region of the partial spike gene of 12 human coronavirus (HCoV)-NL63 isolates from France. To construct the trees, the 2 prototype strains NL63-Amsterdam1 and NL-AY518894 were included. HCoV-229E is used as an outgroup. A) Nucleotide sequence alignments created with ClustalX 1.83; bootstrap values ≥70 are indicated. B) Predicted amino acid sequences; bootstrap values ≥50 are indicated.

infection and the heterogeneity of the virus isolate circulating in France. In this study, 9.3% of the samples were positive for HCoV-NL63. These results suggest that HCoV-NL63 can frequently cause infections, particularly in young children.

Because of availability, only samples that tested negative for other respiratory viruses were included in this study. Consequently, we could not identify co-infections. Nevertheless, by using multiplex RT-PCR, which simultaneously detected the classical human coronaviruses (OC43, 229E, and NL63), we detected 1 co-infection by HCoVs NL63 and OC43 and 3 HCoV-OC43–positive samples. These additional cases of HCoV-OC43 infection in our patients can be explained by the fact that multiplex RT-PCR was performed directly on respiratory samples, whereas in previous routine tests, samples were first instilled into a cell culture system (HUH7 cell line) and RT-PCR was then performed on cell culture supernatant.

Detecting human respiratory coronaviruses requires molecular techniques because of difficulties in virus culture and lack of an assay to detect intracellular antigens or others serologic assay. A multiplex RT-PCR is therefore a useful tool to simultaneously test for various HCoVs from a clinical sample. This study showed that the clinical sensitivity of multiplex RT-PCR was equivalent to simple RT-PCR, allowing clinical studies and routine testing.

No HCoV-229E was detected in samples. HCoVs 229E and NL63 both belong to antigenic group 1. The percentage amino acid sequence identities between the S, M, and N proteins of HCoVs NL63 and 229E are 54.7%, 61.5%, and 43.2%, respectively (2). A cross-protective immune response could explain why these 2 human coronaviruses do not circulate at the same time. We detected HCoV-NL63 in respiratory specimens in February with a frequency of 18%. These results correlate with the fact that human coronaviruses circulate primarily in the winter. However, HCoV-NL63 was found in nasal aspirates each month of our study.

The clinical symptoms associated with HCoV-NL63 still need to be determined. In the patients in our study, symptoms included not only respiratory symptoms but also lower respiratory tract diseases such as bronchiolitis, bronchitis, and pneumonia. Human coronaviruses, except SARS-CoV, generally cause disease much like the common cold, but they have also been associated with more severe lower respiratory tract conditions, especially in frail patients (4,16). Whether HCoV-NL63 is also responsible for coldlike illnesses in healthy adults, as has been described for HCoVs 229E and OC43, must be determined. The same can be said about the very recently described coronavirus CoV-HKU1. This virus was identified in a 71-year-old patient with chronic obstructive airway disease who was hospitalized with pneumonia (3).

Digestive problems were noted in approximately one third of patients. No clear evidence exists that human coronaviruses, except SARS-CoV, cause enteric illness, but previous studies have suggested that these viruses may be involved in enteric diseases (17–20). Further studies must be conducted to detect coronaviruses in stool samples and clarify the origin of these digestive symptoms.

The S protein of coronaviruses is a major determinant of cell tropism and pathogenicity and a major inducer of neutralizing antibodies (21). Furthermore, heterogeneity of the S gene has been observed for different HCoV-NL63 strains. We amplified part of the S gene to study the variability of our isolates. Phylogenetic analysis showed that several different isolates are cocirculating in France, similar to the situation in the Netherlands, Australia, Canada, and Belgium.

In conclusion, HCoV-NL63 can be found in patients with upper and lower respiratory tract illness, particularly in hospitalized children. This observation is the first of HCoV-NL63 infection in France, and several isolates of HCoV-NL63 were found to circulate in our country. The sensitive multiplex RT-PCR for the HCoVs NL63, 229E, and OC43 that we developed is a useful tool to facilitate the routine detection of these pathogens.

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Dr. Vabret is a virologist at the University Hospital of Caen, France. Her main research interests include the circulation and molecular analysis of human respiratory coronaviruses.

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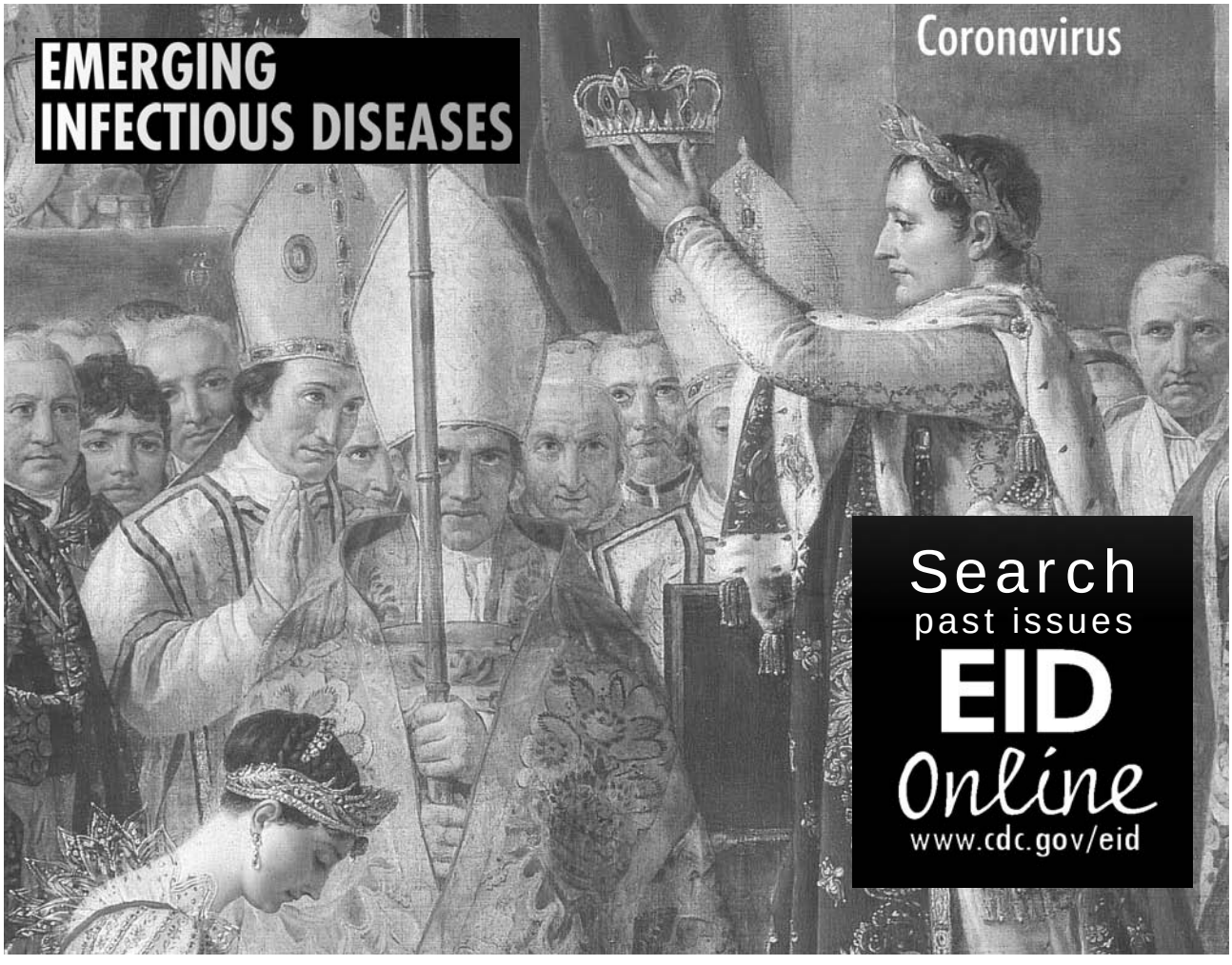

HIV-1 Genetic Diversity in Antenatal Cohort, Canada

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We studied HIV genetic diversity in a cohort of 127 pregnant, HIV-infected women who received prenatal care at Sainte-Justine Hospital in Montreal, Canada, between 1999 and 2003. Clade assignments were derived by phylogenetic analysis of amplified *pol* sequences. Genotyping was successful in 103 of 127 women, 59 (57.3%) of whom were infected with clade B HIV-1, and 44 (42.7%) with nonclade B viruses, including subtypes A, C, D, F, G, and H. Four sequences remained unassigned. Forty-three of 44 women infected with non-clade B viruses were newcomers from sub-Saharan Africa, and subtype identity was consistent with those circulating in their countries of origin. These results highlight the epidemiologic importance of non-B HIV-1 in antenatal populations in a large North American urban center, underscore the influence of population movements on clade intermixing, and identify a group of patients who could be targeted for surveillance and drug therapy followup.

HIV-1 exhibits considerable genetic diversity resulting from the high mutation rate of reverse transcriptase, high viral turnover, viral genomic recombination, and immune and therapeutic selection pressures (1–3). This diversity is a challenge for viral load determination, drug resistance testing, and AIDS vaccine development (1,4–6). Three phylogenetic groups of HIV-1, main (M), outlier (O), non-M, non-O (N), are recognized (2,7). Most HIV-1 infections are caused by group M viruses that comprise 9 clades (A–D, F–H, J, and K) and >13 intersubtype recombinants known as circulating recombinant forms (CRFs) (8). Clade B is most common in North America, Europe, and Australia. However, in the last decade, prevalence of infection with nonclade B viruses has increased in France, Belgium, Spain, and Switzerland (9–12), in large part after migration from or international travel into HIV-endemic areas (13). Nonclade B viruses also circulate in Cuba (14) and the United States (15,16). We measured HIV-1 subtype diversity in a multiethnic cohort of pregnant, HIV-infected women to determine whether nonclade B HIV-1 is emerging in Canada after population movement, and whether antenatal cohorts are suitable sentinel sites to monitor the introduction of nonclade B viruses into Canada.

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Patients and Methods

Patients

One hundred twenty-seven HIV-infected women receiving prenatal care at Centre Maternel et Infantile sur le SIDA, Sainte-Justine Hospital, Montreal, from October 1999 to September 2003 were included in the study. Inclusion criteria were 1) age ≥18 years, 2) a request for prenatal care, 3) positive HIV-1 serologic results, and 4) informed consent. Standardized clinical followup, including antiretroviral (ARV) prophylaxis and treatment, was provided to all women and their children. This cohort study was conducted according to the guidelines of the Ethics Review Board of Sainte-Justine Hospital.

Clinical Parameters

HIV-1 serologic status was determined by using the AxSYM HIV 1/2 gO method (Abbott Diagnostics, Wiesbaden, Germany) and confirmed by Western blot. HIV-1 viral load was measured by using the Versant HIV-1 RNA 3.0 assay (bDNA, Bayer, Pittsburgh, PA, USA). CD4+ T-cell counts were measured by flow cytometry. Standardized data collection assessed sociodemographic

variables and previous and current ARV treatment. Numeric variables were compared by using the Kruskal-Wallis test. Categoric variables were examined by using the Fisher exact test (SPSS version 11.0, SPSS, Inc., Chicago, IL, USA).

HIV-1 Genotyping

In cases in which viral load was >1,000 RNA copies/mL plasma, HIV-1 genotyping was performed by using a protocol (Virco BVBA, Mechelen, Belgium) based on sequencing of a 1,497-bp fragment of the HIV-1 *pol* gene (position 2253-3749). In cases in which viral load was <1,000 copies/mL, viral RNA was extracted from plasma, and a 524-bp *pol* segment (position 2597–3120) was amplified by using primers 3069R (5′-GGA TGG CCC AAA GGT TAA ACA-3′) and 3591F (5′-ATC CTA CAT ACA AAT CAT CCA T-3′) and the QIAamp 1-step reverse transcription–polymerase chain reaction (RT-PCR) method (Qiagen, Mississauga, Ontario, Canada). PCR conditions were 40 cycles consisting of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min, followed by extension at 72°C for 10 min. Amplicons were cloned into pPCR-Script (Stratagene, La Jolla, CA, USA) and sequenced by using dye terminator chemistry (Beckman-Coulter, Palo Alto, CA, USA).

Sequences were aligned with references (2001) representing different HIV-1 subtypes (http://hiv-web.lanl.gov) (8) by using Clustal X version 1.81 (17). Kimura 2-parameter distance matrices were assembled (transition/transversion ratio of 2) (18,19). Phylogenetic reconstructions were built according to the neighbor-joining method, and 1,000 bootstrap resamplings were performed to assess tree topology (MEGA version 2.1) (20). Clade assessment was based on reliable grouping (>80% bootstrap) with reference sequences (8). RIP version 1.9 (www.hiv.lanl.gov/content/hiv-db/RIPPER/rip_test.html) (21) was used to examine potential intersubtype recombinants, with gap stripping on, a window size of 200 characters, and a significance threshold of 90%.

Results

One hundred twenty-seven women 18.3–42.6 years of age (median 30.9, interquartile range [IQR] 7.2) were included in the study: 40 (31.5%) from North and Central America, 35 (27.6%) from the Caribbean, 1 (0.8%) from Asia, and 51 (40.2%) from sub-Saharan Africa. Median HIV-1 viral load at the time of inclusion in the study was 3.24 log RNA copies/mL of plasma (IQR 1.97) and median CD4+ cell count was 403 cells/μL (IQR 248). Of the 127 patients, 66 (52.0%) had not received ARV therapy before study inclusion, 8 (6.3%) had interrupted therapy, and 53 (41.7%) were treated with a regimen consisting of 1 (n = 2), 2 (n = 9), 3 (n = 39) or 4 (n = 3) ARV drugs.

The HIV-1 *pol* gene was successfully amplified and sequenced in 103 (81.1%) of 127 patients, a rate comparable with findings of other studies (22). Seventy-three results were obtained with the Virco procedure, and 30 were obtained with an alternative RT-PCR method. Unsuccessful amplification was associated with low viral load: patients with a viremia level of <500 copies/mL accounted for 23 (95.8%) of 24 in whom gene amplification was unsuccessful, in comparison with 27 (26.2%) of 103 in the rest of the study group ($p<0.0004$, Fisher exact test). This is consistent with the finding that a larger proportion of patients with unsuccessful gene amplification were treated with ARV therapy at the time of inclusion in the study (75.0% versus 34.0%, $p<0.0004$, Fisher exact test). Despite this limitation, sequence information was obtained in more than half of patients with a viremia level of <500 copies/mL (27/50), and in one third of patients with a viremia level of <50 copies/mL (8/24).

Phylogenetic analysis based on a 524-bp *pol* fragment (position 2597–3120) was used to identify the HIV-1 clade. In all cases, grouping based on the 524-bp fragment was consistent with that obtained when all available 1,497-bp sequences were analyzed separately (data not shown). In aggregate analysis, sequences derived from 59 (57.3%) of 103 patients formed a well-defined cluster with clade B reference sequences (Figure, left panel and data not shown). Of these 59 patients, 27 (45.8%) were of Canadian origin, 27 (45.8%) were from Haiti, 2 (3.4%) from Mexico, 1 (1.7%) from Jamaica, 1 (1.7%) from the Dominican Republic, and 1 (1.7%) from the United States. Phylogenetic overlap between these sequences was considerable, and bootstrap support for clustering based on country of origin was <50% (Figure, left panel).

In addition, 44 (42.7%) of 103 patients were infected with nonclade B viruses. Nine (20.5%) of the amplified sequences were similar to reference sequences from clade A, including CRF01-AE. Within this cluster, independent grouping of sequences derived from patients TV641, TV731, and TV783 was only supported by low bootstrap values (Figure, right panel). Sequences from 12 patients (27.3%) clustered alongside clade C references (93% bootstrap), with TV833 the distal taxon. Five (11.4%) grouped with clade D. Two (4.55%) grouped with clades F1 and F2, with TV633 closest to the CRF05-DF reference. One sequence (2.27%) grouped with clade H (99% bootstrap), and 11 (25.0%) with clade G. Among these, 8 sequences formed a well-supported CRF02-AG subcluster (97% bootstrap), while TV909 grouped closest to clade G reference (96% bootstrap). TV737 and TV695 formed a distinct G clade subcluster (100% bootstrap) (Figure, right panel). The 938-nucleotide (nt) fragments of the envelope (*env*) gene V1-V3 region were amplified, sequenced, and analyzed in samples from patients TV737 and TV695. These

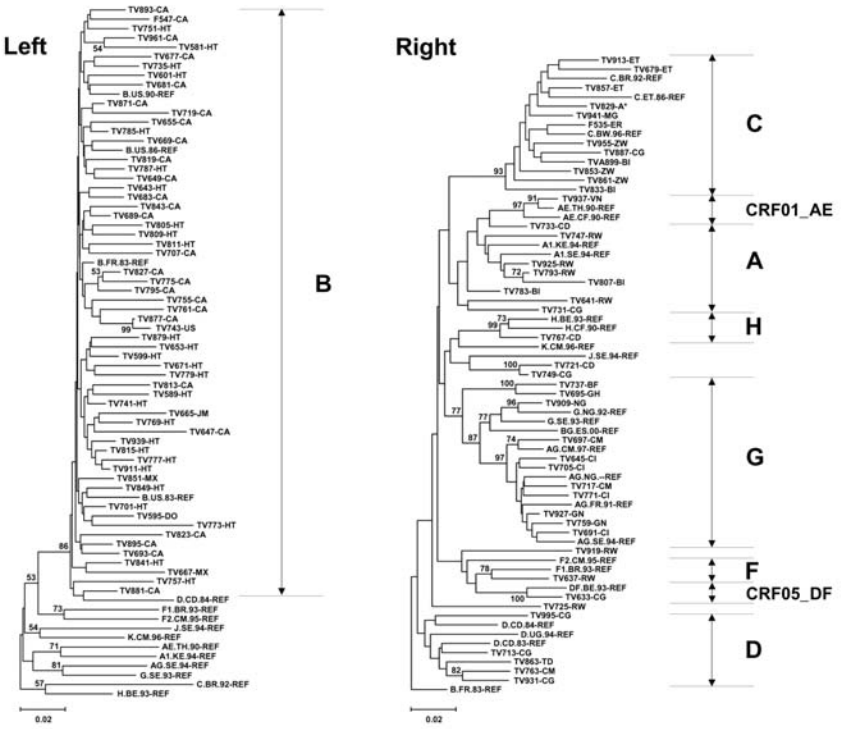

Figure. Phylogenetic analysis of pol sequences derived from pregnant women infected with HIV-1. Trees were constructed by using the neighbor-joining method as described in Patients and Methods. A transition/transversion ratio of 2 was used and 1,000 bootstrap resamplings were performed. Left panel: Subgrouping with clade B HIV-1. Right panel: Grouping with non-B HIV. Reference sequences (REF) were obtained from the Los Alamos National Laboratory database (2001) (8). The scale bar represents 0.02 nucleotide substitutions per site. Letter codes indicate country of origin. All nucleotide sequence information was submitted to GenBank (accession no. DQ059647-DQ059749). CRF, circulating recombinant form.

segments clustered closely with one another (96% bootstrap) but loosely with clade G references (41% bootstrap), which confirmed that these 2 isolates fall outside of the subtype G crown group (data not shown). Finally, TV721 and TV749 clustered loosely with the J reference (61% bootstrap), while TV725 and TV919 grouped outside major clades, although all belonged to the M group (100% bootstrap) as determined by phylogenetic analysis using group N, O, U, and SIVcpz alignments (8) (not shown).

In patients in whom the 1,497-nt sequences were available, the potential intersubtype mosaic nature of viruses with uncertain clade assignment was examined using RIP (21). This analysis indicated that TV731 and TV783 had significant homology with the A1 + A2 consensus, TV833 was homologous to the clade C reference, and TV737 and TV909 closely resembled the clade G consensus (>90% confidence), which confirmed initial assessments. The recombinant nature of TV633 was also supported, with significant homology to clades D and F (putative crossover at position 2795–2796), while TV695 showed highest resemblance to clade G in its 5′-terminal portion and clade C at the 3′ end (>90% confidence), with a potential breakpoint at position 3169–3170. In addition, TV721, TV725, TV749, and TV919 did not show significant homology with any of the sequences in the reference alignment, which prevented assessment of their putative intersubtype nature and their assignment to existing M group clades (Figure, right panel and data not shown). TV721 and TV749 were compared with HIV sequences in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/ BLAST/). The closest homology to TV721 was isolate A2-225.692 from Uganda (23), with 92% identity over a 522-nt segment. The closest homology to TV749 was isolate 97CM.MP806 from Cameroon (24), with 89% identity over an 884-nt segment. When the 938-nt segments of the *env* gene V1-V3 region were amplified and sequenced, TV721 and TV749 clustered closely with one another (100% bootstrap) and with clade G and J references (92% bootstrap) (data not shown). This finding suggests that TV721 and TV749 represent either complex mosaic recombinants or a new subtype of the HIV-1 M group.

In all but 1 patient (43 [97.7%] of 44), those infected with nonclade B viruses were newcomers from Africa, including 34 (77.3%) asylum seekers. Nine patients originated from West Africa: Côte d'Ivoire (n = 4), Burkina Faso (n = 1), Guinea (n = 2), Ghana (n = 1), and Nigeria (n = 1). Twenty-five originated from central Africa: Congo (n = 7), Democratic Republic of Congo (n = 3), Rwanda (n = 7), Burundi (n = 4), Cameroon (n = 3), and Chad (n = 1). Four originated from East Africa: Ethiopia (n = 3) and Eritrea (n = 1). Four originated from southern Africa: Zimbabwe (n = 3) and Madagascar (n = 1). One patient declined to specify her country of origin. Geographic clustering was observed on the cladogram, with West African sequences grouping among clade G, and East and southern African sequences grouping with clade C. The highest HIV-1 genetic diversity was observed in patients from central Africa (Figure, right panel), as previously reported (25).

Median viral load and CD4+ cell count at the time of inclusion in the study were not significantly different in patients infected with clade B virus versus those infected with nonclade B virus, although more patients infected with clade B virus received ARV therapy. In patients not treated, median CD4+ cell count was 91 cells/μL lower in those infected with nonclade B virus, which suggests more advanced disease (Table). Comparison of duration of infection between subgroups was not possible.

Discussion

HIV-1 clade diversity was characterized among a cohort of HIV-infected women receiving prenatal care in a tertiary care hospital serving a cosmopolitan population. Results indicate that 59 (57.3%) of 103 patients in whom genotyping was successful were infected with clade B HIV-1. This finding is compatible with the wide circulation of clade B in North and Central America and the Caribbean, from which 40 (31.5%) and 35 (27.6%), respectively, of the 127 patients in our cohort originated, and the relatively high prevalence of HIV-1 infection among patients from Haiti in the Montreal area (1,26). Additionally, 42.7% of patients in whom genotyping was successful were infected with nonclade B viruses, a proportion much greater than the rate reported in 312 HIV-infected US blood donors (2%) (16) and in a recent Canadian public health surveillance report (8.9%) (27). To our knowledge, this is the highest prevalence of non-B HIV infection reported in any North American study group, including US military personnel (16,28,29). Sequences were identified that belonged to every clade of the HIV-1 M group except J and K. This level of genetic diversity was not previously reported in a North American study group, with the exception of the Centers for Disease Control and Prevention surveillance registry (22), and is as extensive as that observed in Cuba (14). Four of the *pol* segments obtained clustered ambiguously among reference sequences, which suggests that they represent either novel HIV-1 M group clades or complex recombinants. However, additional characterization, including full-genome sequencing, would be required to settle this issue. Based on our results, infection with multiple HIV-1 subtypes cannot be reliably assessed.

A total of 97.7% of non-clade B viruses were found in African women and, in all cases, clade identity was consistent with variants circulating in the patient's area of origin (1). No significant difference was found between the proportions of African women in patients with unsuccessful amplification (8 [33.3%] of 24) versus those in whom amplification was successful (43 [41.7%] of 103, p = 0.496, Fisher exact test), which is indicative of no selection bias. Recent armed conflicts in the African subcontinent have led to an influx into Canada of newcomers from HIV-endemic areas (30,31). Among our study group, dates of arrival into Canada of patients infected with nonclade B HIV-1 correspond with the migration of refugees after the Rwandan genocide and the civil war in the former Republic of Zaire and neighboring Congo (data not shown) (30,31). Nonclade B viruses have spread in Europe and Cuba as a consequence of international travel and immigration from Africa (9–14). Our study demonstrates that multiple HIV-1 clades are being introduced under similar circumstances in a large, North American urban center. From a public health standpoint, antenatal cohorts could represent an important sentinel site to monitor the influx of novel HIV-1 variants in industrialized countries.

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Table. Viral and immune parameters in study participants*

	Overall					Treatment naive	
	Median (IQR) viral load (log copies/mL)†	% viral load <2.7 (n)‡	% viral load <1.7 (n)‡	Median (IQR) CD4+ count (cells/μL)†	% receiving ARV therapy (n)‡	Median (IQR) viral load (log copies/mL)†	Median (IQR) CD4+ cell count in treatment-naive patients (cells/μL)†
HIV-1 B clade	3.61 (1.71)	28.8 (17)	11.9 (7)	360 (285)	44.1 (26)	3.95 (1.38)	418 (278)
HIV-1 non-B clade	3.52 (1.39)	22.7 (10)	2.27 (1)	351 (220)	20.5 (9)	3.53 (0.94)	327 (208)
p value	0.927	0.508	0.316	0.476	0.0102§	0.143	0.107

*HIV-1 viral load and CD4+ cell counts were measured as described in Patients and Methods. Significance of differences between groups was tested by Kruskal-Wallis test or Fisher exact test. Analysis was carried out on the whole study group (N = 103) or restricted to those who did not receive treatment (n = 61). IQR, interquartile range; ARV, antiretroviral.
†Kruskal-Wallis test.
‡Fisher exact test.
§Statistically significant ($p<0.05$) by directional test.

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Multidrug-resistant *Salmonella* Typhimurium in Four Animal Facilities

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In 1999 and 2000, 3 state health departments reported 4 outbreaks of gastrointestinal illness due to *Salmonella enterica* serotype Typhimurium in employees, clients, and client animals from 3 companion animal veterinary clinics and 1 animal shelter. More than 45 persons and companion animals became ill. Four independent investigations resulted in the testing of 19 human samples and >200 animal samples; 18 persons and 36 animals were culture-positive for *S.* Typhimurium. One outbreak was due to multidrug-resistant *S.* Typhimurium R-type ACKSSuT, while the other 3 were due to multidrug-resistant *S.* Typhimurium R-type ACSSuT DT104. This report documents nosocomial transmission of *S.* Typhimurium and demonstrates that companion animal facilities may serve as foci of transmission for salmonellae between animals and humans if adequate precautions are not followed.

Salmonella spp. infect an estimated 1.4 million persons annually in the United States. Although most infections are self-limiting with diarrhea, vomiting, abdominal cramps, and fever, severe infections are not uncommon. Estimates suggest that ≈15,000 people are hospitalized and >500 deaths occur annually due to *Salmonella* infections (1). Food animals are the primary reservoir for human nontyphoidal *Salmonella* infections; person-to-person transmission of nontyphoidal salmonellae is uncommon in the United States. Transmission of salmonellae to humans typically occurs by ingesting meat, dairy products, and other foods contaminated by animal feces or by cross-contamination from foods contaminated with salmonellae. Zoonotic transmission of *Salmonella* spp. can also occur through direct exposure to the feces of reptiles, farm animals, pets, pet treats, and other animals (2–10).

Antimicrobial agents such as fluoroquinolones and third-generation cephalosporins (e.g., ceftriaxone) are commonly used to treat severe human *Salmonella* infections. Resistance to these and other antimicrobial drugs, as well as multidrug resistance, has increased over the last several decades (11), partly as a consequence of antimicrobial drug use in food animals (2). The use of antimicrobial agents in companion animals (e.g., dogs and cats) may also contribute to the development of antimicrobial resistance in salmonellae, but the impact of this contribution is unknown. Antimicrobial drug use in companion animals, therefore, could increase the likelihood of zoonotic transmission of multidrug-resistant salmonellae by generating drug-resistant strains as well as by making animals more susceptible to resistant infections (12).

Salmonella outbreaks with illness in animals and humans have been reported in both equine and companion animal veterinary facilities (13–19). These reports

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commonly describe poor hand washing by employees, eating in work areas, and prior antimicrobial drug therapy in humans or animals. Case reports of companion animals infected with salmonellae have included multidrug-resistant isolates (15,18–22).

In late 1999 and early 2000, 3 state health departments reported to the Centers for Disease Control and Prevention (CDC) outbreaks of gastrointestinal illness in employees and clients of 3 companion animal veterinary clinics and 1 companion animal shelter. We review these outbreaks, which demonstrate that companion animal veterinary facilities can serve as foci of transmission of salmonellae to animals and humans. Recommendations to prevent further outbreaks of salmonellosis associated with companion animal veterinary facilities were reviewed.

Veterinary Clinic, Idaho

In September 1999, several kittens being treated for diarrhea at a veterinary clinic (clinic A) died; stool specimens were not collected. Within 2 days of the kittens' deaths, an employee who had cared for the kittens became ill with diarrhea. Days later a second employee who cared for the kittens became ill, as did other employees who had no direct contact with the kittens. Within 2 weeks, 10 (50%) of 20 employees of clinic A had experienced diarrhea and abdominal cramps. The median age of the ill persons was 31 years (range 19–44 years). The median duration of illness was 7 days (range 4–12 days). Four persons sought medical care. No one was hospitalized.

Stool specimens from 5 ill employees yielded salmonellae, which was serotyped at the Idaho Department of Health and Welfare (IDHW) as *Salmonella enterica* serotype Typhimurium. The IDHW tested all *S.* Typhimurium isolates received during the outbreak period by pulsed-field gel electrophoresis (PFGE) and for chloramphenicol resistance by using disk diffusion. Isolates were tested at CDC for resistance to 17 antimicrobial agents by using broth microdilution (Sensititre, Trek Diagnostic Systems, Cleveland, OH, USA) with Clinical and Laboratory Standards Institute (formerly NCCLS) interpretive criteria used to determine antimicrobial susceptibility (23). The 5 isolates were resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, sulfamethoxazole, and tetracycline (R-type ACKSSuT); 2 isolates were additionally resistant to gentamicin, clavulanic acid, cephalothin, and ceftriaxone. Isolates were indistinguishable by PFGE. Phage testing was performed at CDC according to the methods of the Laboratory of Enteric Pathogens, Central Public Health Laboratory, Health Protection Agency, UK, and all 5 isolates reacted but did not conform (RDNC).

All ill employees had eaten meals in the clinic in the days before illness onset. A breakroom was provided for employees, yet all reported eating on work surfaces instead. The ill employees had no known common exposures outside clinic A. No additional *S.* Typhimurium isolates from Idaho were found with chloramphenicol resistance or with a related PFGE pattern during the outbreak.

Animal Shelter, Minnesota

In Minnesota, *S.* Typhimurium isolates are routinely subtyped at the Minnesota Department of Health (MDH) Public Health Laboratory by PFGE and tested for antimicrobial susceptibility. As part of integrated human-animal surveillance, *S.* Typhimurium isolates from the Minnesota Veterinary Diagnostic Laboratory (MVDL) are routinely forwarded to MDH for PFGE subtyping and antimicrobial susceptibility testing.

On December 2, 1999, five *S.* Typhimurium isolates from cats originating from the same county, submitted by a regional animal shelter (shelter A), were subtyped at MDH; all had the same PFGE pattern. The following day, a human *S.* Typhimurium isolate routinely submitted to MDH was determined to have the same PFGE pattern as the cat isolates. Three additional human cases were identified retrospectively with dates of illness onset from August to November; 2 reported recently adopting a kitten from shelter A. A review of recently diagnosed feline salmonellosis cases from shelter A identified 9 cases; all 9 cases were fatal and originated from multiple sources, but all ill cats were housed at shelter A. Dates of death for the kittens ranged from September through late October (Figure 1). Six of the 9 kittens had been adopted; 5 of the 6 were returned to shelter A because of illness.

From August 1999 to March 2000, a sample of 7 human isolates and 9 feline *S.* Typhimurium isolates were identified as indistinguishable by PFGE. Figure 2 displays PFGE patterns for 4 human and 6 feline isolates. All 16 isolates were resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (R-type ACSSuT). Three cat and 2 human isolates were phage typed at CDC; all were definitive type 104 (DT104). Six of the 7 human case-patients with the PFGE outbreak subtype of *S.* Typhimurium had a discernable connection to shelter A; 4 had adopted kittens from shelter A during August–October 1999. Two additional patients were children who attended the same daycare center as a child who became ill 77 days after adopting 2 kittens from shelter A. This child had been treated with multiple antimicrobial drugs in the month before the onset of her salmonella gastroenteritis, including cephalexin, amoxicillin, trimethoprim-sulfamethoxazole, and ciprofloxacin. Neither of her 2 kittens developed diarrhea, but the outbreak strain of *S.* Typhimurium R-type ACSSuT DT104 was recovered from the stool of one of these cats 115 days after adoption.

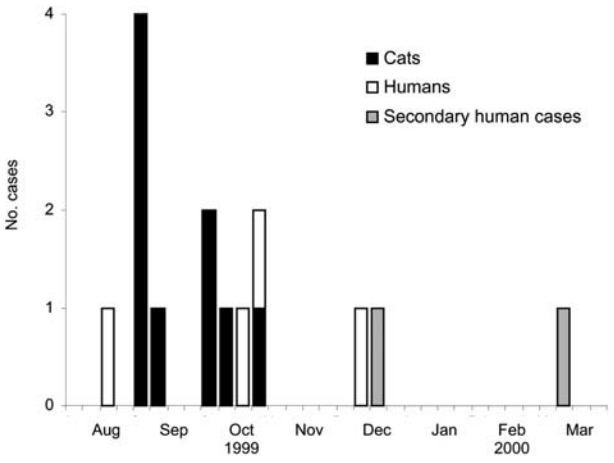

Figure 1. Date of death among cats and week of illness onset among human case-patients, Minnesota, 1999.

The median age of the ill persons was 6 years (range 11 months–23 years). The median duration of illness was 8 days (range 5–11 days). All 7 persons sought medical care; 1 child was hospitalized for 4 nights. One of the ill persons was treated with ciprofloxacin but continued to shed *Salmonella* spp. in stool for at least 214 days after illness onset.

After the institution of enhanced infection control procedures by the shelter staff in October, no further cases of salmonellosis occurred at the facility. In December 1999, environmental samples from drains, kennels, and additional cats housed at the shelter were all negative for *Salmonella* spp.

Clinic B, Washington

In late 1999, twelve cats were brought to a companion animal veterinary clinic (clinic B) with diarrhea, vomiting, anorexia, and lethargy. All 12 cats lived in different homes and all roamed outdoors. Stool specimens collected from 6 of the 12 yielded *S. enterica*; all isolates were serotyped at the National Veterinary Services Laboratory (NVSL) as Typhimurium. Two of 3 persons who became ill had handled ill cats. The third person had brought his cat to clinic B during the outbreak for treatment of an unrelated illness. Stool specimens from the 3 ill persons and 6 cats yielded *S.* Typhimurium, R-type ACSSuT DT 104. Isolates were indistinguishable from each other by PFGE (Figure 3).

The Field Disease Investigation Unit (FDIU) from the Washington State University College of Veterinary Medicine performed a comprehensive investigation. Medical chart reviews demonstrated that 7 (58%) of the 12 ill cats had been seen at clinic B for an unrelated reason 3–37 days (median 5 days) before gastrointestinal illness onset; 4 (57%) of these 7 cats had been treated for their original symptoms with an antimicrobial agent to which the outbreak strain of *S.* Typhimurium was later found to be resistant.

As part of this investigation, 2 controls were selected from clinic B records for each ill cat. For each case, the next cat owner listed alphabetically in clinic B's files, as well as the next cat seen at the clinic after the ill cat, was selected as a control. Distance was measured from owner's homes to the nearest creek. Affected cats owners' homes, regardless of whether the cat's infection was potentially nosocomial, were significantly closer to 1 of 2 creeks traversing the community than were control client's homes (rank sum test, $p<0.01$). Water samples collected from the creeks 2 months after the outbreak were negative for *S. enterica*.

Stool specimens for bacterial culture were collected from additional animals treated at clinic B, including 43 hospitalized cats, 37 outpatient cats, and 23 cats from households with proven infected cats. Salmonellae were isolated from 5 (12%) of 43 hospitalized cats, including the asymptomatic clinic blood donor cat and from 3 (8%) of 37 cats residing in households with infected cats; no isolates were obtained from sampled outpatient cats. The 6 original culture-positive cats were periodically sampled and shed salmonellae 3–60 days before becoming culture-negative. As part of the investigation, specimens from the remaining 6 original cats (from which no previous specimens had been cultured) were collected; these specimens were cultured and confirmed to shed the outbreak strain of *S.* Typhimurium. Fecal cultures of 26 animals from a local animal shelter were negative for *Salmonella.*

Seventy-two environmental samples were collected from clinic B, from homes of clinic B clients and employees, and from a local animal shelter. The outbreak strain of

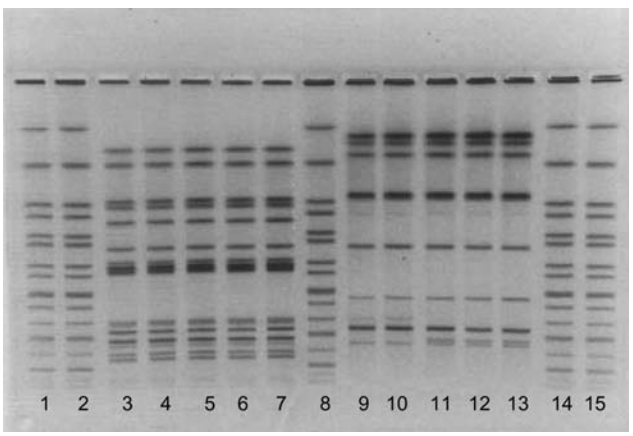

Figure 2. Pulsed-field gel electrophoresis patterns of human and feline isolates from Minnesota outbreak,1999. Lanes 1, 2, 8, 14, and 15 contain *Xbal*-digested DNA from the standard strain H9812; lanes 3, 4, 9, and 10 contain human isolates; lanes 5-7, and 11-13 contain feline isolates. Lanes 3-7 were digested with *Xbal* and lanes 9-13 with *Blnl*.

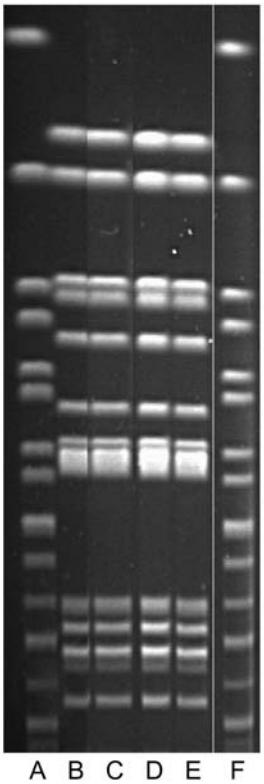

Figure 3. Pulsed-field gel electrophoresis patterns associated with the Washington state outbreaks, 1999 and 2000. Lanes A and F are standards; lane B is cat, clinic B; lane C is cat, clinic C; lanes D and E are human isolates. (For confidentiality reasons, Washington Department of Health did not identify which human isolates were from which outbreak.) Human and cat isolates are indistinguishable.

S. Typhimurium was isolated from the floor of the boarding area and from the floor and door handles in the clinic B isolation ward. The outbreak strain was additionally isolated from an environmental sample (a vacuum cleaner bag) from the house where 2 of the infected cats lived (24). *Salmonella* spp. were not isolated from the homes of clinic B employees, nor from the local animal shelter.

The additional *Salmonella* isolates from animals and the environmental samples were serotyped at NVSL as *S.* Typhimurium and phage typed as DT104. Agar disk-diffusion testing determined the isolates to be R-type ACSSuT. The original 6 animal isolates, the animal isolates collected during the investigation, and the human isolates were indistinguishable by PFGE.

Ten of the ill cats received empiric antimicrobial therapy for their gastrointestinal illness. When antimicrobial susceptibility results became available, the antimicrobial agent of the 7 cats still undergoing treatment was changed to enrofloxacin. Although clinical signs of the affected cats resolved promptly after initiation of 10-day courses of enrofloxacin therapy, stool cultures of specimens from 3 cats taken 14–24 days after completion of the course of enrofloxacin yielded the outbreak strain of *S.* Typhimurium.

Clinic C, Washington

In early 2000, several cats were brought to a companion animal veterinary clinic (clinic C) with diarrhea; no stool specimens were collected. Three days after their cat was treated for vomiting and diarrhea, 2 children became ill with diarrhea. Six days after treating an ill cat, a clinic C employee also became ill with diarrhea. The duration of illness for the 3 ill persons was 5–7 days, and all 3 persons sought medical care. One child was hospitalized for 2 nights, but salmonellae were never cultured from this child's stool. Stool specimens obtained from the ill clinic employee, an asymptomatic clinic employee, and the other child yielded salmonellae, which were identified as *S.* Typhimurium at the Washington State Department of Public Health Laboratory. Clinic C was the only known association between the 3 ill persons.

During this period, stool specimens submitted from 1 dog and 3 cats with diarrhea yielded salmonellae; isolates were serotyped as *S.* Typhimurium by NVSL. Two days before diarrhea onset, the 4 animals had been treated as outpatients at clinic C for unrelated reasons; 3 of the 4 received amoxicillin before diarrhea onset.

The FDIU performed an investigation in clinic C, including bacterial cultures of stool specimens from additional animals (n = 96) and environmental samples from clinic C and employee and client homes (n = 66). *S.* Typhimurium was isolated from 1 cat and 2 dogs (1 asymptomatic). The cat exhibited anorexia and dehydration; a stool specimen was collected. This cat had not been seen at clinic C during the outbreak, but several of its housemates had been seen at clinic C for conditions other than diarrhea during the outbreak. One of the 2 dogs boarded at the clinic during the outbreak and had a single episode of bloody diarrhea; its asymptomatic housemate had not been seen by clinic C.

None of the environmental samples collected from clinic C yielded salmonellae. The outbreak strain of *S.* Typhimurium was identified in the contents of the home vacuum cleaner bags from 2 clinic C employees and the asymptomatic owner of a culture-confirmed cat (24). The *S.* Typhimurium isolates from the 2 ill persons, 7 animals, and the 3 vacuum bags were all demonstrated at CDC to be *S.* Typhimurium R-type ACSSuT DT104. These isolates were additionally indistinguishable by PFGE at the FDIU Laboratory, Pullman, Washington (Figure 3) by using standard protocols (25) and were indistinguishable from those of the clinic B outbreak.

Discussion

Four outbreaks of multidrug-resistant *S.* Typhimurium associated with companion animal veterinary clinics or shelter facilities occurred in the United States in late 1999 and early 2000. In each facility, employees, clients, or both, became infected after animal illness. An outbreak in an Idaho clinic was caused by multidrug-resistant *S.* Typhimurium R-type ACKSSuT with 2 isolates demonstrating additional resistance to ceftriaxone, an antimicrobial agent commonly used to treat children with severe *Salmonella* infections. Outbreaks in 2 Washington clinics and a Minnesota animal shelter were caused by multidrug-resistant *S.* Typhimurium R-type ACSSuT DT104.

In 1999, *S.* Typhimurium R-types ACSSuT, AKSSuT, and ACKSSuT were the most prevalent multidrug-resistant phenotypes among *Salmonella* isolates in the United States. Twenty-one percent of all *Salmonella* isolates of human origin tested at the National Antimicrobial Resistance Monitoring System (NARMS) in 1999 were multidrug-resistant; 6% were *S.* Typhimurium R-type ACSSuT, 2% were R-type AKSSuT, and 1% were R-type ACKSSuT (11).

In each outbreak discussed, the veterinary facility or animal shelter was the only common exposure for infected persons, which demonstrated that infected animals brought to companion animal veterinary clinics and animal shelters can be foci for nosocomial transmission to other animals and for zoonotic transmission to humans. These outbreaks illustrate 1) the hazards of occupational zoonotic transmission of *Salmonella* spp. from ill animals to clinic employees, 2) the hazards of zoonotic transmission of *Salmonella* spp. to clients/pet owners, 3) the risk for nosocomial transmission of *Salmonella* spp. between animals within veterinary facilities and animal shelters, and 4) the potential for environmental contamination to serve as an ongoing source of infection.

The use of antimicrobial agents prescribed by veterinarians may contribute to increased transmission of multidrug-resistant *Salmonella* spp. between animals by lowering the infectious dose required for infection to occur or by increasing the duration of illness when an infected animal is treated with an ineffective drug (12,26,27). The risk for *Salmonella* transmission between animals in veterinary facilities is likely increased by the presence of animals with increased susceptibility to multidrug-resistant *Salmonella* infection due to treatment with antimicrobial agents for other conditions. Fluoroquinolone antimicrobial therapy did not eliminate fecal shedding of susceptible strains of salmonellae in 3 cats and 1 person from whom follow-up cultures were obtained. This finding is similar to other results in humans, which demonstrate that fluoroquinolone treatment is associated with longer duration of carriage (28).

Although person-to-person transmission of nontyphoidal *Salmonella* spp. is rare in the United States, this mode of transmission appears likely in the Minnesota outbreak in which an ill child apparently infected classmates in a daycare center. Several instances of probable secondary transmission to animals within client households after apparent primary nosocomial infection were demonstrated during these outbreaks. The isolation of *Salmonella* spp. from client-owned vacuum cleaner bags illustrated the potential for such secondary transmission. Additional isolation of the outbreak strain from environmental surfaces in the Washington clinic B investigation reinforces the findings of previous studies, which demonstrated the potential to transmit salmonellae through environmental contact (29,30).

Veterinarians should expect, at least occasionally, to evaluate animals infected with *Salmonella* spp. Following these outbreaks, recommendations for infection prevention and control were formulated to help prevent future outbreaks of salmonellosis in association with companion animal facilities (31). Recommendations include wearing gloves while cleaning cages and treating animals, then immediately removing the gloves and washing hands when the task is completed. No eating or drinking should be allowed in animal treatment and holding areas, and feces-contaminated areas should be immediately cleaned and disinfected. Clear warnings of the risk for transmission of *Salmonella* spp. should be given when pets with probable salmonellosis are encountered. Veterinarians should consider culturing the stools of animals with diarrhea and should be aware of the increased risk for infection with multidrug-resistant salmonellae in animals who are given antimicrobial drugs for other conditions. Because use of antimicrobial agents contributes to increasing resistance and facilitates transmission of multidrug-resistant salmonellae, promoting guidelines aimed at improving appropriate use of antimicrobial agents may help prevent transmission of multidrug-resistant *Salmonella* infections in veterinary facilities (31).

Although recommendations have been formulated and disseminated (31), outbreaks of multidrug-resistant *Salmonella* spp. occurred in an animal shelter in Idaho in 2003–2004 (18) and in New York in 2003 (19). Continued outbreaks suggest that more outreach and education regarding the potential for nosocomial and zoonotic outbreaks such as these should be directed to the veterinary community.

Additionally, states should consider integrating human and veterinary surveillance systems and educating the veterinary community on their public health role. In our report, the outbreak in 1 state may have been undetected without the routine comparison of human and veterinary laboratory data. Considering recent outbreaks of zoonotic

diseases such as West Nile virus and monkeypox infections, the continued threat of avian influenza, and the number of agents of bioterrorism that are zoonotic in nature, the integration of human and veterinary surveillance systems is of utmost importance in our public health infrastructure.

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Spoligotyping and *Mycobacterium tuberculosis*

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We evaluated the clinical usefulness of spoligotyping, a polymerase chain reaction–based method for simultaneous detection and typing of *Mycobacterium tuberculosis* strains, with acid-fast bacilli–positive slides from clinical specimens or mycobacterial cultures. Overall sensitivity and specificity were 97% and 95% for the detection of *M. tuberculosis* and 98% and 96% when used with clinical specimens. Laboratory turnaround time of spoligotyping was less than that for culture identification by a median of 20 days. In comparison with IS*6110*-based restriction fragment length polymorphism typing, spoligotyping overestimated the number of isolates with identical DNA fingerprints by ≈50%, but showed a 100% negative predictive value. Spoligotyping resulted in the modification of ongoing antimycobacterial treatment in 40 cases and appropriate therapy in the absence of cultures in 11 cases. The rapidity of this method in detection and typing could make it useful in the management of tuberculosis in a clinical setting.

The last decade has seen a dramatic resurgence in the incidence of tuberculosis throughout the world and an increased need for more rapid methods to diagnose and prevent dissemination of this disease (1). Well-equipped clinical laboratories can detect tuberculosis cases within 14 to 21 days by using liquid culturing systems such as BACTEC (Becton Dickinson, Sparks, MD, USA). Moreover, several studies have verified the usefulness of nucleic acid amplification–based methods for diagnosis of *Mycobacterium tuberculosis* infections in <24 hours (2–4). Concomitantly, recently characterized molecular markers for typing mycobacterial strains have greatly facilitated and improved the study of tuberculosis epidemiology (5–8).

Restriction fragment length polymorphism (RFLP) typing with insertion element IS*6110* as a probe has become the most widely used method for differentiating strains of *M. tuberculosis* isolates (7,8). However, because the application of RFLP typing is restricted to mycobacterial cultures, 20–40 days are required before sufficient mycobacteria are available to obtain sufficient DNA needed for this method. This time restriction limits the usefulness of RFLP typing, especially in studying possible nosocomial transmission of tuberculosis in a clinical setting.

Spoligotyping, a new method for simultaneous detection and typing *of M. tuberculosis* complex bacteria, has been recently developed (9–11). This method is based on polymerase chain reaction (PCR) amplification of a highly polymorphic direct repeat locus in the *M. tuberculosis* genome. Results can be obtained from a *M. tuberculosis* culture within 1 day. Thus, the clinical usefulness of spoligotyping is determined by its rapidity, both in detecting causative bacteria and in providing epidemiologic information on strain identities. Implementing such a method in clinic settings would be useful in surveillance of tuberculosis transmission and in interventions to prevent further spread of this disease.

The aims of this study were to evaluate 1) the reliability of spoligotyping when used with clinical specimens, 2) the potential usefulness of the method in distinguishing *M. tuberculosis* from other nontuberculous mycobacteria (primarily *M. avium*), and 3) the feasibility and impact of spoligotyping in managing tuberculosis in clinical settings.

Patients and Methods

Specimen Collection

We conducted a 2-year survey of suspected cases of tuberculosis with spoligotyping of acid-fast bacilli (AFB)–positive specimens collected consecutively from January 2000 to December 2001 in the Microbiology Laboratory at L. Sacco Hospital in Milan. Three types of specimens were included. The first was material scraped

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from slides prepared from all Ziehl-Neelsen–positive clinical specimens, which were obtained from patients admitted to the hospital. These clinical specimens included sputum, bronchoalveolar lavage, bone marrow aspirate, feces, cerebrospinal fluid, and urine. The second was samples obtained from liquid culture medium containing growing mycobacteria (BACTEC, Becton Dickinson). The third was a mycobacterial colony grown on solid medium (Lowenstein-Jensen). The hospital microbiology laboratory conducted isolation (both in solid and liquid media), identification, and antimicrobial susceptibility testing by using standard methods (12) on all specimens. The results of spoligotyping were immediately provided to the physicians treating the patients.

Clinical Characteristics

Demographic and epidemiologic data were obtained from the medical records of all patients with AFB-positive specimens, including medical history of mycobacteriosis, HIV status, dates and results of mycobacterial smears, signs, symptoms, radiographs of patients with tuberculosis, and CD4+ cell counts (for HIV-infected patients). Data regarding the response time of the method used, possible variation in treatments following spoligotyping results, and patient clinical responses were also obtained.

Isolation of DNA

DNA was isolated from AFB-positive slides as previously described (13). Briefly, stained microscopic preparations were washed in xylol and absolute ethanol, scraped with a sterile blade, and collected in a microcentrifuge tube in 1 × phosphate buffer. The samples were centrifuged for 10 min at 13,000 rpm. The pellets were resuspended in 100 μL lysis buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.45% Tween 20, 0.45% Nonidet P40, and 10 mg/mL proteinase K) and incubated for 3 h at 56°C or overnight at 37°C. The samples were then incubated for 15 min at 95°C and centrifuged for 15 min at 13,000 rpm, and the supernatants were transferred to a new microcentrifuge tube and used in PCR. Mycobacteria were grown in culture, and their DNA was isolated as previously described (6).

RFLP Fingerprinting Analysis and Spoligotyping

Isolates of *M. tuberculosis* were genotyped by RFLP using the IS*6110* probe as a genetic marker, as previously described by van Embden et al. (6). Spoligotyping was performed on genomic DNA by using the standard method described by Kamerbeek et al. (9). Control samples were used in these procedures as previously described (14).

Computer-assisted Analysis of Typing Patterns

Gel Compar software version 4.1 (Applied Maths, Kortrijk, Belgium) was used to compare the hybridization patterns obtained by spoligotyping and RFLP fingerprinting. The software clustered strains with the same genotypic pattern and defined similarity dendrograms joining the obtained clusters. The results of the analysis were compared with our database containing all DNA patterns derived from tuberculosis cases analyzed (the database contains RFLP data from >4,500 different isolates in our region).

Statistical Analysis

The sensitivity and specificity of spoligotyping in distinguishing *M. tuberculosis* from nontuberculous mycobacteria were calculated in comparison with culture results that excluded analysis of patients without culture confirmation. The sensitivity and specificity of spoligotyping in typing *M. tuberculosis* isolates were calculated in comparison with IS*6110* clustering.

Results

Patient Characteristics

Three hundred fifty AFB-positive slides from 164 episodes of suspected mycobacteriosis in 148 patients were analyzed. One hundred seven slides were obtained from fresh material: sputum (n = 65), stool (n = 19), lymph node aspirate (n = 12), bronchoaspirate (n = 4), urine (n = 3), skin biopsy (n = 2), biliar liquid (n =1), and pericardial fluid (n = 1). One hundred five samples were obtained after growth of mycobacteria from liquid medium, and 138 samples derived from culture of different materials (mainly blood, but also sputum, bone marrow aspirate, cerebral spinal fluid, and others) were obtained after growth on solid medium. The characteristics of the 148 patients are shown in Table 1.

Sensitivity and Specificity of Spoligotyping versus Culture

Culture confirmation was obtained in 317 (90.6%) of 350 AFB-positive slides from 138 of 164 episodes of suspected mycobacteriosis (Table 2). *M. tuberculosis* was isolated from 188 specimens from 77 patient episodes. Among these, isoniazid resistance was detected in 12 patients (15.6%), rifampin resistance in 11 patients (14.3%), streptomycin resistance in 4 patients (5.2%), and ethambutol resistance in 2 patients (2.6%). We also observed 6 patients with multidrug-resistant tuberculosis (resistance to at least isoniazid and rifampin). Fifty-six patients (72.7%) were infected with a strain susceptible to all 4 drugs.

One hundred ninety-eight of 350 AFB slides showed positive results by spoligotyping. Culture results confirmed the diagnosis of tuberculosis according to spoligotyping positivity in 182 (96.8%) of these 198 specimens.

Table 1. Characteristics of 148 patients with acid-fast bacilli in biologic specimens*

Age, y	
Median (range)	34 (3–88)
Mean	39.3
Sex, no. (%)	
Male	105 (70.9)
Female	43 (29.1)
Type of patient, no. (%)	
Infectious diseases	124 (83.8)
Pneumology	10 (6.8)
Internal medicine	10 (6.8)
Other	4 (2.7)
HIV status, no. (%)	
Negative	52 (35.1)
Positive	96 (64.9)
CD4+ cell count/μL†	
Median	47.5
Mean (range)	105 (1–589)
Previous tuberculosis, no. (%)	20 (13.5)
Previous MAC infection, no. (%)	10 (6.8)
No tuberculosis or MAC infection, no. (%)	118 (79.7)

*MAC, *Mycobacterium avium* complex.
†Data available for 90 of 96 HIV-infected patients.

We did not observe definitive growth of mycobacteria in cultures from 10 patients. The PCR products obtained from 6 specimens of nontuberculous mycobacteria (2 *M. xenopi*, 2 *M. fortuitum*, and 2 *M. avium*) hybridized with *M. tuberculosis*–specific oligonucleotides, which indicated that that false-positive spoligotyping results were possible. However, mixed infections with 2 different mycobacteria cannot be ruled out.

The spoligotyping response was negative in specimens from 152 patients: 23 specimens showed no growth in culture, 123 were nontuberculous mycobacteria, and 6 showed growth characteristic of *M. tuberculosis* (Table 2). Three of these 6 false-negative spoligotyping results were from slides with very high concentrations of AFB, and a positive result was obtained when we repeated the test at a higher dilution (1:10).

In comparison with culture results, the sensitivity of spoligotyping was 98% for clinical specimens, 91% for slides obtained from liquid medium, and 100% for slides obtained directly from a mycobacterial colony on Lowenstein-Jensen solid medium. The corresponding specificities were 96% (clinical specimens), 98% (liquid medium), and 94% (solid medium), respectively (Table 2).

Clinical Application of Spoligotyping

Under optimal conditions, spoligotyping requires <24 hours for results. However, in the present study, response time was evaluated, taking into consideration routine processing time in the laboratory. The time from receipt of clinical specimens to obtaining spoligotyping results was 1–26 days (median 6 days). However, spoligotyping results from clinical specimens were obtained a median of 20 days (mean ± SD, 22.9 ± 18.6) sooner than those obtained by culture confirmation of tuberculosis and a median of 29 days (35.0 ± 25.2) sooner than those obtained by susceptibility testing. In contrast, RFLP typing results were obtained after a median of 75 days (range 24–160) (Table 3).

The use spoligotyping in determining treatment for 164 episodes of suspected mycobacteriosis was evaluated (Figure 1). In 25 episodes, patients did not begin antimycobacterial treatment because clinicians judged the AFB results to be not suggestive of true mycobacteriosis; none of these patients had clinical and radiologic features of tuberculosis (virtually all of these were infections with *M. gordonae* and *M. xenopi* isolates). Four patients died with-

Table 2. Comparison between spoligotyping and culture results in 350 acid-fast bacilli–positive samples

Mycobacteria grown in culture	No. episodes	Results of spoligotyping from clinical samples		Results of spoligotyping from liquid medium		Results of spoligotyping from solid medium		Total	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
M. tuberculosis	77	54	1	53	5	75	0	182	6
M. avium	28	0	15	0	20	2	31	2	66
M. gordonae	15	0	0	0	12	0	14	0	26
M. xenopi	8	0	2	1	5	1	6	2	13
M. kansasii	2	0	1	0	0	0	2	0	3
M. chelonae	2*	0	2	0	2	0	2	0	6
Other	6†	1	1	0	4	1	4	2	9
No growth	26	9	21	1	2	0	0	10	23
Subtotal		64	43	55	50	79	59	198	152
Total	164‡	107		105		138		350	
Sensitivity, %§		98.2 (71.1–98.4)		91.4 (75.7–90)		100		96.8 (86.2–97)	
Specificity, %§		95.5 (67.7–97.7)		97.7 (95.6–97.8)		93.6		95.3 (88.4–96.1)	

*Automated DNA sequencing rather than culture growth of *M. chelonae* confirmed one of these cases.
†Other, 1 *M. fortuitum*, 1 *M. asiaticum*, and 4 nontyped mycobacteria.
‡16 patients had ≥2 episodes of mycobacterial infections.
§Sensitivity and specificity were calculated without including the culture-negative specimens. Values in parentheses are ranges that include specimens with no growth.

Table 3. Time required for obtaining results with clinical specimens by spoligotyping compared with 3 other methods*

Procedure	Days required, median (range)
Spoligotyping	6 (1–26)
Culture confirmation	28 (6–64)
Susceptibility testing	37 (23–81)
RFLP typing	75 (24–160)

*RFLP, restriction fragment length polymorphism.

in a few days after admission without receiving any antimycobacterial drug.

Clinicians waited until spoligotyping results were obtained before choosing the appropriate therapy for 31 other patients. Subsequently, 20 patients began standard antituberculosis regimens (isoniazid, rifampin, pyrazinamide, and/or ethambutol), whereas the 11 other patients began treatment for infection with *M. avium* (clarithromycin, ethambutol, rifabutin, and/or ciprofloxacin). In all cases, the choice of the treatment based on spoligotyping was not changed after culture and susceptibility test results were obtained.

Empiric antimycobacterial therapy was given to the remaining 104 patients before spoligotyping results were obtained. Seventy-six of these patients received antituberculosis treatment against *M. tuberculosis* infections, and 28 received therapy for infection with *M. avium* based on clinical presentation. Therapy was subsequently modified as a result of the spoligotyping results in 14 of these 104 patients. In 5 patients in whom spoligotyping results were negative and subsequent cultures showed nontuberculous mycobacteria, antituberculosis treatment was replaced with treatment for infection with *M. avium*. In contrast, in 8 patients in whom spoligotyping results were positive and subsequent cultures were positive for *M. tuberculosis*, treatment for infection with *M. avium* was replaced by antituberculosis treatment. In 1 patient, the homology of the spoligotyping pattern with patterns of other patients included in our data bank demonstrated that the isolate originated from an outbreak caused by multidrug-resistant strains. Consequently, therapy was modified to include second-line antituberculosis drugs, which previous in vitro susceptibility data had shown were active against the specific strains. Subsequent in vitro susceptibility data confirmed the spoligotyping results. False-positive spoligotyping results in 9 patients did not result in errors in prescribing treatment. These patients continued ongoing treatment, primarily on the basis of previous spoligotyping results or because of a positive response to treatment.

Analysis of Different Isolates from the Same Patient

We analyzed 34 spoligotyping-positive specimens from 6 patients with successive episodes of culture-confirmed tuberculosis (≥3 months apart). In these patients, spoligotyping was modified to distinguish relapses from new infections. After the samples were decoded, all episodes were classified as true relapses, and no new infections were detected. The banding patterns of the successive specimens matched those of their corresponding initial isolates. The subsequent RFLP results confirmed those obtained with spoligotyping.

Genotyping

The reproducibility of spoligotyping was demonstrated by the identity of results obtained with clinical samples and corresponding cultures from different anatomic sites in the same episode (18 patients), and from episodes of recurrent tuberculosis in the same person (5 patients). Thirty-nine distinct spoligotyping patterns were observed; 55% of the specimens were grouped into 10 clusters, and the others had unique spoligotypes. Sixty-one different RFLP patterns were seen in 64 isolates. Of these 61 patterns, 3 were shared by 2 isolates, while the remaining 58 patterns (95%) were observed in only 1 isolate.

One of the 3 clusters identified by RFLP was a false cluster because it showed a 1-band pattern that correctly matched 2 different spoligotypes. In the remaining 2 RFLP clusters, complete concordance with spoligotyping was observed. The remaining 8 clusters detected by spoligotyping were not confirmed by RFLP analysis. Therefore, although it demonstrated 100% sensitivity, spoligotyping overestimated the number of clustered isolates by ≈50% (specificity 47.5%). Conversely, RFLP analysis had 100% specificity, but lower sensitivity. However, most of the isolates classified as clustered by spoligotyping but not by RFLP showed >50% similarity in their IS*6110* patterns (Figure 2). Using spoligotyping of clinical samples without culture confirmation, we were able to diagnose an

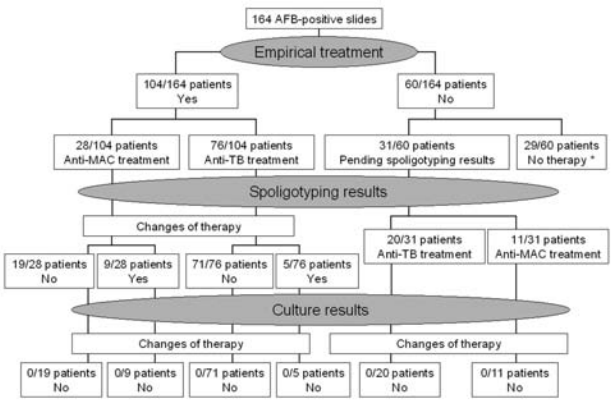

Figure 1. Clinical and therapeutic implications of spoligotyping results in treating suspected mycobacterial diseases. AFB, acid-fast bacilli; pts, patients; MAC, *Mycobacterium avium* complex; TB, tuberculosis. *Twenty-five patients did not begin treatment because they did not have clinical and radiologic features of tuberculosis. Four patients died within a few days after admission without receiving any antimycobacterial drug.

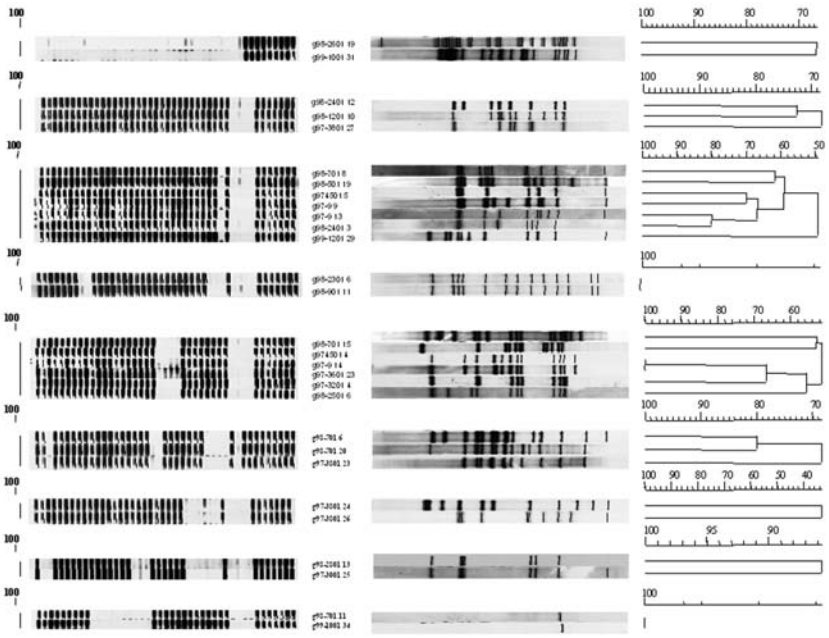

Figure 2. Spoligotype dendrograms generated by clustered *Mycobacterium tuberculosis* strains after computer analysis compared with the corresponding dendrograms of IS*6110* DNA fingerprints.

M. bovis infection and rapidly identify 2 cases of recurrent tuberculosis in patients with the same spoligotyping pattern in both followup specimens and the initial *M. tuberculosis* strain isolated several months earlier.

Discussion

Recent characterization of biologic markers for typing *M. tuberculosis* strains has greatly facilitated and improved tuberculosis epidemiology. IS*6110* RFLP typing is the most widely used method for differentiating *M. tuberculosis* strains (15–19). However, RFLP analysis requires growth of mycobacterial colonies, which involves a consistent lag time between epidemiologic events and typing results. Thus, time remains a limiting factor in obtaining a highly effective method for epidemiologic surveys and preventing the spread of disease.

The development of new PCR-based typing methods (4,5,9,10,20–23) has allowed rapid mycobacterial identification to be combined with epidemiologic typing results. Thus, molecular epidemiologic information can be combined in the context of epidemic events and tuberculosis transmission. Spoligotyping appears to have the specific characteristics needed to satisfy these issues of epidemics and tuberculosis transmission (24–28). This method permits the concomitant identification and differentiation of *M. tuberculosis* strains and avoids the timing problems associated with the slow growth of these bacteria (10,11). However, although spoligotyping can be used with clinical specimens (9), the usefulness of any PCR-based typing method in the clinical setting has yet to be demonstrated. In addition, although previous studies have investigated the effect of specific methodologic issues on the performance of several typing techniques (20–22,24–30), we have demonstrated the usefulness of a PCR–based technique in the clinical setting for diagnostic purposes, as well as for epidemiologic studies of tuberculosis transmission.

We have also shown that spoligotyping can be satisfactorily used with clinical samples. The performance of spoligotyping was satisfactory with all clinical specimens used, and its specificity and sensitivity were 98% and 96%, respectively. These features are comparable with those of commercial PCR methods used for detecting *M. tuberculosis* from clinical samples (19). Moreover, the opportunity to combine rapid diagnostic information and molecular epidemiologic data represents an important advance in the epidemiologic control of tuberculosis.

Several molecular typing studies have compared different methods for *M. tuberculosis* typing. IS*6110*-based RFLP has been found to be more discriminative than direct repeat–based spoligotyping (20–31). Although our data confirm that spoligotyping vastly overestimates the number of clustered isolates, this method has a lower discriminatory power than IS*6110*-RFLP. However, spoligotyping also has a higher negative predictive value, thus enabling the clinician to exclude a particular clustered strain as a cause of infection, if known drug-resistant variants are present.

This study showed that spoligotyping can provide useful data to clinicians in different settings. Although coinfection with *M. tuberculosis* and nontuberculous mycobacteria cannot be ruled out, the ability to differentiate between *M. tuberculosis* and other mycobacteria was demonstrated in 27% of the patients. Some patients began therapy after evaluation of spoligotyping results, while

others changed treatments because spoligotyping did not confirm the initial diagnosis. Time of response for spoligotyping was shorter than that of culture confirmation of tuberculosis by a median of 20 days and that of susceptibility results by a median of 29 days. In addition, a median of 6 days was needed for obtaining spoligotyping results, compared with 75 days for RFLP typing results.

Comparison of molecular typing patterns identified 6 patients with reactivation of tuberculosis caused by the same strain of *M. tuberculosis*, a finding that can differentiate between relapse and new infection in a new episode of tuberculosis. In 1 patient, we found that the spoligotyping pattern was identical to that of other strains that belonged to a cluster of multidrug-resistant tuberculosis. This observation resulted in the modification of antituberculosis treatment 34 days before susceptibility data were available. Moreover, information obtained by spoligotyping was relevant and useful in therapeutic management of ≈33% of the patients.

The clinical utility of spoligotyping may not be fully apparent by analyzing the results of this study, primarily because of the lack of clustered episodes of tuberculosis during the study period. However, spoligotyping would have been useful during a period or in a setting characterized by the emergence of *M. tuberculosis* outbreaks (8,32,33). We have also shown that the usefulness of spoligotyping is increased when results are compared with data on other tuberculosis patients and a DNA database on *M. tuberculosis* strains.

In conclusion, this study underscores the need to implement rapid molecular epidemiologic methods in managing tuberculosis epidemics. We have shown that spoligotyping is a useful method for screening and epidemiologic control of tuberculosis dissemination, particularly when results are required quickly, such as in outbreaks, or in the management of transmission of multidrug-resistant tuberculosis, especially in restricted high-risk situations such as prisons, schools, and hospitals.

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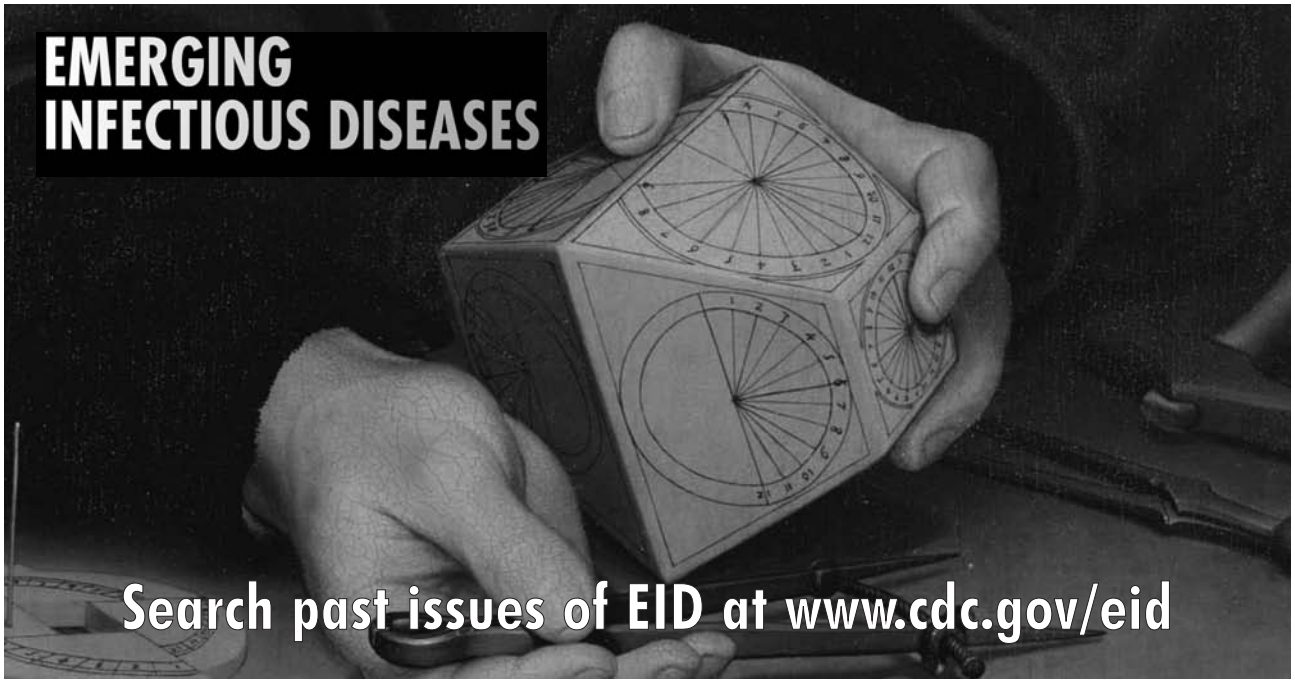

Modeling Control Strategies of Respiratory Pathogens

Babak Pourbohloul,*[1] Lauren Ancel Meyers,†‡[1] Danuta M. Skowronski,* Mel Krajden,* David M. Patrick,* and Robert C. Brunham*

Effectively controlling infectious diseases requires quantitative comparisons of quarantine, infection control precautions, case identification and isolation, and immunization interventions. We used contact network epidemiology to predict the effect of various control policies for a mildly contagious disease, such as severe acute respiratory syndrome, and a moderately contagious disease, such as smallpox. The success of an intervention depends on the transmissibility of the disease and the contact pattern between persons within a community. The model predicts that use of face masks and general vaccination will only moderately affect the spread of mildly contagious diseases. In contrast, quarantine and ring vaccination can prevent the spread of a wide spectrum of diseases. Contact network epidemiology can provide valuable quantitative input to public health decisionmaking, even before a pathogen is well characterized.

Public concern regarding emerging infectious diseases is on the rise. The 21st century began with the emergence or reemergence of zoonotic diseases like severe acute respiratory syndrome (SARS) (1), avian influenza (2), monkeypox infection (3), West Nile virus disease (4), mad cow disease (5), anthrax due to bioterrorist attacks (6), and unusual influenza epidemics (7). In addition to these new threats, public health officials face a large number of disease outbreaks every year in hospitals, schools, and other small communities. While development of vaccines and diagnostic tools proceeds at an unprecedented pace, development of tools for determining optimal intervention strategies lags behind.

In response to this problem, we have found that mathematical models of disease transmission can be used to evaluate and optimize control strategies. Such quantitative predictions can be empirically tested through randomized comparative trials, and mathematical models increasingly contribute to public health decisions regarding policy and intervention (8–13).

We use contact network epidemiology to compare intervention strategies for airborne[2] infectious diseases, including emerging diseases such as SARS, for which epidemiologic data are limited. These methods are based on explicit mathematical models of the heterogeneous patterns of interpersonal contacts that underlie disease transmission in a community, be it a hospital, school, or city (12–21). This approach differs from fully mixed compartmental models that assume that each person can infect every other person with equal probability (8). Some compartmental models have been modified to include population heterogeneity and have provided insights into the long-term effects of intervention strategies (8–11). For communities with extensive heterogeneity in contact patterns, however, network models more explicitly capture patterns of disease transmission and thus enable more accurate and detailed predictions of the effect of control measures on the magnitude and distributions of outbreaks.

Methods

Contact network models capture and estimate interpersonal contacts that lead to disease transmission within a community (22). Contacts can take place within households, schools, workplaces, hospitals, and other public venues. Each person in a community is represented as a

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[2]For the purposes of this manuscript, "airborne" refers to respiratory pathogens that are spread through respiratory secretions and can be either airborne, such as tuberculosis, or dropletborne, such as SARS.

vertex in the network, and each contact between 2 people is represented as an edge connecting the vertexes. The number of edges emanating from a vertex is the degree of that vertex. This quantity indicates the number of contacts who potentially transmit disease to or acquire disease from a person. The variation of degree across the entire network, i.e., the degree distribution, is fundamental to determining the probability for spread of disease through a network of contacts. Given the degree distribution of the contact network, one can analytically predict the fate of an outbreak.

Contact network epidemiology allows us to assess the vulnerability of a population to an infectious disease on the basis of the structure of the network (its degree distribution) and on the average transmissibility (T) of the disease (12,13). T is the average probability that transmission will occur from an infected person (vertex) to an uninfected person. This parameter summarizes multiple aspects of transmissibility, including the contact intensity between persons, duration of infectiousness, and the host's susceptibility to the infectious pathogen (12,13).

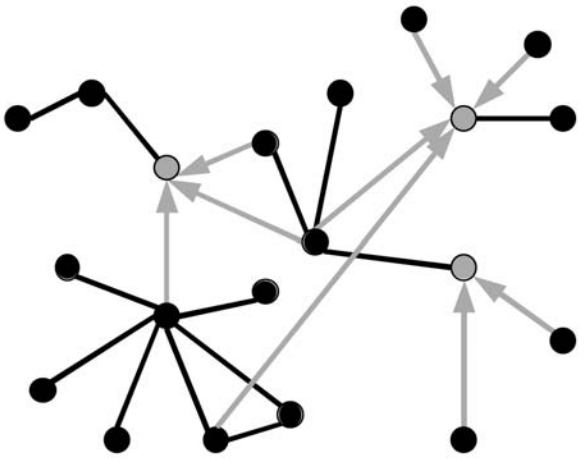

Figure 1. Schematic diagram of a directed network. Each black vertex represents a member of the general population; gray vertexes represent healthcare workers.

Contact Network Parameter Estimation

We built an urban contact network model with 2,000 households with an average household size of 2.6 (5,154 persons) based on demographic information for the Greater Vancouver Regional District, British Columbia, Canada. We used publicly available data from sources such as Statistics Canada to estimate the distribution of ages, household sizes, school and classroom sizes, hospital occupancy, workplaces, and public spaces (23–27).

Most of the edges in the network are undirected, meaning that transmission may occur in either direction (black edges in Figure 1). For example, 2 persons living in the same household will have equal opportunities to infect each other. The remaining edges are directed, meaning that a person may infect another person but the converse is not true (gray edges in Figure 1). For example, suppose person A is healthy and has no reason to go to the hospital until he or she is infected with SARS. At that point, person A will likely come into contact with and potentially spread SARS to caregivers at the hospital. In contrast, if a caregiver at the hospital acquired SARS while person A remained healthy in the community, then no opportunity would exist for transmission in the opposite direction. To model the directional flow of infected patients into a hospital, we include directed edges from persons in the population at large to caregivers in the hospital.

In an urban setting, not all encounters are equally likely to lead to disease transmission. We capture this heterogeneity in 2 ways. First, in the simulated urban network, the probability of a contact between 2 persons depends on the location and nature of their overlapping daily activities. For example, persons in the same household are connected to each other with probability 1, while persons who encounter each other in a public space are connected to each other with a probability from 0.003 to 0.300. Second, after these connections are determined, we assign a distinct transmissibility, T_{ij}, for each pair of connected persons i and j, that depends on the nature of their contact. For a given disease, the distribution of transmissibilities is based on empiric estimates for the diversity in infectious periods and the per day probability of transmission between persons who come into contact with each other. For more details, please refer to the online Appendix, available from http://www.cdc.gov/ncidod/EID/vol11no08/04-0449_app.htm.

Modeling Control Strategies

In any given network exists a critical transmissibility value, T_c, which indicates whether a large-scale epidemic is probable. Any disease with average transmissibility $<T_c$ cannot cause sustained transmission within a population and will thus be limited to small outbreaks. Such diseases die out because of the probabilistic nature of transmission before the disease has a chance to spread to the population at large. In this case, we can mathematically predict the expected size of small outbreaks, s. Diseases with average transmissibility $>T_c$ will spark large-scale epidemics with probability S, which can also be estimated. The value of T_c depends on the contact patterns within a community. Roughly speaking, when abundant opportunities exist for transmission, disease will spread easily, and the epidemic threshold will be low. The equations for s, S, and T_c, which are entirely in terms of the degree distribution and average transmissibility T, are presented in the online Appendix.

The epidemic potential of disease is commonly estimated by using the basic reproductive number R_0, the number

of secondary infections arising from a single infection in a relatively naïve population (8,28). This quantity is linearly related to the transmissibility of the disease, i.e., $R_0 = \gamma T$, where γ depends on the structure of the network (equations 1 and 8 in online Appendix). When T is at the epidemic threshold ($T = T_c$), then $R_0 = 1$. Public health interventions aim to reduce the number of new infected cases, ideally decreasing the effective reproductive number of the disease below the epidemic threshold, $R_{eff}<1$.

The difference between average transmissibility T and the basic reproductive ratio R_0 is important. While both have threshold values that distinguish epidemic from nonepidemic scenarios ($R_0 = 1$ and $T = T_c$), T is determined by the transmission characteristics of the pathogen and the nature of human interactions, but not the numbers of contacts in a community, whereas R_0 depends on all of these factors, particularly on the numbers of interactions within the community. For example, consider a single airborne pathogen spreading through a hospital, where abundant close contacts exist, and through a rural community, where close contacts are rare. The per contact probabilities of transmission (T_{ij}) may be similar in these settings because they are determined by the pathogenesis of the strain in the host, while the numbers of contacts are different. Therefore, the average transmissibility T will be similar in the 2 locations, while R_0 will be substantially higher in the hospital than in the rural setting.

The heterogeneous spread of SARS worldwide suggested context-dependent patterns of transmission with relatively rapid spread through hospitals and relatively slow spread through communities (29). A notable exception to this pattern, the large cluster of SARS cases outside a healthcare setting in the Amoy Gardens apartment complex in Hong Kong, seems to have spread through aerosolization of virus-laden sewage rather than direct person-to-person contact (30). When contact patterns within a community are extremely heterogeneous, explicitly modeling community structure and T makes more sense than assuming a universal R_0. We take this approach to evaluating disease control strategies in an urban setting (31).

A primary public health goal is to bring disease from a value above an epidemic threshold to a value below the threshold, thereby eliminating the threat of a large-scale epidemic. This goal can be achieved through interventions that directly affect the transmissibility of the pathogen (T) or through interventions that modify patterns of interaction so that the epidemic threshold (T_c) is increased. We call these 2 forms of intervention transmission-reduction and contact-reduction, respectively, and depict them graphically in Figure 2. The solid curves represent the predicted size of an outbreak and the probability of an epidemic for an entire spectrum of T from 0 to 1 in an urban setting. All airborne pathogens have a transmissibility value within this range; 0 = no transmission, and 1 = every contact leads to transmission. Thus, any disease can be mapped to a unique value on the curve.

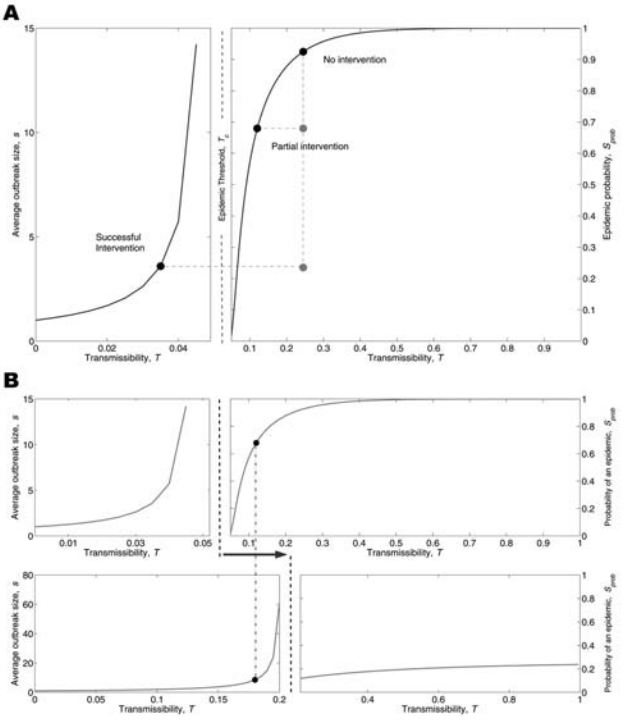

Figure 2. Transmission- vs. contact-reduction intervention. A) Transmission-reduction intervention: solid curves show the average size of an outbreak (left panel) and the probability of a large-scale epidemic (right panel). The horizontal axes cover the spectrum of disease transmissibility (from 0 to 1) such that a single disease is associated with a unique value on either the left curve (if $T<T_c$) or the right curve (if $T>T_c$). The epidemic threshold T_c separates the 2 zones. For better visualization, we chose 2 different scales for horizontal axes of the 2 panels. Consider a disease with T = 0.245 (top black circle). A transmission-reduction intervention causes the black circle to slide on a new position on the curve. A successful intervention is the one that lowers T to a value $<T_c$. B) Contact-reduction intervention: solid curves in the top panel show the epidemiologic vulnerability of the original network. Contact-reduction interventions alter the structure of the contact network and shift the epidemic curves to the right (solid curves in bottom panel). The 2 dashed vertical lines show the critical transmissibility threshold for the old (left) and new (right) networks. Consider the disease denoted by the black circle: the contact-reduction intervention raised the epidemic threshold above transmissibility of the disease and thereby eliminated the possibility of an epidemic.

In our simulated urban contact network, the critical transmissibility threshold is $T_c = 0.048$. An outbreak of disease with $T = 0.245$ will almost certainly spark an epidemic in the absence of intervention (top circle in Figure 2). This value of T is equivalent to an $R_0 = 5$ for this contact network and thus corresponds to a moderately infectious disease like smallpox (32,33). A successful intervention

either reduces T so that it lies below T_c (Figure 2A) or modifies the structure of the network so that T_c rises above T (Figure 2B). The first strategy can be achieved by interventions that reduce the probability of transmission per contact, such as face masks, gloves, gowns, handwashing, and other infection control precautions that prevent the exchange of respiratory droplets without eliminating contact.

The second strategy involves modifying the contact network itself. Interventions such as quarantine and closing schools and other public places effectively eliminate potential contacts (edges) between persons. Interventions such as immunization and the prophylactic use of antibacterial or antiviral drugs are tantamount to removing persons (vertexes) from the contact network and therefore also alter the network structure. We mathematically assess the effect of such strategies by deleting edges and vertexes from the contact network and predicting the new probability of an epidemic and expected distribution of cases within the community.

Results

We evaluated a variety of commonly implemented public health interventions by changing the contact patterns within the network, transmissibility of the disease, or both. For each strategy, we calculated several epidemiologic quantities: 1) the epidemic threshold, T_c, which may be raised by contact-reduction interventions, 2) the transmissibility of the disease, T, which may be reduced by transmission-reduction interventions, 3) if $T<T_c$, the expected size of a small outbreak, s, 4) if $T>T_c$, the probability of a large-scale epidemic, S_{prob}, and 5) if $T>T_c$, the expected size of an epidemic, S, should one occur. Based on calculations of these quantities, Figures 3–5 report the effect of various interventions applied to a moderately contagious disease just above the epidemic threshold (left panel), where we believe SARS to lie (34) and a moderately infectious disease such as smallpox (right panel). Gray entries correspond to unsuccessful interventions; white entries indicate strategies that are predicted to successfully move the pathogen below the epidemic threshold and thereby prevent a large-scale epidemic.

Transmission Reduction

Although general use of face masks may have a moderate effect, its success hinges on correct use and level of compliance. For instance, face masks that are 75% effective will only prevent a large-scale epidemic of a SARS-like disease if ≥60% of the general population complies perfectly (Figure 3). If persons use face masks incorrectly or only partially, this intervention will be less likely to protect persons and the population as a whole. For moderately contagious diseases like smallpox, face masks alone will not protect large urban areas from an epidemic. Figure 3

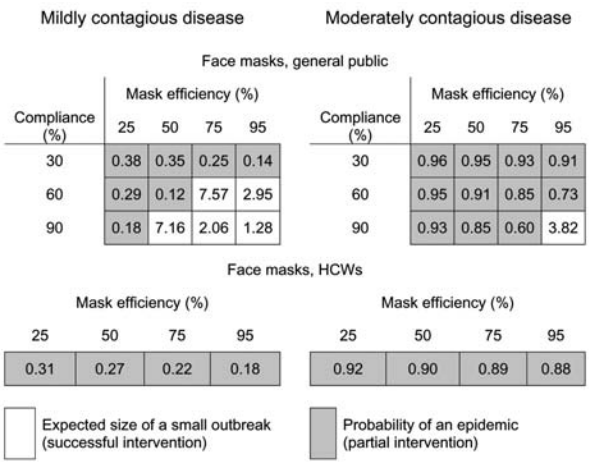

Figure 3. Comparing the effect of face masks for the general public and healthcare workers (HCWs). Mask efficiency is the percent reduction in transmissibility to or from a person correctly using a mask. Compliance is the fraction of the population adopting the intervention. Results are for a mildly contagious disease with a transmissibility T = 0.075 and a moderately contagious disease with a transmissibility T = 0.245. The equivalent basic reproductive number for these diseases are R_0 = 1.545 and R_0 = 5.047, respectively. Without intervention, both of these diseases have T above the epidemic threshold for the community (T_c = 0.048) and thus may ignite a large-scale epidemic. The probabilities that such epidemics will occur (without intervention) are S_{prob} = 0.50 and S_{prob} = 0.97, respectively. Some interventions may not bring T below the epidemic threshold and thus only reduce the probability of an epidemic (gray boxes), while others succeed in containing transmission to a small outbreak (white boxes). Gray boxes give the probability of an epidemic, and white boxes give the expected size of an outbreak. Outbreak size may not be an integer since s is an average taken from all possible outbreaks in the community.

also suggests that use of face masks by healthcare workers, while important for personal protection, offers limited protection to the population and does not predictably preclude an epidemic.

One of the factors that influences the transmissibility T is the duration of infectiousness. The duration of effective infectiousness may be shortened, but not eliminated, by isolating persons immediately after diagnosis. Although isolating an infected person will physically remove him from the network, the person may already have had a chance to infect others before being identified and isolated. For example, an infectious person who is isolated after the second day of a 6-day infectious period will have had 2 days in which disease could be transmitted to close contacts. Thus, isolation can be effective for diseases with low transmissibility but only if case identification occurs early in the infectious period. For such diseases, an isolation strategy that on average reduces the infectious period by 50% will prevent a large-scale epidemic (Figure 4). Isolation will not preclude an epidemic for a highly trans-

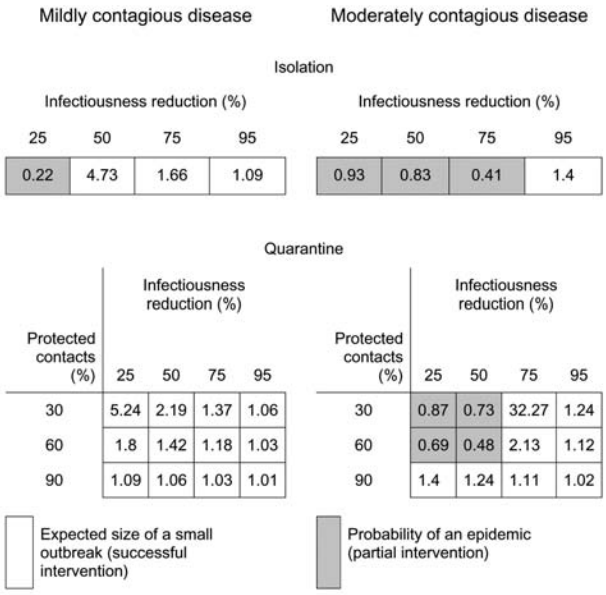

Figure 4. Comparing the effect of isolation and quarantine. Isolation alone reduces the infectious period by a specified percentage. Quarantine involves both isolation and sequestering a fraction of all case contacts. See the Figure 3 caption for further details.

missible disease unless clinical and diagnostic tools can be applied early and confidently, which may not be the case for an emerging infectious disease.

Contact Reduction

Contacts between infected and susceptible persons can be eliminated during an outbreak through measures such as quarantine, closing public venues, and ring vaccination, or they can be eliminated preventatively through general vaccination strategies. Figure 4 predicts that simultaneous case-patient isolation and quarantine of close contacts substantially improves containment. For a mildly contagious disease, an outbreak can be controlled with a combination of isolation that reduces the infectious period by 25% and quarantine that successfully sequesters 30% of all case-patient contacts. Much more rigorous isolation and quarantine are required for a highly contagious disease. Such interventions require a strong surveillance infrastructure, reliable rapid diagnostic tests, and social acceptance.

In Figure 6, we show that such predictions can readily be translated into values of R_{eff}. Interventions that bring a population under the epidemic threshold are those that decrease R_{eff} below 1. We emphasize that the predictions in Figures 4 and 6 are specific to the underlying model of contact patterns in an urban setting and that, contrary to common interpretations, R_{eff} (or R_0) is not a universal constant but instead critically depends on structure of the host community.

Vaccination

A general vaccination strategy is one in which a substantial proportion of the population is vaccinated at random. The success of this measure depends on proportion (coverage), vaccine efficacy, and disease transmissibility. The availability of a vaccine, therefore, does not guarantee prevention unless both delivery and vaccine-induced immunity are sufficient. For example, Figure 5 shows that a mildly contagious disease like SARS may be thwarted by partial coverage (≈75%) with a moderately efficacious vaccine (≈60% vaccine efficacy). Under this strategy, a moderately contagious disease can become epidemic unless a population receives 95% coverage with a 100% efficacious vaccine.

Ring vaccination of close contacts, on the other hand, is a very effective approach overall. This intervention, like quarantine, involves both transmission and contact reduction. Identifying the index patient results in a reduced infectious period. Subsequent identification and protection of his or her contacts through vaccination further limits the potential spread of the pathogen. Figure 5 considers the effect of ring vaccination on the population as a function of the effectiveness of patient isolation and the fraction of contacts that are successfully immunized. Partial protection of contacts may stem from inadequate contact tracing or an ineffective vaccine. For example, vaccinating 80% of

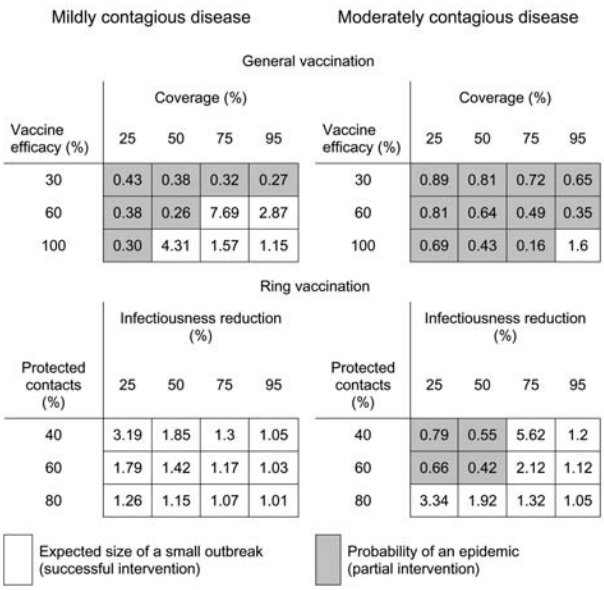

Figure 5. Comparing general vaccination and ring vaccination strategies. General vaccination protects a percentage of persons chosen randomly from the population with an efficacy determined by the vaccine itself. Ring vaccination involves isolating the patient (and the associated reduction in the infectious period) followed by targeted vaccination of contacts. The degree to which contacts are successfully protected depends on the success of contact tracing and the efficacy of the vaccine. See the Figure 3 caption for further details.

Mildly contagious disease

Quarantine

Protected contacts (%)	Infectiousness reduction (%) 25	50	75	95
30	0.81	0.54	0.27	0.05
60	0.44	0.3	0.15	0.03
90	0.08	0.06	0.03	0.01

Moderately contagious disease

Protected contacts (%)	Infectiousness reduction (%) 25	50	75	95
30	2.7	1.86	0.96	0.2
60	1.49	1.03	0.53	0.11
90	0.29	0.2	0.1	0.02

R_{eff}<1 R_{eff}>1

Figure 6. Intervention projections in terms of R_{eff}. This figure presents the results in the lower panel of Figure 4 expressed in terms of effective reproductive number rather than the projected size of an outbreak. If R_{eff}<1 outbreaks will die out, while if R_{eff}>1, epidemics may ensue. Note that the shading indicates epidemic potential and coincides perfectly with the shading in Figure 4.

close contacts with a 50% efficacious vaccine is equivalent to vaccinating 40% of close contacts with a 100% efficacious vaccine. Ring vaccination can be a successful strategy for a mildly contagious disease with even a moderate surveillance infrastructure or a partially efficacious vaccine. However, ring vaccination requires more successful case identification, contact tracing, and vaccination when implemented against a highly contagious disease. Ring vaccination is only applicable to diseases with relatively long incubation periods that allow contacts to be identified, vaccinated, and develop a protective immune response. Thus, this strategy is more appropriate for diseases like smallpox (incubation period 12 days) than SARS (incubation period 2–7 days).

Variation in Outbreak Size

The white entries in Figures 3–5 report the expected (average) size of small outbreaks for diseases below the epidemic threshold. Any particular outbreak, however, may not be exactly equal to this average size. In the left panel of Figure 7, we show the average and standard deviation of outbreak sizes over the range of transmissibility values below the epidemic threshold. For each value of T, we estimate the standard deviation by using 1,000 simulated epidemics on the original urban network (without intervention). For low T, outbreaks tend to be small and close to the average outbreak size s. As T increases toward the epidemic threshold, the distribution of outbreak sizes widens substantially, and s becomes less informative. Given this variability, public health strategies should be based on bringing populations substantially under the epidemic threshold.

Sensitivity Analysis

Our mathematical predictions are based on a single simulated urban network with 2,000 households with an average of 2.6 people per household. To address the sensitivity of the predictions to the particular pattern of contacts in the network, we stochastically generated 100 urban networks of equal size and predicted the probability of an epidemic for the range of T above the epidemic threshold. Since each of these 100 networks has a unique degree distribution, the value of the epidemic threshold varies. In particular we find that the average epidemic threshold is 0.04822 with a 95% confidence interval of 0.04656–0.04988. Recall that the network used in the analysis above has an epidemic threshold T_c=0.048. The right panel of Figure 7 shows the mean probability of an epidemic across these 100 networks with 95% confidence intervals. The probabilities for the particular network that we studied lie very close to the mean probabilities. The narrow confidence intervals suggest that our predictions are fairly robust to the particular architecture of the urban network. We further consider the effect of network size on these predictions in the online Appendix.

Discussion

Using contact network epidemiology, we evaluated various airborne infection control policies for a simulated urban setting like Vancouver. This approach explicitly captures the heterogeneous patterns of interpersonal contacts

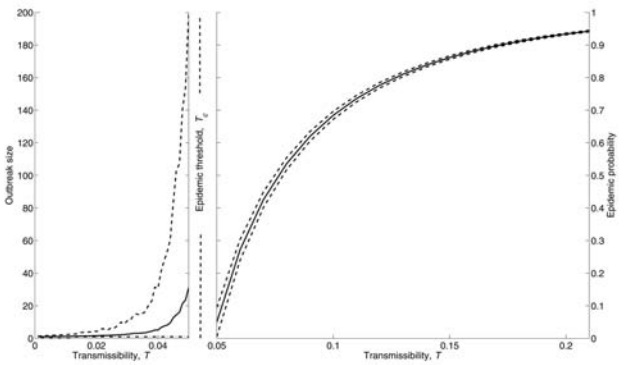

Figure 7. Left panel: variation of outbreak sizes as a function of transmissibility. We generated 1,000 epidemics for each of 20 values of T from 0 to the epidemic threshold. The solid curve represents the mean of outbreak size (μ), the dashed curve represents 1 standard deviation above the mean ($\mu + \sigma$), and the dotted line at the bottom shows the minimum size of an outbreak, which is always equal to 1, meaning that after the introduction of the first infected case the disease did not spread further. Right panel: sensitivity of epidemic probability to network stochasticity. We generated 100 different networks, each with 2,000 households. Because of the stochastic nature of contact formation during network generation, these 100 networks contain different numbers and configurations of edges and therefore have different degree distributions. The solid curve shows the mean probability of an epidemic across the 100 networks for transmissibilities above the epidemic threshold, and the dashed curves are 95% confidence limits for the mean probability of an epidemic.

that lead to disease transmission and allows rapid mathematical prediction of the probability and distribution of an epidemic. This analysis does not depend on computationally intensive simulations. Furthermore, the approach allows one to quantitatively compare strategies that directly reduce the transmissibility of a pathogen or limit opportunities for a pathogen to spread. Although each strategy has been considered on its own, these methods can easily predict the effect of combined interventions for an entire spectrum of airborne infectious diseases, including SARS, smallpox, influenza, and meningococcal meningitis, among others.

Although the qualitative results of this analysis are applied to urban settings, the work is meant to be a proof of concept rather than to provide specific quantitative recommendations for urban control of communicable diseases such as SARS and smallpox. Until we have developed contact network models for a wide range of communities and assessed their generality, contact network epidemiology will need to be applied on a case-by-case basis. For example, hospitals can use these methods to improve control of nosocomial airborne infections. To start, each facility should model its particular network of patient–healthcare worker interactions, then calculate the effect of measures such as respiratory droplet precautions, grouping patients in cohorts, modifications to healthcare worker assignments, and vaccination (12).

The success of contact network epidemiology depends not only on realistic models of contact patterns but also on reliable estimates of the average transmissibility of the pathogen, *T*. As a respiratory pathogen begins to spread through a population, epidemiologists can rapidly identify the mode and rate of disease transmission. These data can provide critical input for intervention strategies. Historically, the rate of disease transmission has been measured and reported in terms of the basic reproductive number R_0, based on the doubling time of case counts in the early phase of an outbreak or epidemic. The value of R_0, however, may vary substantially, depending on the population in which it is measured. For example, recent estimates of R_0 for SARS ranged from 1.2 to 3.6 (34–36). In contrast, *T* is not subject to the particular patterns of interaction within a community and can be reliably estimated in diverse settings. Measuring *T* is only slightly more involved than measuring R_0. For each case, one must measure not just the number of secondary cases, but also the total number of contacts of the case-patient during the infectious period and then divide the first value by the second.

Just as enormous molecular and technological resources are often mobilized to develop vaccines and diagnostic tools for emerging infectious diseases of public health importance, we should also harness the powerful quantitative mathematical tools that help assess disease interventions. When an airborne pathogen strikes, public health officials should be able to make scientifically grounded decisions about the competing medical, economic, and social implications following deployment of control measures. We illustrate that contact network epidemiology can provide detailed and valuable insight into the fate and control of an outbreak. Integrating these tools into public health decision making should facilitate more rational strategies to manage emerging diseases, bioterrorist events, and pandemic influenza in situations in which empiric data are not yet available to guide decision making.

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Dr. Pourbohloul is the director of the Division of Mathematical Modeling at the University of British Columbia Centre for Disease Control. He is leading a research group, funded by the CIHR, in the application of network theory to the prediction and control of SARS and other respiratory infections.

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Estimating Foodborne Gastroenteritis, Australia

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We estimated for Australia the number of cases, hospitalizations, and deaths due to foodborne gastroenteritis in a typical year, circa 2000. The total amount of infectious gastroenteritis was measured by using a national telephone survey. The foodborne proportion was estimated from Australian data on each of 16 pathogens. To account for uncertainty, we used simulation techniques to calculate 95% credibility intervals (CrI). The estimate of incidence of gastroenteritis in Australia is 17.2 million (95% confidence interval 14.5–19.9 million) cases per year. We estimate that 32% (95% CrI 24%–40%) are foodborne, which equals 0.3 (95% CrI 0.2–0.4) episodes per person, or 5.4 million (95% CrI 4.0–6.9 million) cases annually in Australia. Norovirus, enteropathogenic *Escherichia coli*, *Campylobacter* spp., and *Salmonella* spp. cause the most illnesses. In addition, foodborne gastroenteritis causes ≈15,000 (95% CrI 11,000–18,000) hospitalizations and 80 (95% CrI 40–120) deaths annually. This study highlights global public health concerns about foodborne diseases and the need for standardized methods, including assessment of uncertainty, for international comparison.

The pattern of foodborne disease has changed substantially in industrialized countries in recent decades. Outbreaks are more likely to be far reaching, and some are even global in scale because of widespread food distribution and changes in methods of food preparation (1). Further changes in the incidence of foodborne disease and the pattern of food-related illness can be anticipated from global warming (2). As a result of changed conditions in food production and better laboratory detection techniques, new foodborne pathogens continue to be identified (3). In particular, we are now faced with the emergence of antimicrobial drug–resistant bacteria and a number of viruses not previously recognized (4,5).

Foodborne disease is a public health concern in all parts of the world. In the United States, foodborne disease causes an estimated 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths each year (6). In the United Kingdom, an estimated 2.37 million cases of foodborne gastroenteritis occurred in 1995 (7). Previous estimates of foodborne gastroenteritis in Australia have ranged from 1–2 million (8) to 4 million episodes per year (9). The effect of such large numbers of persons with gastrointestinal illness is considerable. A recent national survey of gastroenteritis in Australia found that one third of working adults miss ≥1 days of work when they have gastroenteritis, and another third of cases result in a caregiver missing work (10). In Australia in 2003, 99 reported outbreaks of foodborne disease affected 1,686 people and caused 6 deaths (11). Any evidence of food contamination can also have a major effect on food industry and trade. The food industry in Australia generates >$29 billion in food production, with >20% of products exported, and $57 billion in food processing (12).

Transmission of Pathogens Causing Gastroenteritis

Infectious gastroenteritis is caused by many pathogens, each with unique characteristics requiring different laboratory tests for identification. Transmission of pathogens to humans may occur from contaminated foods or water, or from infected persons, environments, or animals. Some pathogens that cause gastroenteritis, such as *Bacillus cereus*, are always thought to be the result of contaminated food, whereas others, such as rotavirus, are largely transmitted by nonfoodborne routes. Most enteric pathogens have multiple modes of transmission (13).

Many clinical cases of gastroenteritis are assessed as "presumed infectious" and do not have a pathogen isolated, even when a stool sample is tested (14). These include

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cases in which a known pathogen is present but is not identified and cases in which the pathogen is totally unknown (15). Numerous pathogens that were unknown only a few decades ago are now considered commonplace, including *Campylobacter* spp., Shiga toxin–producing *Escherichia coli*, and norovirus (16). More unknown pathogens are likely to be major causes of illness, some of which will become apparent with time and investigation.

Estimating the Level of Foodborne Gastroenteritis

As part of the activities of OzFoodNet, the foodborne disease surveillance network operating in Australia since 2000, we undertook a study to estimate the amount of foodborne gastroenteritis in contemporary Australia. In the absence of an internationally agreed-upon methodology, the approach used in the United States provided the best method for obtaining internationally comparable estimates (6). Two components are required to estimate the extent of foodborne gastroenteritis by this method: 1) the total amount of gastroenteritis in the country and 2) the proportion of gastroenteritis that is foodborne. The product of these 2 estimates gives the total number of cases of foodborne gastroenteritis.

Uncertainty is inherent in data used in such calculations. Variability may be quantified by statistical concepts like standard error and confidence intervals, but other components of uncertainty are due to paucity of data. Since some information is always available, however, we can quantify each component of uncertainty by a plausible probability distribution using all relevant information available. We can then use simulations of these distributions to generate an interval that contains the credible estimates of the number of foodborne cases of gastroenteritis. These interval estimates are akin to credibility intervals used in Bayesian inferences.

Our objective for this study was to use Australian data to calculate the number of cases, hospitalizations, and deaths due to foodborne gastroenteritis in Australia in a typical year, around the year 2000, accounting for uncertainty in the estimate.

Methods

The calculations to estimate incidence, hospitalizations, and deaths are described below and in the Figure. Details of the data sources and the simulation technique used to account for uncertainty are shown in the online Appendix (available from http://www.cdc.gov/ncidod/EID/vol11no08/04-1367_app.htm).

We considered the definition of foodborne to include any infectious gastroenteritis caused by eating food, including food contaminated just before eating. The proportion of infectious gastroenteritis cases that are due to foodborne transmission in the community and their proportion among hospitalizations and deaths, were assessed by using data from multiple Australian sources about individual known pathogens.

Gastroenteritis caused by known pathogens was studied to estimate the proportion of all infectious gastroenteritis that is foodborne. Of 25 pathogens with the potential for foodborne transmission, 16 (those listed in Table 1) were considered relevant. Pathogens not considered relevant were either not foodborne (*Clostridium difficile*), did not cause gastroenteritis (*Brucella* spp., *Listeria* spp., *Toxoplasma gondii*, hepatitis A virus), or were only acquired overseas (*Salmonella* Typhi, *Vibrio cholerae*, *Cyclospora cayetanensis*, *Trichinella spiralis*).

For each of the 16 pathogens, yearly estimates were made of the total number of cases of gastroenteritis in the community; this number was based on data collected in the National Notifiable Diseases Surveillance System, published results from the Water Quality Study conducted in Melbourne in 1998 (17,18), laboratory data, or outbreak data. Necessary adjustments were made for underreporting in the Australian surveillance system, incomplete population coverage, and the proportion of infections acquired overseas (online Appendix). We also estimated, for each of the pathogens, the total number of hospitalizations and deaths based on data from the National Hospital Morbidity Database (NHMD) as well as the proportion of cases due to foodborne transmission, which was estimated from outbreak data, the literature, and because Australian data were lacking, a Delphi process involving 10 foodborne disease experts in Australia. The number of foodborne episodes for each pathogen was then obtained by multiplying the estimate of the foodborne proportion by the estimate of the total number of cases, hospitalizations, and deaths. The overall proportion of infectious gastroenteritis in the community due to foodborne transmission was then estimated by dividing the sum of the foodborne cases due to the 16 known pathogens by the sum of all cases due to the 16 known pathogens (Figure). The equivalent calculation was also done for hospital admissions due to known pathogens to give the proportion of hospitalizations for infectious gastroenteritis due to foodborne transmission.

We assumed that the proportion of gastroenteritis that is foodborne is the same among cases caused by known pathogens as among those caused by unknown pathogens. Adjustments were made for the proportion estimated as acquired overseas for certain pathogens (online Appendix).

Incidence of Foodborne Gastroenteritis

The total amount of infectious gastroenteritis in Australia in 1 year was estimated from the National Gastroenteritis Survey 2001–2002. This computer-assisted telephone survey ran during 12 months from September

2001 to August 2002 in all states and territories of Australia. Ethics clearance was obtained from the Australian Department of Health and Ageing Ethics Committee and from other state health departments and university committees. Random digit dialing was used to select households, and then the person with the most recent birthday was selected as the respondent. The response rate was 67%, and the final sample was 6,087 persons. Data were collected on symptoms of gastroenteritis in the previous 4 weeks. The case definition excluded persons who identified a noninfectious cause for their symptoms, and an adjustment was made for persons with gastrointestinal symptoms secondary to a respiratory infection (19). The case definition was ≥3 loose stools or ≥2 episodes of vomiting or, if respiratory symptoms were present, ≥4 loose stools or ≥3 episodes of vomiting in a 24-hour period in the previous 4 weeks.

The final estimate of the total number of cases of foodborne gastroenteritis in the community is the product of the proportion foodborne and the total number of cases of infectious gastroenteritis. The incidence per person per year was estimated by using population data from the Australian Bureau of Statistics (20).

Hospitalizations for Foodborne Gastroenteritis

The total number of hospitalizations for gastroenteritis was estimated from the National Hospital Morbidity Database (21), which records all admissions to hospital in Australia. Data from 1993/1994 to 1998/1999 were examined for International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes for gastroenteritis as either a principal diagnosis of as any of a further 9 additional diagnoses. Additional diagnoses refer to cases in which gastroenteritis was a contributing factor to, but not the only reason for, admission. These data were used to estimate the average number of diagnoses per year for each of 14 of the known pathogens. No ICD code for *Aeromonas* infection was in the hospital separation dataset, and hospitalizations due to *E. coli* infections did not distinguish between enteropathogenic and Shiga toxin–producing types, leading to a study of 14 rather than 16 known pathogens in hospital data. Diagnoses coded "gastroenteritis, presumed infectious" were also examined. Adjustments were not made for underreporting of individual pathogens, since any missed diagnoses for specific pathogens should be included in this unknown category.

The final estimate of the total number of hospitalizations due to foodborne gastroenteritis was the product of the proportion of hospitalizations among known pathogens that is foodborne and the total number of hospitalizations for infectious gastroenteritis (including cases of unknown but presumed infectious causes).

Table 1. GE due to known pathogens in Australia in a typical year circa 2000*

Pathogen	Median no. GE cases (95% CrI)	Median proportion foodborne (95% CrI)	Median no. foodborne (95% CrI)
Bacteria			
Aeromonas spp.	39,400 (31,700–47,200)	0.25 (0.12–0.38)	9,800 (4,100–15,400)
Bacillus cereus	6,900 (0–16,000)	1 (1.00–1.00)	6,900 (0–15,800)
Campylobacter spp.	277,000 (89,800–463,000)	0.75 (0.67–0.83)	208,000 (67,000–350,000)
Clostridium perfringens	43,000 (440–86,000)	1 (1.00–1.00)	43,000 (400–86,000)
STEC	3,000 (0–6,500)	0.65 (0.48–0.82)	1,900 (0–4,200)
non-STEC *E. coli*	1,152,000 (797,000–1,507,000)	0.5 (0.32–0.68)	563,000 (295,000–831,000)
Salmonella spp.	92,000 (26,000–158,000)	0.87 (0.81–0.93)	81,000 (23,000–138,000)
Shigella spp.	3,200 (0–6,900)	0.1 (0.04–0.16)	300 (0–700)
Staphylococcus aureus	14,100 (0–29,800)	1 (1.00–1.00)	14,200 (0–29,800)
Vibrio parahaemolyticus	1,080 (0–2,600)	0.71 (0.54–0.88)	740 (0–1,850)
Yersinia spp.	2,200 (0–4,500)	0.75 0.63–0.87)	1,620 (0–3,400)
Total bacteria	1,639,000 (1,175,000–2,103,000)	0.58 (0.44–0.72)	950,000 (590,000–1,310,000)
Viruses			
Norovirus	1,832,000 (1,361,000–2,302,000)	0.25 (0.12–0.38)	446,000 (193,000–700,000)
Rotavirus	241,000 (98,000–384,000)	0.02 (0.01–0.03)	4,700 (700–8,600)
Astrovirus/adenovirus	190,000 (63,000–316,000)	0.1 (0.02–0.18)	17,500 (0–36,800)
Total viruses	2,280,000 (1,740,000–2,820,000)	0.21 (0.11–0.31)	470,000 (210,000–730,000)
Parasites			
Cryptosporidium parvum	271,000 (255,000–287,000)	0.1 (0.02–0.18)	25,000 (0–54,000)
Giardia lamblia	430,000 (232,000–628,000)	0.05 (0.01–0.09)	20,400 (0–41,100)
Total parasites	704,000 (442,000–966,000)	0.14 (0.04–0.24)	66,000 (18,000–114,000)
Total	4,640,000 (3,750,000–5,510,000)	0.32 (0.24–0.40)	1,480,000 (1,030,000–1,920,000)

*GE, gastroenteritis; CrI, credibility interval; STEC, Shiga toxin–producing *Escherichia coli*.

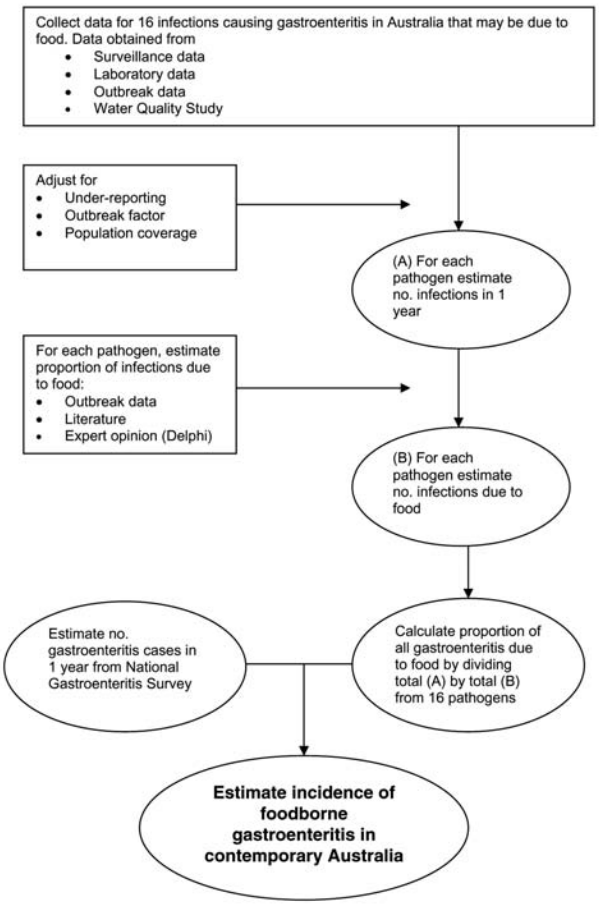

Figure. Scheme of data sources and calculations used to estimate the number of cases of foodborne gastroenteritis in the community in 1 year in Australia around the year 2000.

Deaths Due to Foodborne Gastroenteritis

Deaths in the hospital were determined for gastroenteritis as either a principal diagnosis of as any of a further 9 additional diagnoses (data from 1993/1994 to 1998/1999). The final estimate of the total number of deaths due to foodborne gastroenteritis was the product of the proportion foodborne and the total number of deaths in the hospital due to infectious gastroenteritis.

Accounting for Uncertainty in Data

Where suitable data were available, 95% confidence intervals (CI) were calculated. Otherwise, uncertainty in the data was accounted for by using simulation techniques. Plausible probability distributions were generated by using all available information, and the interval between 2.5th and 97.5th percentiles gave the 95% credible interval (95% CrI). The median was taken as the point estimate. This technique is explained in the online Appendix. Calculations were carried out with the Statistical Package for the Social Sciences, version 11.50 (SPSS Inc., Chicago, IL, USA).

Results

Incidence

The case definition applied to 450 (7%) of 6,087 respondents to the National Gastroenteritis Survey. When weighted to the Australian population by age and sex, this number extrapolated to 17.2 million (95% CI 14.5–19.9 million) cases of gastroenteritis in Australia in 1 year, or 0.92 (95% CI 0.77–1.06) cases per person per year (13). This number includes all causes of infectious gastroenteritis.

Among the 16 known pathogens were an estimated 4.6 million (95% CrI 3.8–5.5 million) cases of gastroenteritis due to all modes of transmission. Of these, an estimated 1.6 million (95% CrI 1.2–2.1 million) were due to bacterial infections, 2.3 million (95% CrI 1.7–2.8 million) were due to viral infections, and 0.70 million (95% CrI 0.44–0.97 million) were due to parasites (Table 1).

Among known pathogens, 1.5 million (95% CrI 1.0–1.9 million) cases were acquired through food. Enteropathogenic *E. coli*, noroviruses, *Campylobacter* spp., and *Salmonella* spp. accounted for 88% of all foodborne disease in this group of pathogens (Table 1). The proportion of gastroenteritis due to foodborne transmission was estimated at 32% (95% CrI 24–40%). The product of the total number of cases of gastroenteritis (17.2 million; 95% CrI 14.5–19.9 million) multiplied by the proportion that was foodborne (0.32, 95% CrI 0.24–0.40) produced an estimate of 5.4 million cases of foodborne gastroenteritis in 1 year in Australia (circa 2002), with a 95% CrI of 4.0–6.9 million cases.

Hospitalizations

Among hospitalizations for gastroenteritis due to the 14 known pathogens were 10,070 (95% CrI 8,630–11,470) diagnoses of gastroenteritis; an estimated 3,640 (95% CrI 2,600–4,670) of these cases were due to eating contaminated food. The overall proportion of hospitalizations estimated to be from foodborne gastroenteritis was 0.36 (95% CrI 0.30–0.41) (Table 2).

The total number of hospital diagnoses for gastroenteritis was estimated as 41,000 (95% CrI 33,000–49,000). The number due to foodborne transmission was 14,700 (95% CrI 11,400–18,000) (Table 3).

Deaths

The NHMD 1993/1994 to 1998/1999 showed 1,302 deaths (157–311 per year) in patients with a code for a principal or additional diagnosis of infectious gastroenteritis in the 6 years. The average was 217 (standard deviation 51) per year. Of these 1,302 deaths, 287 occurred in patients with a principal diagnosis of infectious gastroenteritis. Application of the proportion of hospital diagnoses due to foodborne gastroenteritis (36%, 95% CrI

Table 2. Estimated hospital diagnoses of gastroenteritis due to foodborne pathogens in Australia, circa 2000*

Agent	ICD-9-CM code	No. hospital diagnoses per year (95% CrI)†	% foodborne (95% CrI)	No. hospital diagnoses from foodborne transmission per year (95% CrI)‡
Bacteria		4,960 (3,360–6,310)	70 (65–75)	3,480 (2,440–4,500)
Aeromonas spp.	NA	NA	25 (12–38)	–
Bacillus cereus	008.59	29 (0–66)	100	29 (0–66)
Campylobacter spp.	008.43	3,140 (1,754–4,546)	75 (67–83)	2,260 (1,250–3,300)
Clostridium perfringens	005.2	1 (0–3)	100	1 (0–3)
Escherichia coli	008.00–04	102 (53–154)	50 (32–68)	50 (23–86)
Salmonella spp. (nontyphoidal)	003	1,330 (1,130–1,530)	87 (81–93)	1,060 (900–1,240)
Shigella spp.	004	320 (270–370)	10 (4–16)	19 (8–31)
Staphylococcus aureus	005.0	21 (17–25)	100	21 (17–25)
Vibrio parahaemolyticus	005.4	4 (2–6)	71 (54–88)	3 (1–5)
Yersinia enterocolitica	008.44	34 (24–44)	75 (63–87)	25 (17–35)
Viruses		3,940 (3,740–4,140)	2 (1–3)	100 (60–140)
Astrovirus/adenovirus	008.62/008.66	190 (130–250)	10 (2–18)	19 (4–37)
Norovirus	008.63	17 (2–32)	25 (12–38)	4 (0–9)
Rotavirus	008.61	3,740 (3,540–3,920)	2 (1–3)	70 (40–110)
Parasites		1,160 (950–1,390)	6 (2–9)	64 (19–116)
Cryptosporidium spp.	007.4	200 (0–400)	10 (2–18)	14 (0–49)
Giardia lamblia	007.1	1,000 (900–1,100)	5 (1–9)	49 (7–90)
Total known§		10,070 (8,630–11,470)		3,640 (2,600–4,670)
Miscellaneous and unknown agents		30,800 (22,700–38,400)	36 (30–41)¶	11,000 (8,000–14,000)
Miscellaneous agents (not listed above)		2,800 (2,400–3,200)		1,000 (800–1,200)
Unknown#		28,000 (20,000–35,700)		10,000 (6,800–13,200)
Total known and unknown		41,000 (33,000–49,000)	36 (30–41)	14,700 (11,400–18,000)

*ICD-9-CM, International Classification of Diseases, Ninth Revision, Clinical Modification; CrI, credibility interval; NA, not applicable.
†Includes principal and 9 additional diagnoses. Simulated distribution based on raw yearly National Hospital Mortality Database data 1993/1994–1998/1999.
‡Adjusted for 1) proportion foodborne and 2) proportion overseas acquired.
§36% (95% CrI 30%–41%) known pathogens foodborne (not overseas acquired).
¶Apply % foodborne in known pathogens.
#Includes codes 0051–3 and 8–9, 00849, 0085, 0088, 009, 00841–2, 00846–9,and 00869.

30%–41%) to the number of deaths in which the diagnosis included infectious gastroenteritis (217, 95% CrI 120–320) provided an estimate of 76 (95% CrI 41–120) deaths due to foodborne gastroenteritis each year (Table 3).

Discussion

The estimates from this study demonstrate the considerable prevalence of foodborne disease in contemporary Australia and justify the attention given to foodborne disease surveillance and food safety. The uncertainty estimates indicate that even the lower boundary of the credible interval is still high, with at least 4 million cases of foodborne gastroenteritis, and possibly as many as 7 million per year. This means that on average, every Australian can expect to experience an episode of foodborne illness about every 3 to 4 years. Hospitalizations are uncommon at 8 per 10,000 people each year, and ≈4 deaths per million persons occur per year.

Similar studies have been done in United States (6) and the United Kingdom (7). The Australian estimate of incidence is remarkably similar to that reported for the United States, but higher than in the United Kingdom. In the United States, 36% of all gastroenteritis was estimated to be due to foodborne transmission, and incidence was estimated at 0.28 cases per person per year. In the United Kingdom, 26% of gastroenteritis was estimated to be due to foodborne transmission, and incidence was estimated at 0.04 cases per person per year in 1995. The importance of using a standardized method when comparing results of the amount of foodborne gastroenteritis across countries or times cannot be overemphasized. Evidence suggests that a prospective cohort study design may produce a lower incidence of community gastroenteritis than a cross-sectional design. The UK study included a quality control substudy to compare the incidence based on a retrospective recall method with incidence from a prospective diary method; the estimates of incidence were 0.6 and 0.2 cases per person per year, respectively (14). Prospective studies that require participants to supply a stool sample every time they report gastroenteritis might tend to cause an underestimate because of unwillingness to provide a sample; on the other hand, in a retrospective recall method, respondents might "telescope" events into a shorter time frame. A prospective study done in the Netherlands (22) also found

Table 3. Infectious and foodborne gastroenteritis in Australia in a typical year, circa 2000*

Measure	All causes estimate (95% CrI)	Foodborne transmission estimate (95% CrI)
No. cases per year ($\times 10^6$)	17.2 (14.5–19.9)	5.4 (4.0–6.9)
Known pathogen	4.6 (3.7–5.5)	1.5 (1.0–1.9)
Cases per person per year	0.92 (0.77–1.06)	0.29 (0.23–0.35)
No. hospital diagnoses per year ($\times 10^3$)	40.9 (32.7–48.6)	14.7 (11.4–17.7)
Known pathogen	10.1 (8.6–11.5)	3.6 (2.6–4.7)
Hospital diagnoses per 10,000 persons per year	22 (17–26)	8 (6–9)
Deaths per year	217 (120–320)	76 (40–120)
Deaths per 10,000 persons per year	0.12 (0.06–0.17)	0.04 (0.02–0.06)

*CrI, credibility interval.

a lower incidence than that seen in the United Kingdom. Other variations in methods also exist across countries, such as differences in surveillance systems and the quality of outbreak data available to estimate the proportion of cases that are foodborne. Not only may the study design influence the final estimate, but also the definition of gastroenteritis. Even a seemingly small difference in the definition of gastroenteritis can lead to a considerable difference in the final estimates (23).

The definition of community gastroenteritis used in this Australian study refers to moderate-to-severe illness, with at ≥3 loose stools or ≥2 episodes of vomiting in a single day. To improve the specificity of our definition for enteric illness, we excluded patients with concomitant respiratory symptoms unless they had more severe symptoms of diarrhea or vomiting. Previous studies have found similarly high rates of respiratory symptoms amongst cases of gastroenteritis (24). A definition inclusive of milder illness would lead to a higher estimate of foodborne gastroenteritis, and a definition that included only more severe illness would lead to a lower estimate.

We also took account of those with concurrent respiratory symptoms in our definition of community gastroenteritis, although most studies estimating the amount of gastroenteritis have not considered this. The United States study (6) adjusted for those with respiratory illness by excluding a proportion of case-patients who were thought likely to have symptoms secondary to respiratory infections rather than a primary enteric infection. The UK definition of gastroenteritis was different from the Australian definition in several ways. While differing arguments can be raised about the best definition of gastroenteritis, the main concern is to have a consistent, reasonable definition for comparative purposes.

Of the 5.4 million (95% CrI 4.0–6.9 million) cases of foodborne gastroenteritis, 28% were attributed to known pathogens. This finding compares with 18% in the United States (6) and 41% in the United Kingdom (7). The Australian data used to estimate the pathogen-specific numbers of community cases of gastroenteritis were variable in quality. *Salmonella* notifications have been relatively stable over the last 5 years, and characteristics of this illness are fairly well understood. In comparison, reports of illness due to *Campylobacter* have increased steadily during the same time (25). This finding could be due to reporting artifacts or increasing infection rates in the community. The diagnostic laboratory tests have not changed appreciably during this time.

The pathogen-specific estimates in this study that most influenced the final estimate of the proportion of gastroenteritis that is foodborne were those for norovirus and enteropathogenic *E. coli*, as these accounted for the largest numbers. The estimates for both were determined from a high-quality longitudinal study (17). Nevertheless, the sample was limited to a specific subpopulation and geographic location. The high proportion of *E. coli* is similar to findings in the United Kingdom, although we estimated that 50% of cases caused by this pathogen were foodborne compared to 8% of cases in the UK assessment (7) and 30% in the United States (6). In recent years, the capacity of laboratories to identify noroviruses with polymerase chain reaction tests has improved considerably, and this virus is likely to become increasingly recognized (26).

Factors were used to adjust for underreporting when using data from outbreaks and surveillance. Further studies are needed to give more robust estimates of the level of underreporting compared with the true level in the community. The estimates of the proportion of illness due to foodborne transmission for specific pathogens relied largely on outbreak data and opinions of foodborne diseases experts. Outbreak data can be very sensitive to the outcomes from larger events, which could bias the estimate of the proportion foodborne in either direction (27). Foodborne disease experts' experience was based on pathogen characteristics in the laboratory, results of outbreak investigations, and knowledge from case-control studies of sporadic infections. For pathogens estimated to have a large number of cases, such as norovirus, the estimate of the proportion thought to be foodborne can influence the final estimate. Both the UK and Australian estimates were based on outbreak data, but only 11% of Norwalk-like virus (caliciviruses) gastroenteritis was ascribed to foodborne transmission in the UK study, compared with 40% in the United States, and 25% in Australia (6,7). These individual

estimates had some influence on the final estimates of the proportion of all gastroenteritis that is foodborne.

Hospital data in Australia are fairly complete, and only a few hospitals, mostly private, have not contributed records of all admissions to the national database in the last decade (21). Coding of admissions varies over time and place, but a patient with gastroenteritis is likely to be coded for this condition in the first 10 diagnoses (28). Approximately two thirds of diagnoses were coded as the main reason for admission. Additional diagnoses may represent cases with complications or comorbidity that took precedence in the order of coding or cases acquired in the hospital. Some deaths due to gastroenteritis may have occurred in nursing homes, which were not included. Among the known pathogens, bacterial infections accounted for >90% of hospital admissions in Australia, which is similar to the proportion in the United Kingdom (7) but higher than the 60% estimated in the United States (6). Campylobacteriosis followed by salmonellosis accounted for most admissions due to bacterial infections in Australia and the United Kingdom; in the United States this order was reversed. These illnesses are important when considering the severe end of the spectrum of foodborne gastroenteritis.

We used the best available Australian data to conduct this study, but as experienced by others conducting research overseas, the quality of the data inevitably varied. Since the quality of the data cannot be easily improved, we chose to provide estimates that reflect the true state of uncertainty of the data by using a simulation technique that can be easily applied. Taking account of uncertainty informs the data users, including policy makers, that a very precise estimate is not possible. An appreciation of the degree of confidence that can be placed in an estimate is an important part of the responsible presentation of results that may have considerable effects at a policy level. With these stipulations, we are confident that the level of foodborne gastroenteritis is high in Australia. In the future, improvements in data completeness and quality would enhance the robustness of the calculations, but estimates of uncertainty are likely to remain an important component of the results.

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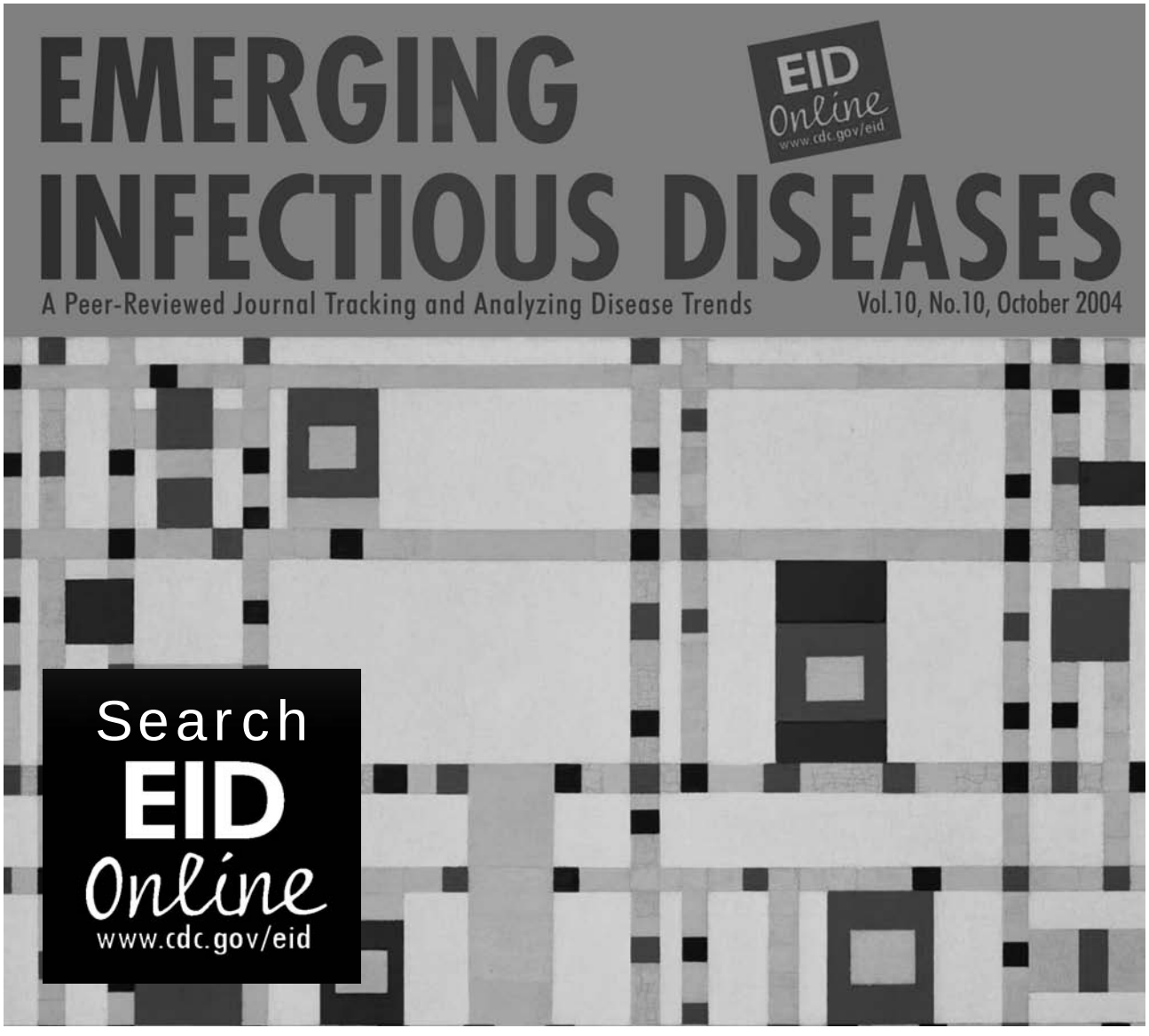

Optimizing Treatment of Antimicrobial-resistant *Neisseria gonorrhoeae*

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The increasing prevalence of ciprofloxacin-resistant *Neisseria gonorrhoeae* has required replacing inexpensive oral ciprofloxacin treatment with more expensive injectable ceftriaxone. Further, monitoring antimicrobial resistance requires culture testing, but nonculture gonorrhea tests are rapidly replacing culture. Since the strategies were similar in effectiveness (>99%), we evaluated, from the health-care system perspective, cost-minimizing strategies for both diagnosis (culture followed by antimicrobial susceptibility tests versus nonculture-based tests) and treatment (ciprofloxacin versus ceftriaxone) of gonorrhea in women. Our results indicate that switching from ciprofloxacin to ceftriaxone is cost-minimizing (i.e., optimal) when the prevalence of gonorrhea is >3% and prevalence of ciprofloxacin resistance is >5%. Similarly, culture-based testing and susceptibility surveillance are optimal when the prevalence of gonorrhea is <13%; nonculture-based testing is optimal (cost-minimizing) when gonorrhea prevalence is ≥13%.

Gonorrhea is the second most frequently reported sexually transmitted disease (STD) in the United States (1,2) infecting an estimated 800,000 people at a cost of $1 billion annually (3,4). In women, untreated gonorrhea can lead to pelvic inflammatory disease (PID) and to profound long-term sequelae such as chronic pelvic pain, ectopic pregnancy, and infertility (5). In addition, gonococcal infections have been shown to facilitate transmission of HIV (6). Gonorrhea is frequently asymptomatic in women, which creates a large pool of undetected infections. These infections go untreated, which may increase the probability of disease progression in the patient and transmission to sexual partners.

An important obstacle in the control of gonorrhea is the emergence of antimicrobial–resistant strains. As ciprofloxacin-resistant *Neisseria gonorrhoeae* strains become more prevalent in the United States (7–10), treatment with cephalosporins such as cefixime and ceftriaxone becomes necessary (10,11). Antimicrobial susceptibility testing requires that *N. gonorrhoeae* be grown in culture. However, the availability of affordable and accurate nonculture tests, along with the convenience of combination nonculture tests for *N. gonorrhoeae* and *Chlamydia trachomatis*, has resulted in the increased use of nonculture-based tests[1] (12). The resulting reduction in the use of culture-based testing poses challenges for monitoring antimicrobial resistance.

To the best of our knowledge, the economic consequences of diagnostic test and treatment selection in the face of rising antimicrobial resistance for *N. gonorrhoeae* have not been explored in the literature. Our objective was to identify the most cost-effective combination of diagnostic test (culture or nonculture) and treatment (ciprofloxacin or ceftriaxone) for gonorrhea when the incidence of ciprofloxacin-resistant *N. gonorrhoeae* infections is increasing.

Methods

The 4 Strategies

We evaluated and compared the cost and disease outcomes associated with 4 strategies, identified from current practice and consultations with experts, for diagnosing and treating gonorrhea in women (Table 1). The 2 treatments evaluated were a single, oral 500-mg dose of ciprofloxacin (for which gonococcal resistance exists in some parts of the United States), and a single, 125-mg dose of ceftriaxone by intramuscular injection (for which we assumed no resistance has been identified). Two of the strategies used

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[1]In 2000, only 18% of gonorrhea tests performed by public health laboratories in the United States were culture-based tests.

Table 1. Strategies modeled

Strategy (ST)	Brief description	Detailed description
ST1	Ciprofloxacin + culture tests + ciprofloxacin susceptibility tests	Prescribe ciprofloxacin to symptomatic patients and culture test all patients. Test 80% of all positive specimens for ciprofloxacin resistance. Recall and treat asymptomatic gonorrhea patients and patients with ciprofloxacin-resistant strains.
ST2*	Ciprofloxacin + nonculture tests	Prescribe ciprofloxacin to symptomatic patients and use nonculture tests on all patients. Recall and treat positive asymptomatic gonorrhea patients.
ST3	Ceftriaxone + culture tests + ceftriaxone susceptibility tests	Prescribe ceftriaxone to symptomatic patients and culture test all patients. Recall and treat asymptomatic gonorrhea patients. Test 20% of positive isolates for resistance to cephalosporin.
ST4*	Ceftriaxone + nonculture tests	Prescribe ceftriaxone to symptomatic patients and use nonculture tests on all patients. Recall and treat asymptomatic gonorrhea patients.

*Since ST2 and ST4 do not use culture-based testing, no antimicrobial susceptibility tests are assumed to be associated with these strategies.

culture-based tests followed by antimicrobial susceptibility testing, while the remaining 2 strategies used nonculture-based combination tests, such as nucleic acid amplification assay or the nucleic acid hybridization test, for detecting both *N. gonorrhoeae* and *C. trachomatis*. Since we know that resistance to ciprofloxacin already exists, we assume that 80% of culture-positive specimens would also be tested for antimicrobial susceptibility when ciprofloxacin was used for treatment (ST1). Ideally, 100% of culture-positive specimens would be tested for antimicrobial susceptibility, but we allow for some losses because of incomplete tracking, handling, and transportation. In the absence of recorded treatment failure caused by antimicrobial resistance to ceftriaxone, we assume that 20% of specimens would be susceptibility tested for surveillance purposes.

For all 4 strategies, we assume that women with symptoms of gonorrhea who go to a healthcare provider will be presumptively treated with the antimicrobial agent indicated by the strategy. In all strategies, all women undergo testing for gonorrhea with either culture- or nonculture-based tests. All strategies assume that attempts will be made to recall and treat asymptomatic women who test positive. Two strategies (ST1 and ST3, Table 1) further assume that those symptomatic women found to be infected by a resistant strain will be recalled and retreated. Successful treatment of the detected patients depends on the effectiveness of the antimicrobial therapy, given the particular susceptibility patterns of gonococcal strains in a geographic location.

Economic Evaluation Methods

To conduct an economic evaluation of the 4 strategies from a healthcare system perspective, we built a decision tree by using DATA 4.0 (TreeAge Software Inc., Williamstown, MA, USA). Online Appendix 1 contains schematics of the tree (http://www.cdc.gov/ncidod/EID/vol11no08/05-0157_app1.htm). The gonorrhea-related health outcomes included in the evaluation of each strategy were PID and PID-associated sequelae (chronic pelvic pain, ectopic pregnancy, and infertility). We also included the probability and associated costs of female-to-male transmission of gonorrhea (assuming that all couples are heterosexual). If the male partner is infected, either urethritis or epididymitis could develop and the infection could be transmitted to another female partner (or the original female partner could be reinfected after she has been cured of the initial infection). In the initial female-to-male transmission of gonorrhea, the additional probability and associated costs of cotransmission of HIV exist. Economic outcomes include all diagnostic test-related costs (i.e., cost of supplies, equipment, and labor), direct medical costs of treatment (for pelvic examination and patient recall), cost of treating PID and sequelae (i.e., inpatient and outpatient medical costs), and costs of transmission of gonorrhea and HIV to sex partners (assuming that all couples are heterosexual).

For each strategy (Table 1), we used the decision tree to calculate the expected cost per case of gonorrhea treated, the expected proportion of cases successfully treated (case-patients with no PID or sequelae), and expected cost per case-patient successfully treated. Average and incremental cost-effectiveness analyses, also conducted for a hypothetical cohort of 1 million women treated with each of the 4 alternative strategies, are contained in online Appendix 2 (http://www.cdc.gov/ncidod/EID/vol11no08/05-0157_app2.htm).

Data

Probabilities

The probabilities used were derived from a review of the published literature, expert opinion, and unpublished data from local, state, and national sources (Table 2). In the base case, the prevalence of gonococcal infection among women was assumed to be 0.5% (range 0%–15%). We assumed that of all gonorrhea-infected women who enter the clinic, 30% (range 20%–60%) display urogenital symptoms for gonorrhea, and 70% do not display such symptoms. We also assume among women who enter the clinic, but are uninfected with gonorrhea, that 20% (range 0%–40%) will be presumptively treated for gonorrhea due to nonspecific urogenital symptoms associated with both

Table 2. Input probabilities

Variable description	Probabilities (%)			Sources
	Base	Range	Distribution*	
Prevalence of gonorrhea in community among women	1.0	0–15	Triangular	2
Prevalence of ciprofloxacin-resistant *Neisseria gonorrhoeae*	0.1	0–20	Triangular	7
Prevalence of ceftriaxone-resistant *N. gonorrhoeae*	0			Assumed
Treatment failure when strain is resistant to antimicrobial agent	100			Assumed†
Treatment failure when strain is not resistant to antimicrobial agent	0			Assumed†
Infected with gonorrhea and symptomatic	30	20–50	Triangular	5,13,14
Infected with gonorrhea but without symptoms‡	70	Residual‡		Calculated
Not infected but with gonorrhea symptoms	20	10–40	Triangular	5,13,15
Not infected and without gonorrhea symptoms‡	80	Residual‡		Calculated
Recalled patient returning to clinic	40	20–80	Triangular	16,17
Sensitivity of nonculture-based tests	95	85–100	Triangular	14,18,19
Specificity of nonculture-based tests	97	95–99	Triangular	14,18,19
Sensitivity of culture-based tests	93	85–95	Triangular	14,18,19
Specificity of culture-based tests	97	95–97	Triangular	14,18,19
Concurrent HIV transmission§	0.066	0–0.5	Triangular	20
Develop pelvic inflammatory disease (PID) and sequelae, among untreated gonorrhea cases	16	10–40	Triangular	5,13,14,21
Development of PID only (no sequelae)¶	70	70–72	Uniform	15,16,21,22
Developing sequelae of PID¶				
Infertility	6	1–6	Uniform	15,16,21
Ectopic pregnancy	8	5–9	Uniform	15,16,21
Chronic pelvic pain	16	15–20	Uniform	15,16,21
Urethritis	50	35–65	Uniform	15,16,21
Epididymitis	2	1–5	Uniform	15,16,21
For strategy 1, % of culture-positive samples tested for antimicrobial susceptibility#	80			Assumed
For strategy 3, % of culture-positive samples tested for antimicrobial resistance#	20			Assumed
Female-to-male transmission of gonorrhea§	50	30–75	Uniform	5,13,23
Male-to-female transmission of gonorrhea§	50	30–75	Uniform	5,13,23

*The probability distributions used in the Monte Carlo sensitivity analysis. Uniform distributions were constructed with the minimum and maximum of the given ranges. Triangular distributions were constructed with the minimum and maximum of the given ranges and the base case as the "most likely" value.
†Assumes 0% drug failure if organism is not resistant.
‡The probability of being infected and without gonorrhea symptoms is the residual value after considering the probability of being infected and with gonorrhea symptoms. Likewise, the probability of being not infected and without gonorrhea symptoms is the residual value after considering the probability of not being infected and with gonorrhea symptoms.
§Describes probability of initially infected woman transmitting disease to male partner, who then has the probability of infecting another female partner (or reinfecting original female partner after she has been cured of initial infection). Further, with the initial female-to-male transmission, the probability of concurrent HIV transmission exists.
¶Rate of PID (only) and rates of PID-related sequelae are given as percentages of those that develop PID.
#See Table 1 for descriptions of strategy 1 (ST1) and strategy 3 (ST3). In ST1, culture-positive samples are tested for ciprofloxacin resistance. In ST3, culture-positive samples are tested for ceftriaxone resistance.

gonorrhea and other STDs (13–15). All women with untreated cases of gonorrhea have a 16% probability of developing PID (15,16).

Previous studies used an estimate that 80% of women notified of a positive test result returned for treatment (16,17). However, in the absence of additional supportive data, we assumed recall rates, for both asymptomatic patients and those infected with a resistant strain, to be 40%. To simplify the model, we further assumed that infection with a resistant strain would lead to complete treatment failure. In reality, antimicrobial resistance is often not absolute, and successful treatment may still occur when a patient is infected with a resistant strain. This assumption biases the results toward switching from ciprofloxacin to ceftriaxone (i.e., from ST1 or ST2 to ST3 or ST4, Table 1). The sensitivity and specificity of the several screening tests were obtained from the peer-reviewed medical literature (14,18,19).

Costs

Table 3 shows the cost estimates used in the model. The direct medical costs included were those associated with diagnostic testing, antimicrobial therapy for gonorrhea, and subsequent sequelae of untreated gonorrhea (15,21–23). Because the perspective of the analysis is that of the healthcare system, we did not include indirect costs, such as lost production, and intangible costs, such as pain and personal trauma.

Previous studies estimated the average clinician time associated with a full pelvic examination, including the

Table 3. Cost estimates

Items	Costs (2001 US$) Base	Range	Distribution*	Sources
Nonculture test for *Neisseria gonorrhoeae*	7	5–20	Triangular	Pers. comm.†
Culture test for *N. gonorrhoeae*	5			Pers. comm.†
Antimicrobial susceptibility tests	20	5–60	Uniform	Pers. comm.†
Weighted cost of symptomatic pelvic inflammatory disease (PID) and sequelae for untreated gonorrhea‡	3,250	3,000–3,500	Uniform	13,15,21,23
Weighted cost of asymptomatic PID and sequelae for untreated gonorrhea‡	2,250	2,000–2,500	Uniform	13,15,21,23
Outpatient case of epididymitis	229	152–277	Uniform	13,15,21,23
Inpatient case of epididymitis	3,604	2,997–4,802	Uniform	13,15,21,23
Clinic time: 5 min (routine checkup)	15	40–70	Uniform	17,24
Clinic time: 30 min (pelvic examination)	60	5–20	Uniform	17,24
Ciprofloxacin, 500 mg, oral	2	1–6	Uniform	25,26
Ceftriaxone, 125 mg, IM§	10	10–15	Uniform	25,26
Onward transmission of gonorrhea to female, per case of gonorrhea¶	60	0–300	Triangular	14,21,22,23
Onward transmission of HIV to male, per case of gonorrhea#	130	0–1,000	Triangular	20,27,28

*The probability distributions used in the Monte Carlo sensitivity analysis. Uniform distributions were constructed with the minimum and maximum of the given ranges. Triangular distributions were constructed with the minimum and maximum of the given ranges, and the base case as the "most likely" value.
†Costs of culture and nonculture diagnostic tests were obtained from Dean Willis and Karla Schmitt, Florida State Department of Health. Costs of susceptibility testing were provided by Norman O'Connor, State of Hawaii Department of Health Laboratories Division, Roman Golash, Illinois Department of Public Health, and Paul Hannah, Orange County Public Health Laboratory, California.
‡Weighted using the probabilities (Table 2) of occurrence of PID (only), infertility, ectopic pregnancy, and chronic pelvic pain. Dollar values of each health outcome taken from listed sources.
§IM, intramuscular injection.
¶Cost of gonorrhea transmitted to female after initial female-to-male transmission. Calculated as a weighted average cost, weighted using the probabilities of onward transmission and the probabilities of occurrence of PID (only), infertility, ectopic pregnancy and chronic pelvic pain in the female (see Table 2 for probabilities). Costs of each outcome were taken from the listed sources.
#Cost of HIV in male patient after initial female-to-male transmission. Calculated as a weighted average cost, weighted by using the probabilities of onward transmission (see Table 2 for probabilities). Cost of a case of HIV is a weighted average cost, weighted by probabilities of HIV-related health outcomes, taken from listed sources.

estimated follow-up cost of scheduling a return visit for a positive test result, by direct observation of activities in a clinic patient-flow analysis (17). The direct medical costs of the time associated with a PID-related clinic visit, either an initial visit or one including a full pelvic exam, were estimated by using the MarketScan database (24). The costs of the various diagnostic tests were obtained through personal communication with health department laboratories in Hawaii, Orange County (California), and Florida (Table 3). The cost of diagnostic tests included the cost of reagents, kits, equipment, supplies, and the laboratory technician's time. For nonculture tests, since *N. gonorrhoeae* testing is routinely performed as part of a dual *N. gonorrhoeae* and *C. trachomatis* test, the incremental cost of performing the *N. gonorrhoeae* test as part of the dual test was used in the base model. However, the considerably higher cost of performing a single *N. gonorrhoeae* nonculture test by itself was incorporated and examined in the sensitivity analysis. The range of costs for antimicrobial agents reflects the prices obtained from both the private sector and public clinics (25,26). We assume directly observed therapy resulting in full treatment compliance, and any residual noncompliance is implicitly assumed as treatment failure. We estimated the average cost of both symptomatic and asymptomatic PID by summing the costs associated with each outcome multiplied by the proportion of persons who will be affected (Table 3). The principal outcomes associated with untreated infection, if symptomatic, include inpatient and outpatient treatment cost of PID and subsequent long-term chronic pelvic pain, surgery, ectopic pregnancy, and infertility. The outcomes associated with asymptomatic or silent PID are long-term sequelae only. The model also incorporated the cost of transmission of both gonorrhea and HIV to the index patient's sexual partners (20,27,28). All cost data were adjusted to 2001 US dollars, by using the medical care component of the consumer price index (29).

Sensitivity Analysis

Univariate sensitivity analyses were conducted to examine the effect of changes in the prevalence of gonorrhea and the prevalence of ciprofloxacin-resistant *N. gonorrhoeae* on the cost per patient successfully treated. Multivariate sensitivity analyses were conducted to determine breakeven points (or threshold values) indicating input values at which any 2 strategies had the same cost per patient successfully treated. Threshold values were calculated to determine the robustness of the baseline results and the relative importance of the input variables on allowing for variation around the baseline.

To determine when a change occurs in the threshold, we simultaneously changed the values of key variables over a range of gonorrhea prevalence in women (0%–15%) and a range of prevalence of ciprofloxacin-resistant gonorrhea (0%–20%). We changed the cost ratio of ciprofloxacin to ceftriaxone from the base case of 1:5 (ciprofloxacin = $2/dose; ceftriaxone = $10/dose; Table 3) to both 1:2 and 1:7.5. Simultaneously, we changed the cost ratio of culture tests to nonculture tests from 1:1.4 (culture tests = $5, nonculture tests = $5, Table 3) to both 1:1 and 1:3. We then simultaneously altered the specificity and sensitivity of the 2 tests. Finally, we conducted a Monte Carlo simulation,[2] in which we simultaneously altered all the input variables by using predefined probability distributions to examine whether they had significant consequences on model results.

Results

Base Case Analysis

All 4 strategies ensured that PID did not develop in >99% of all treated patients, regardless of the assumed prevalence of gonorrhea (Table 4). This finding means that the costs per patient treated are almost the same as the cost per patient successfully treated (i.e., costs per patient with no PID or sequelae) and that relative costs are central in determining cost-effectiveness. However, the high cost-effectiveness ratios (CERs, which estimate the additional cost per additional case of PID averted on comparing a strategy with the baseline or the next-most-effective strategy in average and incremental cost-effectiveness analysis, respectively) generated in cases in which alternative strategies are similar in effectiveness do not offer an intuitive decision-making tool for choosing an optimal strategy. Instead, a cost-minimization approach, which selects as optimal a strategy that minimizes cost per case successfully prevented (i.e., least costly in achieving the same level of effectiveness), provides a more practical and intuitive decision-making tool. Detailed results from incremental cost-effectiveness analyses are contained in online Appendix 2 for those programs that choose to consider the additional CERs in making decision on budgetary allocations.

When the prevalence of gonorrhea is ≤5%, the 2 strategies based on culture and susceptibility testing (ST1 and ST3) are cheaper than the other 2 strategies (ST2 and ST4). For any strategy, increasing the prevalence of gonorrhea from 1% to 10% more than doubled the cost per patient treated. This doubling is primarily due to the increase in the proportion of patients who face additional costs for testing, treatment, or both.

Results from varying the prevalence of gonorrhea and the prevalence of ciprofloxacin resistance simultaneously are shown in Figure 1. If the prevalence of gonorrhea is <1%, ST1 has the lowest cost per patient successfully treated even if prevalence of ciprofloxacin resistance is as high as 20%. Even when prevalence of gonorrhea approaches

[2]Monte Carlo simulation involves specifying a probability distribution of values for model inputs. A computer algorithm then runs the model for several iterations. During each iteration, the computer algorithm selects input values from the probability distributions, and calculates the output (e.g., cost per patient successfully treated). After the final run, the model provides results such as the mean, median, and 5th and 95th percentiles for each specified output.

Table 4. Cost per case treated and percentage of treated cases without PID* on varying prevalence of gonorrhea and ciprofloxacin resistance (base-case values†)

		0.1%		2%		10%	
Prevalence (%) gonorrhea‡	Strategy§	$/case treated¶	% cases with no PID¶	$/case treated	% cases with no PID	$/case treated	% cases with no PID
1	ST1	26.00	99.92	26.03	99.92	26.17	99.92
	ST2	32.76	99.93	32.85	99.93	33.20	99.92
	ST3	26.21	99.92	26.21	99.92	26.21	99.92
	ST4	34.07	99.93	34.07	99.93	34.07	99.93
5	ST1	42.04	99.61	42.20	99.60	42.89	99.60
	ST2	45.70	99.65	46.11	99.64	47.87	99.61
	ST3	41.92	99.61	41.92	99.61	41.92	99.61
	ST4	47.12	99.65	47.12	99.65	47.12	99.65
10	ST1	62.09	99.21	62.41	99.21	63.79	99.18
	ST2	61.86	99.30	62.70	99.29	66.21	99.22
	ST3	61.55	99.21	61.55	99.21	61.55	99.21
	ST4	63.42	99.31	63.42	99.31	63.42	99.31

*PID, pelvic inflammatory disease, which can cause sequelae such as chronic pelvic pain, infertility, and ectopic pregnancy.
†Baseline values given in Tables 2 and 3.
‡When gonorrhea prevalence is 1% and prevalence of ciprofloxacin-resistant *Neisseria gonorrhoeae* is 0.1%, PID would not develop in 98.4% of patients treated. In the absence of any treatment, PID would not develop in 74% (range 60%–90%) of gonorrhea-infected women.
§Strategies modeled were ST1: ciprofloxacin + culture-based tests + ciprofloxacin-susceptibility tests; ST2: ciprofloxacin + nonculture-based tests; ST3: ceftriaxone + culture-based tests + ceftriaxone-susceptibility tests; ST4: ceftriaxone + nonculture-based tests. See Table 1 and text for further details.
¶Cost per patient treated and percentage of patients treated refer to all women who come to the public health clinic and undergo therapy as per 1 of the 4 strategies, regardless of actual infection.

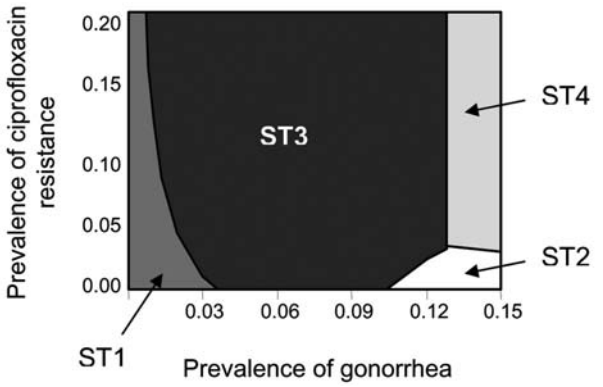

Figure 1. Lowest cost per patient successfully treated on varying prevalence of gonorrhea and prevalence of ciprofloxacin-resistant *Neisseria gonorrhoeae*. Notes: strategy depicted is optimal (lowest cost per patient successfully treated) for given combinations of prevalence of gonorrhea and prevalence of ciprofloxacin-resistant *N. gonorrhoeae*. Since the alternative strategies are similar in effectiveness, cost-effectiveness analysis does not offer a practical decision-making tool. Instead, cost minimization, which selects as optimal a strategy that costs least while achieving the same level of effectiveness (i.e., per case of successful treatment), serves as a more practical and intuitive tool kit for decision making. Case-patients refer to all women who attend a public health clinic and undergo therapy as per 1 of the 4 strategies, regardless of actual infection. The strategies modeled were ST1: ciprofloxacin + culture-based tests + ciprofloxacin-susceptibility tests; ST2: ciprofloxacin + nonculture-based tests; ST3: ceftriaxone + culture-based tests + ceftriaxone-susceptibility tests; ST4: ceftriaxone + nonculture-based tests (see Table 1 and text for further details). Values for input variables other than prevalence of gonorrhea and prevalence of ciprofloxacin-resistant *N. gonorrhoeae* are the base case values given in Tables 2 and 3.

3%, ST1 is the optimal strategy if prevalence of ciprofloxacin resistance is <4%. Strategy 3 (ceftriaxone + culture-based testing) is frequently the most optimal strategy when prevalence of gonorrhea is 3%–12%. With a few exceptions, the 2 strategies that use nonculture-based tests become most optimal only when the prevalence of gonorrhea is >13%. Finally, if ciprofloxacin-resistance levels are ≥3% and gonorrhea prevalence is >13%, a switch to ceftriaxone (ST4) is recommended. Overall, the base-case analysis indicates that culture-based strategies are optimal (lowest cost per patient successfully treated) at lower levels of gonorrhea prevalence, while nonculture-based strategies become optimal as gonorrhea prevalence increases.

Sensitivity Analysis

The model was found to be sensitive to changes in several estimates, including the relative cost of antimicrobial agents and diagnostic tests. For example, if the ratio of cost of ciprofloxacin to cost of ceftriaxone is changed from 1:5 (base case, Figure 1) to 1:2 (Figure 2A) and the costs of tests become equal (Figure 2A), the two strategies that include non-culture tests (ST2 and ST4) are optimal for greater combinations of gonorrhea prevalence and ciprofloxacin-resistance prevalence than in the base case. However, if the ratio of the cost of culture tests to non-culture tests is changed from 1:1 (Figure 2A) to 1:3 (Figure 2B), then the two strategies that include culture tests (ST1 and ST3) become optimal for all combinations of gonorrhea prevalence and ciprofloxacin-resistance prevalence.

Regardless of the relative difference in sensitivity and specificity of the 2 types of tests, strategies containing culture-based tests (ST1 or ST3) are optimal if prevalence of gonorrhea is <6% (Figure 3A). However, when both the sensitivity and specificity of the culture-based tests are set at the minimum values, and the nonculture-based tests are at maximum values, the optimal diagnostic choice

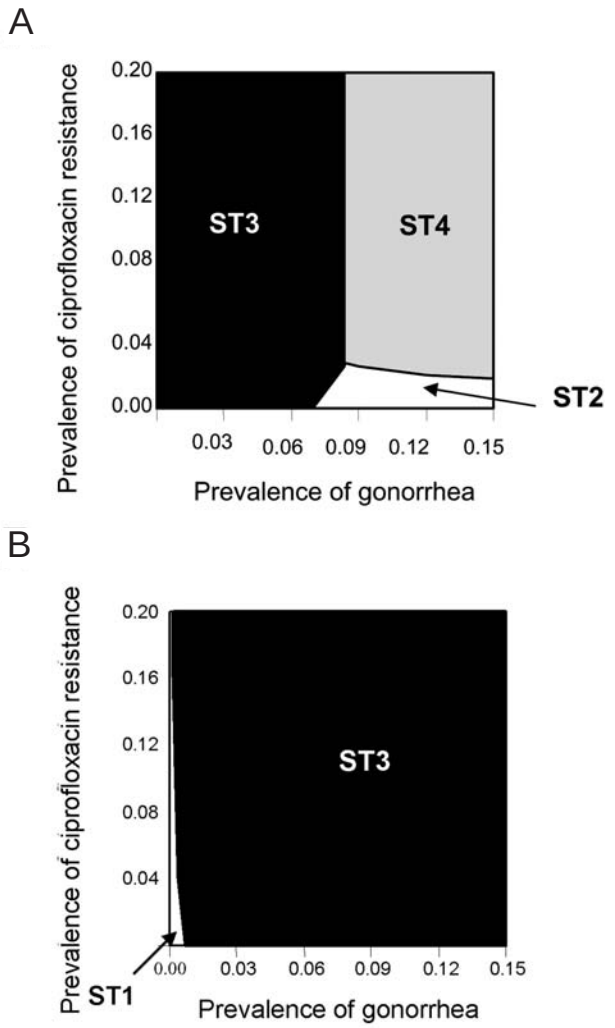

Figure 2. Lowest cost per patient successfully treated on varying relative costs of drugs and tests. A) Cost of culture = $5; cost of nonculture = $5; cost of ciprofloxacin= $5; cost of ceftriaxone = $10. B) Cost of culture = $5; cost of nonculture = $15; cost of ciprofloxacin = $2; cost of ceftriaxone=$15. For notes, see Figure 1 legend.

switches from culture-based (ST1 or ST3) to nonculture-based tests (ST2 or ST4), if prevalence of gonorrhea is ≥8% (Figures 3A). However, when the sensitivity and specificity of culture-based tests are set at their maximum value, and the nonculture-based tests are at their minimum value, the 2 strategies that contain culture-based tests are optimal for all combinations of gonorrhea prevalence and ciprofloxacin-resistance prevalence (Figure 3B).

From the Monte Carlo analysis, for 3 of the combinations of gonorrhea prevalence and ciprofloxacin-resistance prevalence, strategy 1 has the lowest mean cost per patient treated (Table 5). Only when gonorrhea prevalence is 2% and ciprofloxacin-resistance prevalence is 10% does strategy 3 have a lower mean cost per patient treated (Table 5). Note that, for any given combination of gonorrhea prevalence and ciprofloxacin-resistance prevalence, considerable overlap exists among the confidence intervals around the means of the cost per patient treated (Table 5).

Discussion

The absence of any recommended, evidence-based method that can be used to identify the most cost-effective gonorrhea treatment strategy has resulted in ad hoc decision making regarding when to change drug therapy because of antimicrobial resistance. For example, the threshold for changing drug therapy for gonorrhea treatment has often been when prevalence of gonococcal strains resistant to a given antimicrobial agent reaches 5% (30–32). The model indicates that using a single variable to define the breakpoint is inefficient. For example, if gonorrhea prevalence is <1%, our results show that ciprofloxacin would be most optimal even if ciprofloxacin resistance were as high as 20%. The decision regarding when to change from 1 drug to another on the basis of the prevalence of gonorrhea and the prevalence of ciprofloxacin resistance is summarized in a tool kit contained in online Appendix 3 (http://www.cdc.gov/ncidod/EID/vol11no08/ 05-0157_app3.htm).

While we cannot cover every situation and setting, our results clearly illustrate that a single, generic policy regarding when to switch drug treatments (for reasons of antimicrobial resistance) is not necessarily optimal from an economic perspective. However, the sensitivity analyses demonstrate that our model has wide applicability and can, by varying input data, provide answers across a wide range of settings. The current model can readily be adopted to produce a practical and interactive tool kit that would allow for variation across a wide range of input values.

Our analyses identified 2 other important points. First, since all the strategies were similar in terms of effectiveness (i.e., percentage of patients successfully treated), relative costs will be important in determining the most cost-effective strategy. Second, the large variability in key

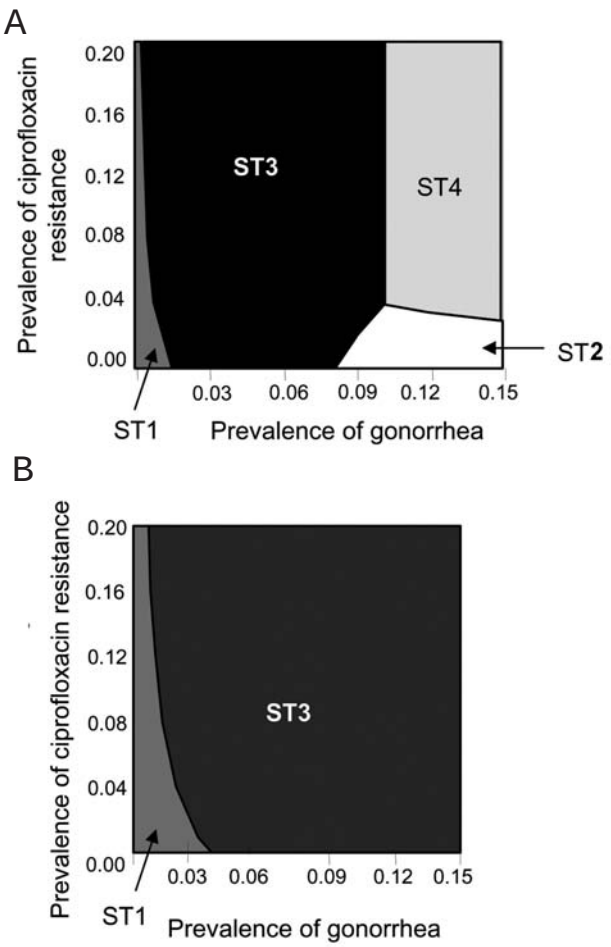

Figure 3. Lowest cost per patient successfully treated on varying sensitivity and specificity of culture- and nonculture-based tests. A) Culture: sensitivity = 75%, specificity = 95%; nonculture: sensitivity = 85%, specificity = 95%. For notes, see Figure 1 legend. B) Culture: sensitivity = 95%, specificity = 97%; nonculture: sensitivity = 85%, specificity = 95%.

variables (i.e., prevalence of gonorrhea, prevalence of ciprofloxacin-resistance, relative costs of drugs and diagnostic tests) across geographic locations and clinical settings makes it unlikely that the same single strategy will be the most cost-effective strategy across all these settings.

The sensitivity of the results to relative costs of diagnostic tests is of concern because the current practice of providing higher reimbursement rates (compared to actual cost) for nonculture serves as a subsidy for nonculture tests. We also did not value all the benefits associated with culture-based tests, specifically the additional knowledge obtained regarding antimicrobial susceptibility. In interpreting the model results, the inadequacies of not accounting for the full benefits of culture should be acknowledged. Likewise, nonculture-based testing, which does not necessarily require a pelvic examination, may confer both cost

Table 5. Monte Carlo simulation* results: mean cost per patient treated and percentage of patients without PID† (5th percentile, 95th percentile)

Prevalence (%) gonorrhea	Strategy‡	Prevalence of ciprofloxacin resistance = 0.1%		Prevalence of ciprofloxacin resistance = 2%	
		$/patient treated	% patients without PID	$/patient treated	% patients without PID
1	ST1	27.34 (21.45, 33.23)	99.92 (99.84, 99.96)	27.44 (21.72, 33.64)	99.92 (99.84, 99.96)
	ST2	39.78 (30.45, 50.19)	99.92 (99.83, 99.96)	39.78 (30.57, 50.95)	99.92 (99.84, 99.96)
	ST3	28.94 (22.92, 35.08)	99.92 (99.84, 99.96)	28.99 (23.25, 35.32)	99.92 (99.84, 99.96)
	ST4	42.00 (31.38, 53.08)	99.93 (99.84, 99.96)	41.99 (32.23, 53.83)	99.92 (99.84, 99.96)
10	ST1	68.73 (46.22, 99.01)	99.18 (98.41, 99.59)	71.65 (47.04, 102.58)	99.16 (98.38, 99.61)
	ST2	77.34 (53.53, 106.86)	99.23 (98.43, 99.63)	77.29 (54.83, 110.64)	99.19 (98.44, 99.61)
	ST3	70.37 (47.06, 98.72)	99.18 (98.41, 99.60)	70.33 (46.85, 101.75)	99.17 (98.39, 99.61)
	ST4	79.69 (55.78, 109.48)	99.23 (98.44, 99.80)	79.68 (55.90, 110.87)	99.21 (98.48, 99.63)

*Monte Carlo simulation involves specifying a probability distribution of values for model inputs (see Tables 2 and 3 for distributions used). A computer algorithm ran the model for 10,000 iterations. During each iteration, the computer algorithm selects input values from the probability distributions and calculates the output (e.g., cost per patient successfully treated). After the final run, the model provides results such as the mean, median, and 5th and 95th percentiles for each specified output.
†PID, pelvic inflammatory disease, which can cause sequelae such as chronic pelvic pain, infertility, and ectopic pregnancy.
‡The strategies modeled were ST1: ciprofloxacin + culture-based tests + ciprofloxacin-susceptibility tests; ST2: ciprofloxacin + nonculture-based tests; ST3: ceftriaxone + culture-based tests + ceftriaxone-susceptibility tests; ST4: ceftriaxone + nonculture-based tests. See Table 1 and text for further details.

advantages and higher patient acceptability (e.g., noninvasive methods for testing may be preferred by some women).

Further, in practice, selection of diagnostic test is often driven by priorities of testing for chlamydia, rather than gonorrhea testing alone. We did not consider all the costs and benefits associated with diagnosis and treatment of both *N. gonorrhoeae* and *C. trachomatis*. In addition, our results apply specifically to adult women and cannot be generalized for men.

With regard to antimicrobial drug selection, Monte Carlo simulations, which were based on assumed distributions and not actual data, show considerable overlap in costs and effectiveness across the 2 antimicrobial choices. Accordingly, caution should be exercised in recommending 1 drug over another, unless the results are backed with more certain and site-specific data on key variables for a given location.

A limitation that should prevent overemphasizing the sensitivity of the results to the relative cost of the drugs is the assumption of 100% treatment failure with ciprofloxacin resistance, which may overestimate the cost of ciprofloxacin resistance and incomplete patient recalls. Our model also assumes that resistance (or other treatment failures) to ceftriaxone is zero (as per the latest surveillance reports), although the model is designed to allow one to relax the assumption and vary the prevalence of ceftriaxone resistance. If one were to assume <100% treatment failure with ciprofloxacin or assume existence of some treatment failure to ceftriaxone including resistance, using ciprofloxacin (ST1 and ST2) would be most cost-effective for even larger ranges of gonorrhea prevalence and ciprofloxacin-resistance prevalence.

If a single strategy has a greater probability of contributing to resistance (because of inappropriate antimicrobial use), measuring the additional cost of increased resistance is beyond the scope of this model. A model limitation also arises from not including a valuation for reserving a class of antimicrobial agents for future use. Our model contains the implicit assumption that when ceftriaxone-resistant gonorrhea becomes problematic, an equally effective and affordable antimicrobial agent will be available to replace ceftriaxone. If the future costs of prematurely depriving physicians and patients of ceftriaxone were included, strategy 1 would become the dominant strategy in Figure 1. Any method used to recommend systemwide switching of therapies because of antimicrobial resistance should take into account that considerable value exists in keeping in reserve an already existing antimicrobial agent for as long as economically feasible.

The overall conclusion from our model is that decisions regarding changes in drug therapies used for gonorrhea treatment require several types of data. Both prevalence of gonorrhea and prevalence of ciprofloxacin-resistant gonococcal strains must be considered. Since prevalence data are dynamic and population-specific, ongoing collection of such data is necessary to allow informed decision making to take place.

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Sheep Feed and Scrapie, France

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Scrapie is a small ruminant, transmissible spongiform encephalopathy (TSE). Although in the past scrapie has not been considered a zoonosis, the emergence of bovine spongiform encephalopathy, transmissible to humans and experimentally to sheep, indicates that risk exists for small ruminant TSEs in humans. To identify the risk factors for introducing scrapie into sheep flocks, a case-control study was conducted in France from 1999 to 2000. Ninety-four case and 350 control flocks were matched by location and main breed. Three main hypotheses were tested: direct contact between flocks, indirect environmental contact, and foodborne risk. Statistical analysis was performed by using adjusted generalized linear models with the complementary log-log link function, considering flock size as an offset. A notable effect of using proprietary concentrates and milk replacers was observed. The risk was heterogeneous among feed factories. Contacts between flocks were not shown to be a risk factor.

Scrapie is a transmissible spongiform encephalopathy (TSE) affecting sheep and goats (1), as is Creutzfeldt-Jakob disease (CJD) in humans or bovine spongiform encephalopathy (BSE) in cattle. Moreover, scrapie is contagious in natural conditions (2). Though genetic determinism is a major feature of scrapie, the infectious agent is nonetheless needed for the disease to develop (3,4).

Known to exist for centuries, scrapie was thought to be a possible origin of BSE, although this hypothesis has not yet been verified. Sheep and goats can be experimentally infected with BSE, resulting in a disease that is impossible to distinguish from natural scrapie (5). Since BSE is implicated in the emergence of variant CJD (6,7), the existence of BSE in small ruminants poses a further risk for human health. Scrapie has become a public health challenge, and its propagation must be stopped; therefore, the risk factors for the introduction of scrapie in sheep must be understood.

In sheep infected with scrapie, the infectious agent is widely distributed in the organism. In particular, the gut-associated lymphoid tissues and the placenta are considered highly important in spreading the disease (8) and can contaminate the environment (9). Because feed is considered to be the main, if not the only, contamination source of BSE in cattle (10,11), it can also be presumed to be a potential risk factor for scrapie in sheep.

A case-control study of infected and scrapie-free flocks was conducted to identify risk factors for scrapie in sheep flocks in France. Various risk factors hypotheses were tested from the most plausible to the weakest.

Materials and Methods

Study Design

A case-control study of infected and scrapie–free flocks was designed (see online appendix for details; http://www.cdc.gov/ncidod/EID/vol11no08/04-1223_app.htm). A flock was defined as having at least 20 adult ewes. To consider the heterogeneity of exposure to scrapie risk, cases and controls were matched according to main sheep breed and location. A "case" was any flock having ≥1 animal that had been shown as scrapie-positive by the French surveillance network from January 1996 to July 2000 (12). Four frequency-matched control flocks were randomly selected from the sheep flocks in which scrapie had never been reported. Flocks that did not meet this criterion were excluded.

The suspected risk factors were grouped into 3 categories corresponding to the main working hypotheses of scrapie dissemination. The first category covered risks for transmission by direct contact between flocks and indirectly through the environment. The second category covered foodborne risks. The third category covered other environmental dissemination risks such as equipment sharing between farms or transmission through hay mites. Table 1 describes the 22 potential risk factors studied.

Data Collection

Information was collected by using a preestablished questionnaire to interview farmers and analyzing farm records. Questions related to potential risk factors covered

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the 4-year period preceding detection of the first clinical case of scrapie in case flocks and the 4-year period preceding the interview for controls. Additionally, information regarding potential confounding factors including flock size, production type (dairy, meat, or mixed), and intensification level of the flock production was recorded

Table 1. Univariate analysis of potential risk factors

Risk factors	Modalities	No. controls (%)	No. cases (%)	Univariate Clog-log model	
				OR*	80% CI
Direct contacts between flocks and indirect environmental contacts					
Purchase of ewes	No	227 (65)	55 (59)	1.0	–
	Yes	123 (35)	39 (41)	1.3	1.0–1.8
Purchase of rams	No	146 (42)	33 (35)	1.0	–
	Yes	204 (58)	61 (65)	1.0	0.8–1.4
Temporary direct contacts between flocks†	No	230 (66)	66 (70)	1.0	–
	Yes	120 (34)	28 (30)	0.7	0.5–1.1
Stay of animals in other flocks with direct contacts	No	319 (91)	87 (93)	1.0	–
	Yes	31 (9)	7 (7)	0.7	0.4–1.3
Stay of animals from other flocks with direct contacts	No	332 (95)	91 (97)	1.0	–
	Yes	18 (5)	3 (3)	0.8	0.4–1.9
Presence of small ruminants in the vicinity of the farm	No	71 (20)	14 (15)	1.0	–
	Yes	279 (80)	80 (85)	1.1	0.7–1.7
Sharing paths	No	149 (43)	39 (41)	1.0	–
	Yes	201 (57)	55 (59)	0.9	0.7–1.2
Other indirect environmental contacts‡	No	311 (89)	85 (90)	1.0	–
	Yes	39 (11)	9 (10)	0.7	0.4–1.2
Feeding					
Purchase of raw materials§	No	213 (61)	66 (70)	1.0	–
	Yes	137 (39)	28 (30)	0.6	0.4– 0.8
Purchase of milk replacers¶	No	287 (82)	66 (70)	1.0	–
	Yes	63 (18)	28 (30)	2.0	1.4–2.7
Purchase of proprietary concentrates¶	No	79 (23)	7 (7)	1.0	–
	Yes	271 (77)	87 (93)	2.2	1.2–3.8
Purchase of milk replacers from factory 1	No	329 (94)	76 (81)	1.0	–
	Yes	21 (6)	18 (19)	3.1	2.1–4.6
Purchase of milk replacers from other factories	No	317 (91)	87 (93)	1.0	–
	Yes	33 (9)	7 (7)	0.9	0.5–1.6
Purchase of proprietary concentrates from factory 1	No	271 (77)	48 (51)	1.0	–
	Yes	79 (23)	46 (49)	2.6	1.9–3.5
Purchase of proprietary concentrates from factory 2	No	292 (83)	85 (90)	1.0	–
	Yes	58 (17)	9 (10)	0.4	0.2–0.7
Purchase of proprietary concentrates from other factories	No	228 (65)	54 (57)	1.0	–
	Yes	122 (35)	40 (43)	1.2	0.8–1.7
Other indirect contacts					
Artificial insemination	No	247 (71)	57 (61)	1.0	–
	Yes	103 (29)	37 (39)	1.0	0.7–1.5
Cesarean section performed by veterinarian	No	163 (47)	28 (30)	1.0	–
	Yes	187 (53)	66 (70)	1.9	1.3–2.7
Ear-tagging	No	236 (67)	55 (59)	1.0	–
	Yes	114 (33)	39 (41)	1.1	0.8–1.6
Sharing of farming devices	No	85 (24)	22 (23)	1.0	–
	Yes	265 (76)	72 (77)	0.7	0.5–1.0
Presence of dogs on the farm	No	325 (93)	84 (89)	1.0	–
	Yes	25 (7)	10 (11)	1.1	0.7–1.7
Purchase of hay	No	247 (71)	70 (74)	1.0	–
	Yes	103 (29)	24 (26)	0.7	0.5–1.0

*OR, odds ratio; CI, confidence interval.
†Contacts by transhumance or common pastures with contacts between animals.
‡e.g., common pastures without direct contacts between animals.
§Purchase of hay excluded because considered in other indirect contacts.
¶Independently of the factories.

Table 2. Multivariate analysis of potential confounding factors

Factors	Modalities*	No. controls (%)	No. cases (%)	Log-linear model	
				OR†	95% CI
Flock size	<133*	100 (29)	11 (12)	1.0	–
	133–236	87 (25)	24 (25)	2.5	1.1 – 5.5
	237–366	77 (22)	33 (35)	4.0	1.8 – 8.6
	>366	86 (25)	26 (28)	3.0	1.3 – 7.0
Type of flock	Dairy*	229 (65)	64 (68)	1.0	–
	Meat	113 (32)	27 (29)	1.0	0.3 – 3.2
	Mixed	8 (2)	3 (3)	1.2	0.3 – 5.5
Intensification criteria	None*	241 (69)	56 (60)	1.0	–
	Production monitoring	38 (11)	16 (17)	1.8	0.8 – 3.8
	Involvement in a breeding scheme	71 (20)	22 (23)	1.1	0.6 – 2.1

*Reference modality.
†OR, odds ratio; CI, confidence interval.

(Table 2). Interviews were conducted from May 1999 to July 2000 with 453 flock owners (98 cases and 355 controls). Nine flocks were excluded because they did not meet the inclusion criteria. A total of 444 flocks (94 cases and 350 controls) were included in the study. Data were encoded and then stored in an Access database (Microsoft Access 97 SR-2, Microsoft Corporation, Redmond, WA, USA).

Study Sample

The flocks were mainly located in 2 departments (Pyrénées Atlantiques, n = 267/444, Aveyron n = 51/444). The others were widely distributed throughout metropolitan France. Ten mixed breeds and 23 pure breeds were included in the study. The flocks were mainly specialized in 1 type of production (66% in dairy production, 32% in meat production) (Table 2). The flock size ranged from 21 to 1,787 ewes (mean 274, SD 198).

Analysis

Data analysis was conducted in 2 steps by using statistical models adjusted for the 2 matching factors through the corresponding cross-variable "strata" (main breed and location) treated as a stratification variable (13). First, to identify the confounding factors to be further analyzed (14), a log-linear model considered 5 factors, including flock size (number of ewes), production type, intensification level of the flock production as potential confounding factors, flock status, and strata. The model introduced the main effect of these 5 factors with all second interaction terms. Flock size was the only potential confounding factor notably associated with the flock status (Table 2). Second, to assess associations between flock status and risk factors, a generalized linear model for binary outcome was set up with the complementary log-log link function (Clog-log model) (14) (see online Appendix, available at http://www.cdc.gov/ncidod/EID/vol11no08/04-1223_app.htm). This model considered the flock size by using the logarithm of the flock size as an offset (15,16). All exposures were considered as binary, and the absence of exposition was the reference modality for each risk factor. Factors notably associated with the flock status at 20% level through univariate analysis (Table 1) were selected for subsequent multivariate analyses. The univariate analysis consisted of the construction of a Clog-log model for each risk factor; strata were systematically introduced as covariate. Furthermore, 2 distinct multivariate models were applied to consider colinearity between feed type and feed factories in the foodborne risk study. The first model (multivariate Clog-log 1) analyzed feed types without regard to factories, whereas the second one (multivariate Clog-log 2) evaluated the risk according to the feed factories that produced milk replacers and proprietary concentrates. Regarding the proprietary feed factories, only the purchase of milk replacers and proprietary concentrates at factory 1 and the purchase of proprietary concentrates at factory 2 occurred frequently enough to be studied separately. Statistical software Splus (S-Plus 2000 Professional Release 2, Mathsoft, Inc., Seattle, WA, USA) was used to analyze the data.

Results

According to the univariate analysis, 8 potential risk factors were selected (Table 1). Six risk factors were related to foodborne risk; the other 2 were related to purchasing ewes, and cesarean sections performed by the veterinarian. The subsequent multivariate model (multivariate Clog-log 1) (Table 3) showed a significant association between the flock status and using milk replacers. In addition, using the multivariate Clog-log 2 model milk replacers and proprietary concentrates from factory 1 were significantly associated with the flock status (Table 3).

Discussion

The main finding of the study was the role of feed as a risk factor for scrapie. This is consistent with what has been shown for BSE in cattle. The use of proprietary concentrates, and more precisely the use of feed containing

Table 3. Multivariate analysis of risk factors

Risk factors	Modalities*	No. cases (%)	No. controls (%)	Multivariate Clog-log 1		Multivariate Clog-log 2	
				OR†	95% CI	OR	95% CI
Direct contacts between flocks and indirect environmental contacts							
Purchase of ewes	No	39 (41)	123 (35)	1	–	1	–
	Yes			1.3	0.9–2.0	1.3	0.8–2.0
Feeding							
Purchase of raw materials (hay excluded)	No	28 (30)	137 (39)	1	–	1	–
	Yes			0.6	0.4–1.0	0.7	0.4–1.0
Purchase of milk replacers	No	28 (30)	63 (18)	1	–		
	Yes			1.9	1.2–3.0	NI	
Purchase of proprietary concentrates	No	87 (93)	271 (77)	1	–		
	Yes			1.5	0.7–3.4	NI	
Purchase of milk replacers from factory 1	No	18 (19)	21 (6)			1	–
	Yes			NI		1.9	1.0–3.5
Purchase of proprietary concentrates from factory 1	No	46 (49)	79 (23)			1	–
	Yes			NI		2.0	1.2–3.3
Purchase of proprietary concentrates from factory 2	No	9 (10)	58 (17)			1	–
	Yes			NI		0.7	0.3–1.5
Other indirect contacts							
Cesarean section performed by veterinarian	No	66 (70)	187 (53)	1	–	1	–
	Yes			1.6	0.9–2.8	1.4	0.8–2.5

*Reference modality = No.
†OR, odds ratio; CI, confidence interval; NI, not in model.

meat and bone meal (MBM), was shown to have a major role in BSE infection of cattle (11). The agent of BSE is not inactivated by MBM processing methods, which were put into place by the industry in the late 1970s (17).

In France, MBM was authorized for small ruminants until July 1994. Moreover, the MBM ban proved to be <100% efficient; hundreds of BSE cases were observed in cattle in France born after the MBM ban of feed for cattle. The exposure period that was investigated in the current study was from 1991 to June 2000, depending on the case. It occurred before the French MBM ban in feed for all farmed animals in November 2000; furthermore, the period investigated was before the MBM ban for small ruminants in France for more than half of the cases. It is, therefore, plausible that sheep may have been contaminated by MBM in feed throughout the 1990s, despite control measures. The results showed that 1 feed company was at risk for proprietary concentrates when others were not. This finding is in agreement with the fact that risk might depend on the type of raw materials used in the factory, as well as the way they were processed and used.

The risk attributable to milk replacers is the first evidence of such a TSE risk in animals. Milk replacers for all farmed species are made of skimmed cow milk enriched with vegetable or animal fats. Milk has not been shown to be at risk for scrapie transmission (18–20). Even if animal fat is not infectious, the animal fats that were incorporated in milk replacers may have been contaminated. Contamination could have occurred during collection at the slaughterhouse by contact with infectious material such as central nervous system or paravertebral ganglia. In France, these fats were prohibited for use in farm animal feed in November 2000.

The same factory was identified as selling both the milk replacers and the proprietary concentrates at risk for scrapie. Most farmers buy both their feed concentrates and milk replacers from the same wholesaler (which, in turn, buys from the same factory). Even if the effect of the 2 factors remained in the multivariate analysis, a confounding effect between these 2 factors cannot be excluded.

The main concern raised by this study is the nature of the infectious agent that was transmitted to sheep by means of feed. It might be scrapie, but it could be also BSE, since cattle were infected by feed during the same period in France. In 2005, BSE in a goat was first reported in France (21); in the United Kingdom, a goat that was thought to have scrapie in 1990 is being reexamined because it is now suspected to have had BSE (http://www.defra.gov.uk/news/2005/050208a.htm). In France, every index case animal from infected small ruminant flocks that has been reported since the surveillance began in 1990 has been biochemically tested to distinguish natural scrapie isolates

from isolates sharing common biochemical features with experimental ovine BSE (validated by the TSEs Community Reference Laboratory of Weybridge, UK [unpub. data]). Among >400 small ruminant field isolates tested in France, only 1 isolate from a goat was indistinguishable from BSE. These arguments suggest that the agent transmitted to sheep by food was scrapie rather than BSE. Moreover, BSE is thought to have been transmitted and amplified by recycling contaminated carcasses into MBM on a regional basis (22). It follows that if the sheep identified as having scrapie did in fact have BSE, this misconception would have occurred in the same regions as BSE in cattle. That the areas of France most at risk for BSE in cattle (23) were different from those where scrapie occurred during the study does not suggest that the infectious agent for sheep was BSE.

Unexpectedly, the other hypotheses concerning the contamination of flocks with scrapie were not confirmed by the present study. In Norway, a matched case-control study showed 3 risk factors, though at a 10% α level: purchasing females, sharing rams, and sharing pastures between flocks (24). However, in a recent Irish study, purchasing breeding sheep through markets was not a risk factor for scrapie at a 5% α level (25). In the Norwegian study, feed did not appear to be a risk factor, whereas in the Irish study, feeding proprietary concentrates to lambs appeared to be protective. In the present study, purchasing ewes may not have emerged as a risk factor merely because of the lack of power of the study. The link between cesarean sections and scrapie occurrence that was observed in the univariate analysis was likely due to a confounding effect with the real risk factors and so became nonsignificant in the multivariate analyses.

Beyond the limits of the study, our results clearly show that in France, and more precisely in southwest France where most of the studied farms were located, the major risk for the introduction of scrapie in a flock during the 1990s was feeding certain proprietary concentrates and, possibly, milk replacers to sheep. Exposing sheep to TSE risk by feeding has certainly decreased since that time because of the complementary control measures taken in 1996 (ban on specified risk materials and cadavers in the processing of MBM) and 2000 (complete ban of MBM and certain animal fats for all farmed animals). However, it is essential to monitor these risk factors over time in France and to extend this kind of study to other countries in which the disease occurs.

The study results show strong evidence that TSEs can spread to sheep through feeding in field conditions, as is the case for cattle. Given the potential risk for humans, the possibility of BSE spreading to sheep must be taken seriously, even though the horizontal transmission of BSE in sheep would occur and stay at a low level (26), should such contamination occur (27). In any case, such findings support the need for a more comprehensive surveillance of TSEs in sheep, as well as the need to systematically examine all scrapie cases for their resemblance to BSE.

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Cost-Benefit of Stockpiling Drugs for Influenza Pandemic

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We analyzed strategies for the use of stockpiled antiviral drugs in the context of a future influenza pandemic and estimated cost-benefit ratios. Current stockpiling of oseltamivir appears to be cost-saving to the economy under several treatment strategies, including therapeutic treatment of patients and postexposure prophylactic treatment of patients' close contacts.

The widespread epidemic of highly pathogenic avian influenza that emerged in east Asia continues today. As the epidemic grows, so does the probability that this virulent virus will acquire genetic traits for increased person-to-person transmissibility, potentially setting the stage for the next global influenza pandemic (1).

The next pandemic will be associated with major adverse health and economic outcomes, with estimated costs reaching US$166 billion in the United States alone (2). The World Health Organization recently encouraged health authorities to consider stockpiling antiviral drugs in anticipation of a pandemic (3). However, the cost-benefit of stockpiling has yet to be assessed, and the optimal strategy for antiviral use is still under debate. The Israeli Ministry of Health appointed a working group to address national preparation for an influenza pandemic. We set out to identify strategies for the use of the antiviral drug oseltamivir in the containment of a pandemic and to construct a mathematical model to appraise the cost and benefit of each strategy in terms of health-related and economic outcomes.

The Study

We estimated the health-related impact of pandemic influenza on the Israeli population, by using rates (illness, physician visits, hospitalizations, and deaths) derived from previous pandemics, according to Meltzer et al. (2). Costs related to these outcomes were calculated from data provided by a major Israeli healthcare organization (4) and by the Israeli Central Bureau of Statistics (5). We calculated direct costs to the healthcare system and overall costs to the economy, the latter including the value of lost workdays but not the potential value of lost lives. Point estimates of variables used in the base-case model are detailed in Table 1 and online Appendix 1 (available from http://www.cdc.gov/ncidod/EID/vol11no08/04-1156_app1.htm).

According to base-case assumptions, a pandemic would result in an estimated 1,618,200 patients (≈25% of the Israeli population), 781,921 physician visits, 10,334 hospitalizations, 2,855 deaths, and 6,536,240 lost workdays. These outcomes would result in an excess of $55.4 million in health-related costs and in overall costs to the economy of $523.5 million (≈0.5% of the Israeli gross domestic product).

We defined 3 strategies for the use of antiviral drugs during a pandemic: therapeutic use, long-term preexposure prophylaxis, and short-term postexposure prophylaxis for close contacts of influenza patients (with index patients under treatment). The first 2 strategies could target either the entire population or only those at high risk for complications. The efficacy of therapeutic treatment was based on currently available evidence regarding epidemic influenza (online Appendix 1). Systematic review and meta-analysis were used to estimate the efficacy of preexposure prophylaxis, while the expected efficacy of postexposure prophylaxis and the number of persons treated under this strategy were estimated by using the results of a recently published stochastic simulation model (6).

The impact of each strategy on health-related outcomes was analyzed in a spreadsheet model by using the formulas summarized in online Appendix 2 (available from http://www.cdc.gov/ncidod/EID/vol11no08/04-1156_ app2.htm). Briefly, the economic benefit of each strategy was calculated by multiplying each of the reductions in adverse outcomes by its estimated economic value. The cost of each strategy was calculated by multiplying the estimated number of treated persons by the discounted cost of a single antiviral course. Oseltamivir was selected as the drug of choice, at a daily dosage of 75 mg for prophylaxis and 150 mg for treatment (7). Oseltamivir stockpiling costs were calculated with prices quoted in March 2004 by the manufacturer's representative in Israel for uncapsulated, water-soluble, bulk active powder with a 10-year shelf life.

We compared the economic outcomes of each of the 5 strategies with nonintervention, estimated stockpiling costs, and calculated cost-benefit ratios. Based on the historic incidence of 3 influenza pandemics over the last century, we adjusted all cost-benefit outcomes for a conservatively estimated probability of 3 pandemics every 100 years and applied a wide range of estimates for sensitivity analyses (online Appendix 1). Table 2 details the cost-benefit ratios of the competing strategies. Therapeutic treatment and postexposure prophylaxis were shown to be cost-saving, with a cost-benefit ratio of 2.44–3.68.

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Table 1. Point estimate and range of values for selected model variables*

Variable	Point estimate	Range
Overall attack rate, %†	25	15–35
Probability of pandemic (per year)	3	1–10
Adult workdays lost, by age, y		
<1–≤18	3.7	2–5
19–64	4.9	3–7
≥65	0.5	0.25–2
Average hospital stay (days) by age, y		
<1–≤18	4.0	2–5
19–64	5.8	2–7
≥65	7.0	4-9
Patients seeking medical care within 48 h, %	80	70–90
Efficacy of antiviral prophylaxis, %		
Preexposure prophylaxis (50 days)	71	57–85
Postexposure prophylaxis (7 days)	36	25–47
Efficacy of antiviral therapy, %		
Reduction in hospitalizations	59	30–70
Reduction in antimicrobial drug use	63	40–80
Reduction in lost workdays under treatment	1	0.5–1.5

*Complete list and references available in online Appendix 1.
†Population attack rate was calculated by stratifying the population by age and risk and applying age- and risk-specific attack rates and ranges (online Appendix 1).

Since many characteristics of the next pandemic viral strain remain unknown, our modeling methods and parameter estimates were designed to consistently underestimate intervention-related benefits, thus yielding minimum estimates of the true cost-benefit ratios. In a series of multivariate sensitivity analyses that used the variable ranges detailed in Table 1, the model proved to be robust. Even under the most unfavorable estimates, prepandemic stockpiling remained cost-saving as long as the estimated probability of a pandemic remained >1 every 80 years. No consistent advantage to either therapeutic or short-term prophylactic use of antiviral drugs could be determined.

Conclusions

In light of recent episodes of human infection with avian influenza, the World Health Organization reiterated its 1997 call for all countries to prepare for the next "inevitable, and possibly imminent" pandemic (3). Strain-specific vaccine, the most effective tool for influenza control, will most likely not be available in the early stages of a pandemic because of its prolonged development time (3), and early control measures will have to employ alternative options, mainly the judicious use of antiviral drugs. These drugs are likely to be in short supply if not preemptively stockpiled (3). Compared with neuraminidase inhibitors, the M2-inhibitor drugs have several major disadvantages, mainly high rates of viral resistance (as shown in recent H5N1 and H9N2 isolates) and adverse effects (8).

Our model suggests that prepandemic stockpiling of oseltamivir is cost-saving to the economy over a wide range of treatment strategies. Stockpiling is also directly cost-saving to the healthcare system, if oseltamivir use is limited to treating patients at high risk. Investment in stockpiling remains cost-saving to the economy as long as the estimated annual pandemic risk remains >1 pandemic every 80 years. In the last 400 years, at least 31 pandemics have been recorded (8), so that regardless of recent events in Southeast Asia, present investments in antiviral agents can be expected to yield a substantial economic return of >$3.68 per $1 invested, while saving many lives.

This favorable cost-benefit ratio can be achieved if stockpiled antiviral drugs are administered either solely as a therapeutic measure or as short-term prophylaxis for exposed contacts, a strategy termed "ring prophylaxis" (9) or "targeted prophylaxis" (6). Only 1 study published to date (6) used dynamic mathematical modeling to examine the expected effectiveness of this latter control measure on the population level; that study suggested that this strategy may significantly reduce illness and death. This epidemiologically directed short-term prophylaxis of close contacts may require antiviral stockpiles considerably larger than necessary for therapeutically treating patients, but our model suggests that this investment may still prove cost-saving, providing that the outbreak dissemination patterns and population attributes correlate with those assumed by Longini et al. (online Appendix 1).

When one considers a ring prophylaxis strategy, the risk of "strategy failure" due to early antiviral stockpile depletion must be considered. If postexposure prophylaxis does not confer sufficient immunity upon exposed contacts who underwent prophylaxis, and if vaccines or additional

Table 2. Cost-benefit ratios of antiviral utilization strategies*

Strategy		Cost-benefit ratio, relative to nonintervention	
		All costs to economy	Direct healthcare costs
NI	No intervention (base case)	Ref.	Ref.
1a	Therapeutic use (all patients)	2.44	0.30
1b	Therapeutic use (limited to patients at high risk)	3.68	1.51
2a	Preexposure long-term prophylaxis of entire population	0.38	0.04
2b	Preexposure long-term prophylaxis, limited to high-risk population	0.37	0.10
3a	Postexposure short-term prophylaxis for all close contacts ("ring prophylaxis"), including treatment of index patients	2.49	0.27

*Ref., reference value of zero divided by zero.

antiviral agents do not become available, rapid consumption of available stocks may leave the population vulnerable to additional outbreak waves, potentially caused by influx of new cases. The probabilities of similar "failure" scenarios are difficult to assess and were not included in our analysis. Application of this strategy for the entire population without surplus antiviral reserves should therefore be considered cautiously and monitored closely.

This study aimed to elicit minimum cost-benefit estimates for investment in a national antiviral stockpile. Among our conservative assumptions, we chose to exclude indirect costs of preventable deaths, which, if added, would have increased cost-benefit ratios up to 6-fold (online Appendix 1). Furthermore, in view of recent events in east Asia, the probability of a pandemic has probably risen to >3 per 100 years, and new strains may prove more pathogenic than previous pandemic strains. In modeling the benefits of therapeutic strategies, we omitted the beneficial effects of decreased viral shedding afforded by neuraminidase inhibitors (7), such as a lower secondary attack rates among untreated contacts. We also ignored the possibility that a fully implemented prophylactic strategy might achieve full containment of the outbreak (probability estimated at ≈6% for 7-day postexposure ring prophylaxis (6), dependent on several factors such as compliance, delay in treatment initiation, and basic reproductive number (6,10). Finally, as witnessed during the epidemic of severe acute respiratory syndrome (SARS), the economic consequences of a rapidly disseminating disease extend well beyond direct costs to the healthcare system and lost workdays. Canada had losses >$1 billion during the SARS epidemic, although the disease directly affected <500 patients (11). From an economic viewpoint, mitigating a pandemic could prevent extensive indirect economic losses.

The conclusions of this study must be considered carefully during the planning of antiviral stockpiling. Drug prices can be expected to change substantially as a result of contractual negotiations with manufacturers (although our results indicate stockpiling may remain cost-saving even if drug costs are more than tripled, as would be the case if preprepared capsules are purchased). Powder-form antiviral drugs have considerable advantages in terms of cost and shelf life, but the logistical aspects of their preparation and distribution should be further assessed to confirm feasibility. Finally, we assumed that strain-specific vaccine would not be available in sufficient quantities during the first stages of the pandemic. Efforts are currently being directed towards shortening this delay. Once available, strain-specific vaccines would likely be the favored intervention, with antiviral agents serving as adjunct treatment.

In summary, prepandemic stockpiling of antiviral drugs can be expected to prove cost-saving. Cost-beneficial strategies for their use may involve treatment of patients, and, if backed by adequate antiviral stockpiles, short-term postexposure prophylaxis of close contacts. These strategies should be considered when planning stockpiling efforts.

Several countries have already begun active stockpiling efforts (12), sufficient in some cases to allow antiviral treatment of up to 25% of the population (13). We believe that antiviral stockpiling should be considered a prudent investment that may help mitigate this impending global threat.

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Staphylococcus lugdunensis Pacemaker-related Infection

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We report the first known case of a device-related bloodstream infection involving *Staphylococcus lugdunensis* small-colony variants. Recurrent pacemaker-related bloodstream infection within a period of 10 months illustrates the poor clinical and microbiologic response even to prolonged antimicrobial drug therapy in a patient infected with this staphylococcal subpopulation.

During the past decade, *Staphylococcus lugdunensis* has emerged as an important pathogen implicated in both community-acquired and nosocomial infections (1,2). Clinical manifestations of infections with these organisms include abscesses (3), meningitis (4), ventriculoperitoneal shunt infection (5), spondylodiscitis (6), prosthetic joint infection (7), catheter-related bacteremia (2), and endocarditis (1). Infections with *S. lugdunensis* tend to have a more fulminant course, with an outcome resembling that of *S. aureus* infections rather than that caused by coagulase-negative staphylococci (8). In addition, these organisms are frequently misidentified as *S. aureus* because of their morphologic appearance with yellow pigmentation and complete hemolysis when cultured on blood agar.

Small-colony variants (SCVs) are mainly reported in *S. aureus*, and interest in infections with SCVs has recently increased after an association between recovery of *S. aureus* SCVs and persistent and relapsing infection has become evident (9). SCVs are a slow-growing subpopulation of the species with characteristics that can associated by a common factor, i.e., alterations in electron transport (10). The generation time for SCVs is up to 9-fold longer than for metabolically normal strains, which results in tiny colonies that are frequently not visible until after 48 to 72 hours of incubation. Consequently, correct identification and susceptibility testing for clinical laboratories are complicated, which may result in diagnostic underestimation and therapeutic failures. While most studies have dealt with SCVs of *S. aureus*, little is known about infections with SCVs of coagulase-negative staphylococci. Recently, 2 cases of bloodstream infections caused by SCVs of *S. epidermidis* and *S. capitis*, respectively, were reported (11). Both infections were related to foreign bodies and observed after pacemaker implantation.

We report the first known case of a device-related bloodstream infection due to *S. lugdunensis* SCVs and other colony variants of this species. Of particular interest, this infection was also observed after pacemaker implantation.

The Case

In July 2003, a 61-year-old man was transferred from a local hospital to our cardiothoracic surgery department with a diagnosis of pacemaker lead infection. Past medical history included nephrectomy in 1996 for cancer of the left kidney and implantation of a universal demand pacemaker (dual chamber pacemaker) for treatment of sick sinus syndrome in 1990. In August 2002, after being in place for 12 years, the pacemaker battery was replaced. Three months later, the patient was admitted to a local hospital with a temperature of 40°C and chills. Laboratory findings included a leukocyte count of 17,500/μL and a C-reactive protein (CRP) level of 90 mg/L. A transesophageal echocardiogram showed thickening of the left coronary aortic valve, and thrombotic material was seen on the ventricular pacemaker lead. A blood culture drawn on admission showed *S. lugdunensis* susceptible by agar diffusion to penicillin, oxacillin, erythromycin, clindamycin, rifampin, and aminoglycosides. Antimicrobial drug therapy was instituted with intravenous ampicillin/sulbactam and gentamicin for 14 days with prompt resolution of clinical symptoms, and follow-up blood cultures remained negative. Three days later, however, a spiking fever and chills developed in the patient. Antimicrobial drug treatment was changed to intravenous vancomycin and rifampin. The patient's condition improved rapidly, and he was discharged after 3 weeks of antimicrobial drug therapy when the CRP value had returned to normal.

Two months later in February 2003, the patient was readmitted to the cardiology department with the presumptive diagnosis of endocarditis. During a transient febrile episode, a blood culture was obtained that again yielded *S. lugdunensis* (Figure 1A). Antimicrobial drug therapy was resumed with intravenous flucloxacillin and gentamicin. All 4 follow-up blood cultures obtained 3 and 4 days later, when the patient was afebrile, were again positive for *S. lugdunensis*. An echocardiogram did not show vegetations or other evidence of endocarditis. Pacemaker removal was strongly suggested, but the patient refused. After 14 days of intravenous treatment, the antimicrobial drug regimen was changed to oral administration of flucloxacillin for 14 days. After a full recovery, the patient was discharged, but removal of the pacemaker system was recommended if clinical symptoms reappeared.

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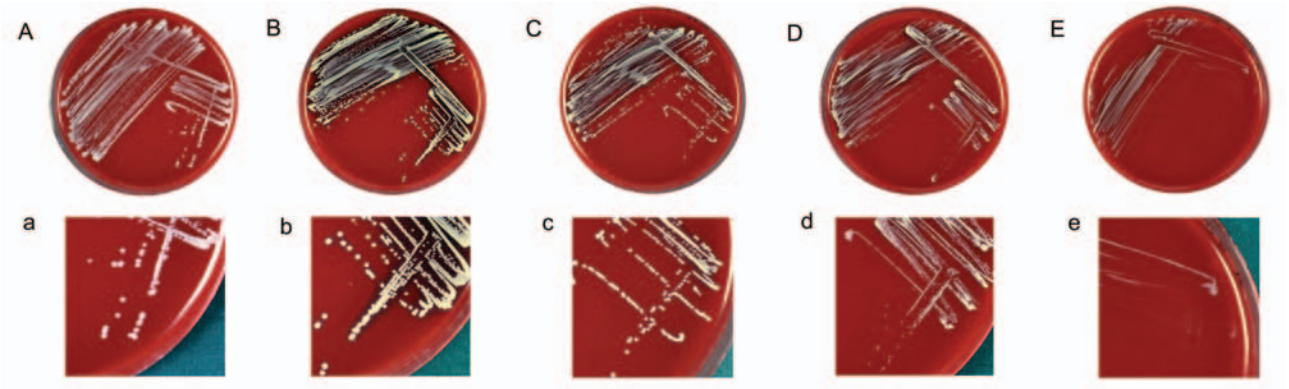

Figure 1. Sheep blood agar plates (A–E) and magnified sectors (a–e) after overnight incubation at 37°C showing different morphotypes of clonal isolates of the *Staphylococcus lugdunensis* strain recovered from blood cultures and the infected pocket of a patient with pacemaker infection. Plates A–D/a–d show *S. lugdunensis* colonies exhibiting the normal phenotype characterized by colonies of different diameter, ranging from 0.8 to 2.5 mm with creamy (A/a) or yellow (B–D/b–d) pigmentation and moderately heavy (B/b), weak (C–D/c–d), or absent (A/a) hemolysis; plate E/e shows the small-colony variant phenotype characterized by tiny (pinpoint), nonpigmented, and nonhemolytic colonies.

Four months later in July 2003, the patient came to the local hospital with recurrent high fever and chills, a leukocyte count of 12,200/μL, and a CRP value of 37 mg/L, but he did not show any peripheral sign of endocarditis. Four sets of blood cultures drawn on admission showed *S. lugdunensis*. A transesophageal echocardiogram showed large vegetations in the right atrium inserting at the ventricular lead but no involvement of cardiac valves. The patient responded promptly to the initiation of antimicrobial drug therapy with intravenous flucloxacillin and gentamicin and became afebrile. He was then transferred to our cardiothoracic surgery department for pacemaker ablation.

Four days later, the complete pacemaker system, including the intracardiac leads, was removed by open heart surgery. The cardiac valves did not show signs of infective endocarditis, but large vegetations adhered to both the atrial and the ventricular lead. Follow-up blood cultures remained negative but thrombotic material scraped from the pacemaker leads was analyzed by culture. After 2 days of incubation, this material yielded nonhemolytic and nonpigmented, as well as yellow-pigmented, hemolytic colonies of variable size, which were gram-positive catalase-positive cocci, consistent with staphylococci. The results of subcultures on solid media suggested a mixed population of staphylococci, with at least 4 different colony morphologies (Figure 1B–E). Four single-colony subcultures of different colony morphotypes also produced colony variations that persisted in serial subcultures of single colonies.

Clumping factor was not present and tube coagulase test results were negative. Identification was initially attempted with the gram-positive identification card provided with the VITEK 2 system (bioMérieux, Marcy l' Etoile, France). The large hemolytic morphotype (Figure 1B) showed a profile consistent with *S. lugdunensis*, with positive results for ornithine decarboxylase, trehalose, and L-pyrrolidonyl-β-naphthylamide. Other morphotypes were repeatedly identified as *S. haemolyticus* (Figure 1C; T index 0.93) and *S. auricularis* (Figure 1D and E; T index 0.46), respectively. The *S. lugdunensis* isolate that grew as tiny (pinpoint), nonpigmented, and nonhemolytic colonies was shown to be a hemin-auxotrophic SCV (Figure 1E). The *S. lugdunensis* isolate (large colony morphotype) was susceptible to all antimicrobial agents in the VITEK GPS-P526 card test (bioMérieux) and did not produce β-lactamase. The other morphotypes did not grow sufficiently to allow antimicrobial susceptibility testing with the VITEK system. However, susceptibility to penicillin and oxacillin was confirmed by an Etest (AB Biodisk, Solna, Sweden) for all colony variants.

The API ID 32 Staph system (bioMérieux) identified all morphotypes as *S. lugdunensis*, which was later confirmed by 16S ribosomal RNA gene sequencing using the RIDOM entries (12). All isolates, including an additional *S. lugdunensis* blood isolate obtained in February 2003 that produced flat, white, and nonhemolytic colonies (Figure 1A), were compared by pulsed-field gel electrophoresis and found to be identical, although the colony morphology was different (Figure 2).

Postoperative recovery was uneventful. Treatment with intravenous flucloxacillin and gentamicin was continued for 14 days. A 72-hour electrocardiogram did not show any need for pacemaker reinsertion. Fourteen days after surgery, the patient was discharged from the hospital, after a total clinical course of 10 months with recurrent infections.

Conclusions

Previous reports have rarely emphasized colony variation as an important feature of *S. lugdunensis*. In the initial description of the species in 1988 (13), colony variation

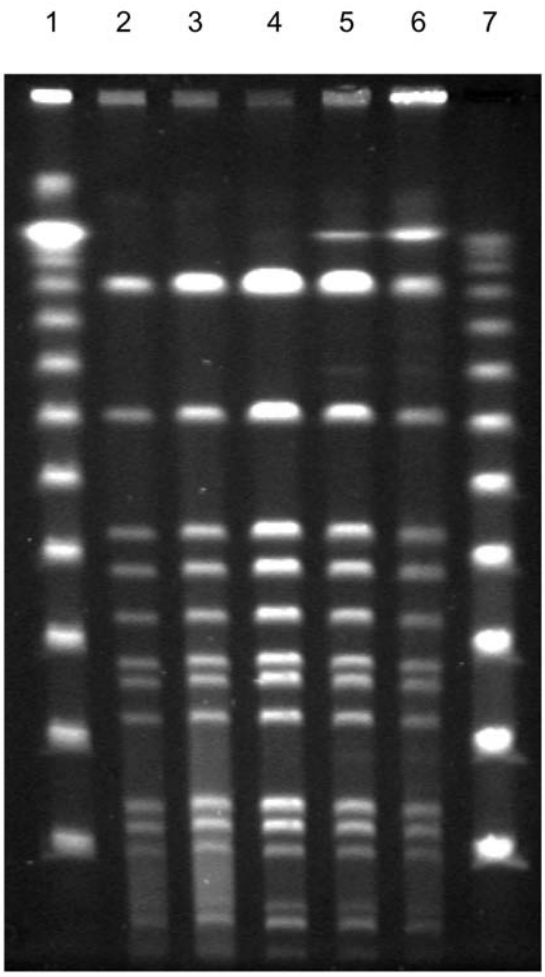

Figure 2. Fingerprint patterns for different *Staphylococcus lugdunensis* colony morphotypes, including small-colony variants (SCVs), after pulsed-field gel electrophoresis after digestion with *Sma*I, showing identical isolates. Lanes 1 and 7, 100-bp ladder; lane 2, blood isolate; lanes 3-5, colony variants; lane 6, SCVs of *S. lugdunensis* obtained from thrombotic material.

was observed in 3 of 11 strains. More recently, Leung et al. reported colony variation of *S. lugdunensis* in a fatal case of endocarditis (14). Unlike other staphylococcal species such as *S. capitis* and *S. hominis*, which show colony variation that disappeared after extended incubation, mixed morphotypes of *S. lugdunensis* were persistently detectable through incubation and subculture (14). The authors speculated that preceding antimicrobial drug therapy may play a role in producing colony variation in *S. lugdunensis* and that previous studies may have underreported the characteristic of colony variation seen in this species.

Some of the aberrant morphotypes described in earlier studies may have in fact been SCVs. Both prior exposure to antimicrobial drugs and the presence of chronic or recurring infections, often with indwelling foreign devices that have been associated with SCVs of *S. aureus*, *S. epidermidis*, and *S. capitis* (1,15), are features commonly observed in infections with *S. lugdunensis* (2,4,5,7,14). In our case, repeated courses of gentamicin therapy may have selected for SCVs. Although the infection showed a rather benign clinical course and did not confirm other reports of *S. lugdunensis* endocarditis in which the infection was more aggressive, it illustrates the chronic, recurrent, and persistent nature of infections with SCVs and the problems associated with delayed identification of *S. lugdunensis* colony variants and interpretation of its clinical significance.

The refusal of the patient to have the pacemaker removed added to the chronic course of the infection. Although these variants were not identified until removal of the device, the clinical importance of SCVs for this persistent infection can be anticipated. Clinical isolates are often a mixed population of parent strains and SCVs. Because of their different generation times, even a small percentage of normally growing organisms may rapidly replace SCVs in liquid medium such as a blood culture during overnight incubation. Thus, SCVs may have gone undetected in previously obtained blood cultures. Increased awareness of colony variation and the possible occurrence of SCVs as a characteristic feature of *S. lugdunensis* should be helpful in earlier recognition of the pathogen and appropriate management of the infection.

Dr. Seifert is professor of clinical microbiology at the Institute for Medical Microbiology, Immunology and Hygiene at the University of Cologne, Germany. His research interests include the molecular epidemiology of nosocomial pathogens, in particular, *Acinetobacter* and *Staphylococcus* species, catheter-related infections, and antimicrobial drug resistance and its mechanisms.

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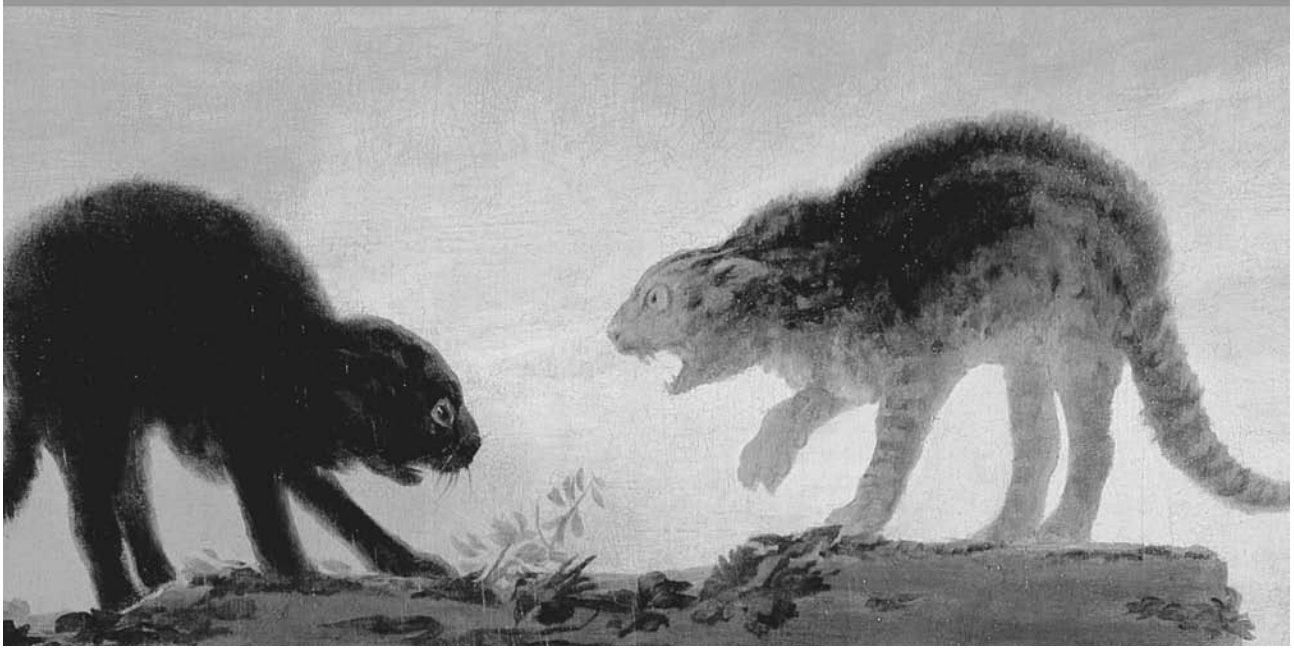

Bartonella quintana in Domestic Cat

Vu Dang La,* Lam Tran-Hung,* Gérard Aboudharam,* Didier Raoult,* and Michel Drancourt*

We recovered *Bartonella quintana* DNA from dental pulp of a domestic cat. This study, the first to detect *B. quintana* in a nonhuman mammal, changes our understanding of the epidemiology of this infection and proposes that cats may be an emerging source of human infection.

The α-proteobacterium *Bartonella quintana* is a fastidious, gram-negative organism; humans are the only known reservoir, and the human body louse, *Pediculus humanus corporis*, is the only known vector (1). Body lice infestation is linked to poor hygiene in homeless persons and persons engaged in war, as has been reported in several circumstances since trench fever was first described during World War I. *B. quintana* causes trench fever, chronic bacteremia, and endocarditis in homeless and alcoholic patients (2) and bacillary angiomatosis in both HIV-infected and immunocompetent patients (3). Rare cases of chronic lymph node infection caused by *B. quintana* were also reported (4,5). These patients were initially diagnosed with cat-scratch disease; they lived in conditions with high hygienic standards and had no evidence of infestation by body lice; they did have close contacts with cats and flea-infested kittens, however. Similarly, the source of *B. quintana* remains unknown in a few patients with *B. quintana* bacillary angiomatosis and endocarditis. Another investigation found a 4.5% prevalence of *B. quintana* in cat fleas collected in France (6). What is missing from these puzzling cases of *B. quintana* infection, however, is documentation of *B. quintana* in a cat. In this study, by using dental pulp of domestic cats to detect *Bartonella* spp. by polymerase chain reaction (PCR) that targets fragments of the *pap31* gene, the 16S–23S internal transcribed spacer (ITS) (6,7), and 2 other genomic regions (8), we identified *B. quintana* in a cat.

The Study

Nine domestic cats collected in Marseille were euthanized for medical indications unrelated to infectious diseases. We collected 32 cuspid teeth from these cats (Table 1), although only 1 tooth from each cat was tested for *Bartonella* DNA. Dental pulp was extracted by using an original protocol involving external decontamination by 70% ethanol and setting the entire decontaminated tooth in sterile resin (Resin Polyester Sody 33, ESCIL, Chassieu, France). After polymerization at room temperature, the apex was removed from the tooth by using a sterilized disk, and the opened canal system was inserted upside down into a sterile Eppendorf tube and centrifuged at 8,000 rpm for 10 min to recover the dental pulp. Total DNA was then extracted according to standard phenol-chloroform protocol. A negative control (sterile water) was processed in parallel exactly as described above.

PCR amplifications were performed in a 25-μL reaction mixture containing 5 pmol of each primer (Eurogentec, Seraing, Belgium), 200 μmol/L each dNTP (Invitrogen, Cergy-Pontoise, France) in 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 μg bovine serum albumin (Roche, Mannheim, Germany), 1 U Taq DNA polymerase (EuroblueTaq, Eurobio, Les Ulis, France), and 2 μL DNA. Primers PAPn1/PAPn2 targeting *pap31* were previously described (7). Primers URBarto1/URBarto2 amplified a 639-bp/722-bp ITS fragment of *B. henselae* and *B. quintana*, respectively. This fragment has 67.7% sequence similarity between *B. henselae* and *B. quintana* (6). We also amplified 2 intergenic fragments, no. 336 (597 bp) and no. 894 (383 bp), which are specific for *B. quintana* and have been incorporated into multispacer typing of *B. quintana* (8). PCR included an initial 3-min step of denaturation at 94°C followed by 41 cycles of 30 s denaturation at 94°C, 30 s primer annealing at 58°C for *pap31* primers (50°C for ITS primers), and 90 s elongation at 72°C. Amplification was completed by holding the reaction mixture at 72°C for 7 min. PCR products separated by 1.5% agarose gel electrophoresis were visualized by ethidium bromide staining, purified by using MultiScreen-PCR Filter Plate (Millipore, Saint-Quentin en Yvelines, France), and sequenced in both directions by using the d-Rhodamine Terminator Cycle Sequencing Ready Reaction

Table 1. Results of cat tooth investigation for *Bartonella* spp.*

Cat	*pap31*	ITS	336	894	Sequencing results
1	+	+	NT	NT	*B. henselae* (1 mutation for *pap31*) and 100% similarity for ITS
2	+	+	+	+	*B. quintana* 100% similarity for 4 genomic regions
3	–	–	NT	NT	
4	–	–	NT	NT	
5	–	–	NT	NT	
6	–	–	NT	NT	
7	–	–	NT	NT	
8	–	–	NT	NT	
9	–	–	NT	NT	

*ITS, internal transcribed spacer; NT, not tested.

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kit (PerkinElmer, Coignières, France). Sequencing products were resolved in an Applied Biosystem automatic sequencer model 3100 (PerkinElmer).

No amplification was observed for the negative controls in any PCR experiment. We obtained *pap31* amplicons with DNA extracted from the teeth of cat 1 and cat 2. A 222-bp sequence derived from the tooth of cat 2 shared complete identity with that of *B. quintana pap31* (GenBank accession no. AF308171), and a 237-bp sequence derived from the tooth of cat 1 shared 99% similarity with that of *B. henselae* ZF-1 (Houston genotype) *pap31* (GenBank accession no. AF321116). One mutation, resulting in a glycine → aspartic acid shift at codon 137, differentiated query and reference sequences (GenBank accession no. AY839861) (Figure 1). ITS amplicons obtained from the same teeth (Figure 2) shared a complete sequence identity with *B. henselae* ITS (GenBank accession no. AF312496) in cat 1 and *B. quintana* ITS (GenBank accession no. AF368395) in cat 2 (Table 1). Sequences of fragment 336 in cat 2 shared 100% similarity with *B. quintana* reference sequence (GenBank accession no. AY660705) with 3 best BLAST scores ≥1,142 (E-value 0) and 82% similarity with *B. henselae* strain Houston-1 with BLAST score of 92 (E-value $2e^{-15}$). Sequence of fragment 894 from the same tooth shared 100% similarity with *B. quintana* reference sequence (GenBank accession no. AY660713) with 5 best BLAST scores ≥385 (E-value ≤e^{-104}).

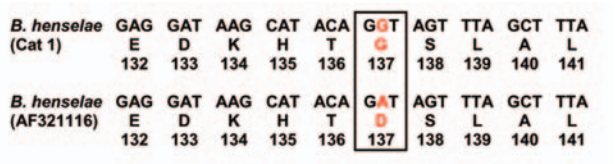

Figure 1. Comparison of *Bartonella henselae pap31* sequences between cat 1 and reference showing 1 mutation.

Conclusions

We found *B. quintana* and *B. henselae* DNA in the dental pulp of 2 domestic cats in France. To prevent contamination, we recovered pulp after the entire tooth was set in sterile resin. No amplification was obtained from controls, and no positive control was used. Amplicons were consistently obtained during separate PCR experiments targeting 4 different regions of the *Bartonella* genome. A unique mutation in the *pap31* sequence derived from a specimen definitely ruled out contamination by modern laboratory *Bartonella* DNA. We previously detected *B. henselae* DNA in dental pulp from 13th- to 16th-century domestic cats (9) and from cats buried for 1 year (10). This study is, however, the first detection of *B. henselae* ZF-1, Houston genotype outside of cat-scratch disease lymph nodes (7).

B. quintana identity was confirmed by amplification of 2 genomic fragments not subject to genomic transfer and by high BLAST scores with 4 different molecular targets. Until now, *B. quintana* has been detected only in humans (2,3,5) and human body lice (1). We unexpectedly recovered *B. quintana* DNA from a cat's dental pulp, which gives a prevalence of 2.5% among 39 cats tested in 3 studies, including this one (9,10). *B. henselae* was found in 23% of cats, and *B. clarridgeiae* was the most prevalent species in cat fleas. These observations agree with a 4.5% prevalence of *B. quintana* recently observed in cat fleas in France (6) (Table 2), whereas it was not detected in biting flies from California (11). We suspected as early as 1994 that cats may play a role in *B. quintana* infection (4). We described 2 patients with either *B. quintana* chronic peripheral (4) or mediastinal adenomegaly (5) who lived in good hygienic conditions and had no evidence of body lice infestation but did have close contact with cats. Ongoing PCR and sequence-based survey of lymph nodes in patients suspected of cat-scratch disease in Marseille found 11.2% *B. henselae* and 1 additional case of *B. quintana* (Table 2). A few additional patients have been reported (12). Likewise, 1 of 14 patients with *B. quintana* bacillary angiomatosis did not have risk factors, including low income, homelessness, and exposure to lice, but did have contact with cats (3,13). The same observation holds true for 3 of 38 patients with *B. quintana* endocarditis who did not have risk factors, including homelessness, alcoholism, and exposure to body lice, but did have contact with cats or cat fleas. These data led us to hypothesize that a *B. quintana* bacteremic domestic cat could be a rare source for *B. quintana* human infection. If confirmed, these data may lead to a recommendation that immunocompromised patients and patients at risk for endocarditis avoid contact with cats.

Present data reinforce the idea that dental pulp is a suitable specimen on which to base PCR detection of bloodborne bacteria. In addition to our work on feline bartonellosis, we detected *B. quintana* in the dental pulp of a homeless patient with previous bacteremia (14) and in a

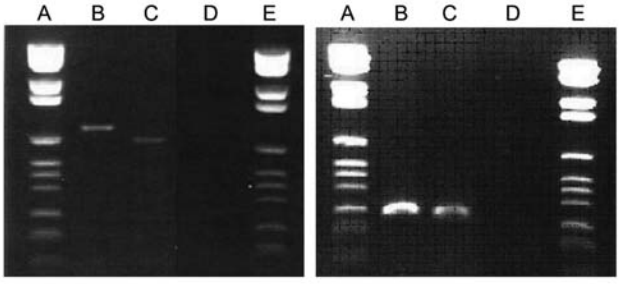

Figure 2. Agarose gel stained with ethidium bromide showing the amplicons intergenic spacer (left panel) and *pap31* (right panel) in cuspid teeth from 2 cats. Lanes A and E, DNA size ladder; lane B, cat 1; lane C, cat 2; lane D, negative control.

Table 2. Prevalence of *Bartonella* spp. in cats, fleas, and gland tissue from humans with suspected cat-scratch disease in Marseille

Bartonella sp.	Cats, n/N (%)*	Fleas, n/N (%)†	Gland tissue, n/N (%)‡
B. henselae	9/39 (23)	9/309 (2.9)	36/321 (11.2)
B. clarridgeiae	0/39 (0)	55/309 (17.8)	0/321 (0)
B. quintana	1/39 (2.5)	14/309 (4.5)	1/321 (0.3)

*Reference 9 and 10.
†Reference 6.
‡D. Raoult, unpub. data.

4,000-year-old cadaver (15). One may speculate on a common ancestor of *B. henselae* and *B. quintana* in cats, with *B. quintana* evolution toward a more specific niche. Further use of cat dental pulp to detect and genotype *B. quintana* may confirm these data and refine cat-based epidemiology and diagnosis of poorly understood clinical forms of *B. quintana* human infection.

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Dr. La is a PhD student at Université de la Méditerranée—Unité des Rickettsies. He studies bacterial colonization and use of dental pulp to detect bloodborne pathogens.

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Malaria and Irrigated Crops, Accra, Ghana

Eveline Klinkenberg,*† P.J. McCall,* Ian M. Hastings,* Michael D. Wilson,‡ Felix P. Amerasinghe,§ and Martin J. Donnelly*

We investigated the prevalence of malaria and associated risk factors in children living in urban Ghana. Malaria prevalence was associated with low hemoglobin concentration, low socioeconomic status, and higher age. Our findings indicate that African urban poor are seriously affected by malaria and that irrigated agriculture may increase this risk.

Malaria is predominantly a rural disease in Africa. Previous studies have shown that *Anopheles* mosquito breeding decreases with increasing proximity to the center of urban areas (1,2). Although the complex factors that contribute to malaria risk are not fully understood (2), availability of vector breeding sites is clearly essential. Urban agriculture, promoted as a means of increasing food security, improving nutrition, and alleviating poverty (3), can, especially when irrigated, create breeding habitats that could increase malaria transmission in cities. This potential risk was indicated by other authors (3–6), but only a limited number of studies have attempted to quantify the impact of urban agriculture on malaria transmission (4,7,8), and virtually all used only entomologic parameters (e.g., the entomologic inoculation rate, an estimate of the number of infected bites received per person per unit of time) in their analyses. Such measures are only proxies of actual malaria risk, and no studies have assessed the malaria parasite prevalence, a direct indicator of the impact of malaria, in communities with and without urban agriculture. By 2025, an estimated 700 million people will live in urban communities in Africa, which is approximately double the current urban population (9). With such rapid expansion, identification of the risk factors for urban malaria requires urgent attention (10).

The Study

From October 2002 to January 2003, we investigated malaria parasite prevalence in central Accra, Ghana, in communities bordering irrigated urban agriculture areas and in control communities (defined as sites located >1 km from an urban agricultural area, based on the likely appetitive flight distance of female mosquitoes) (11) (Figure 1). Communities around the main agricultural sites in Accra were selected and based on them representative control communities in terms of socioeconomic status, housing and crowding were selected. Different types of urban agriculture exist: basic backyard farming in or around the house, cultivation of stable crops such as maize on (temporary) fallow land, and cultivation of ornamental plants, mostly along roadsides. An important part of agriculture in the city is commercial cultivation of vegetables, such as lettuce, onion, and cabbage (Figure 2). These crops are irrigated from wells or streams with watering cans, and crops are sometimes cultivated on raised beds with water-filled furrows. Irrigated farming has the greatest potential to create additional breeding sites, and irrigated, open-spaced vegetable farming has been linked to higher anopheline densities in Kumasi, Ghana (4). The study focused on this

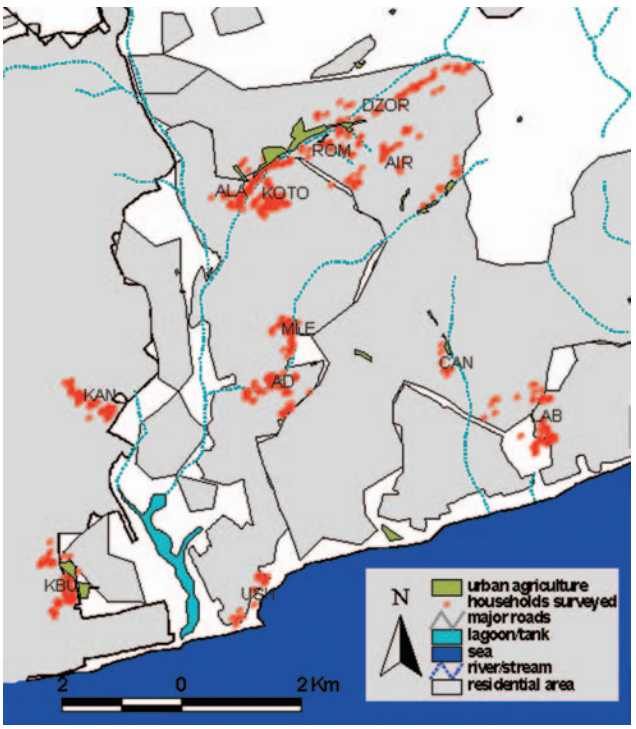

Figure 1. Location of urban agricultural (UA) sites and households surveyed within Accra, Ghana. Communities surveyed are shown with full name, UA or control (C), number of children sampled, and malaria prevalence. AIR, (Airport, UA, n = 77, 19.5%); ALA, (Alajo UA, n = 166, 15.1%); DZOR, (Dzorwulu UA, n = 132, 19.7%); KBU, (Korle Bu, UA, n = 181, 8.8%); KOTO, (Kotobabi, UA, n = 219, 18.3%); ROM, (Roman Ridge, UA, n = 105, 22.9%); CANT, (Cantonments, UA, n = 23, 13.0%); MLE, (Kokomlemle, C*, n = 160, 20.6%); AD, (Asylum Down, C*, n = 160, 11.3%); KAN, (Kaneshie, C, n = 159, 19.5%); LAB, (Labonie/LA, C, n = 175, 9.7%); USH, (Ushertown, C, n = 200, 6.5%). Communities marked C* were originally identified as control communities but small UA sites were later identified close to them.

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Figure 2. Commercial irrigated vegetable production in urban Accra, Ghana. Courtesy of Dr. Guy Barnish, Liverpool School of Tropical Medicine.

type of urban agriculture, which refers to irrigated, open-spaced, commercial vegetable production.

In the selected communities, we conducted a cross-sectional house-to-house survey to assess malaria parasitemia and hemoglobin (Hb) concentration in children 6 to 60 months of age. A team consisting of technicians and trained enumerators went house to house to collect data. Houses were selected arbitrarily and queried regarding the presence of children <5 years of age. For each community, the whole area was covered to account for spatial heterogeneity. If most compounds or houses had children <5 years of age, houses were omitted to obtain the target sample size of 150 children from the community.

Informed consent was obtained from each child's caregiver. Thick and thin blood films were collected and read according to standard World Health Organization protocols. Hb levels were assessed by using a blood hemoglobin photometer (HemoCue, Angelholm, Sweden). Children with parasitemias or Hb levels <8.0 g/dL were provided free treatment at a local clinic. The epidemiologic data were related to proximity to sites of urban agriculture, socioeconomic status based on household assets following a World Bank template (12), and possible confounding factors obtained by questionnaire from the child's caregiver. The location of each house, study site boundaries, landmarks, and urban agricultural areas were mapped by using a hand-held global positioning system. For each household in the urban agricultural communities, the shortest distance to the nearest agricultural site was calculated by using Arcinfo (ESRI, Redlands, CA, USA). Ethical approval was granted by the Liverpool School of Tropical Medicine and the University of Ghana, Legon.

A total of 1,757 children from 938 households in 12 different communities were enrolled in the study. Table 1 shows the baseline characteristics of the children with *Plasmodium*-positive and -negative slides, and Table 2 shows the characteristics for the urban agricultural and control communities. Of 261 infections detected, 258 were *P. falciparum*, 2 were *P. malariae*, and 1 was a mixed infection with *P. falciparum* and *P. malariae*. The average Hb level was 10.82 g/dL (SD 1.47), and 78 (4.5%) of 1,738 children had moderate-to-severe anemia (Hb <8.0 g/dL). Overall malaria parasite prevalence was 14.9% (261/1,757, range 6%–22%) and was higher in communities around urban agricultural sites than in control communities (16.5% and 11.4%, respectively, odds ratio [OR] 1.53, 95% confidence interval [CI] 1.10–2.14, p = 0.008). In a univariate analysis (Pearson chi square for binominal variables and *t* test for continuous variables), Hb concentration (negative association, p<0.001); moderate-to-severe anemia (OR 3.49, 95% CI 1.98–6.11, p<0.001); having netting in front of windows, doors, or both (OR 0.65, 95% CI 0.46–0.92, p = 0.012); socioeconomic status (negative association, p<0.001); and age (positive association peaking at ≈3 years of age, p = 0.002) showed significant associations with presence of malaria parasites. Reported bed net use by a household was 33% (range 6%–53% in different communities) but was not significantly associated with presence of malaria parasites in the blood.

A generalized linear mixed model (GLMM) approach, using an SAS macro (Glimmix 800, SAS Inc., Cary, NC, USA) that allowed a logistic link function, was used to investigate the association between putative predictor variables and malaria parasite prevalence. Covariates with p<0.1 in the univariate analysis were entered in the multivariate model. Household was nested within community, and both variables were treated as random effects. Age was divided into the following groups: 6–12, 13–24, 25–36, 37–48, and 49–60 months. Hb was entered as a continuous variable. Malaria parasitemia was significantly associated with Hb (negative association, p<0.001), age group (positive association, p<0001), and socioeconomic status (negative association, p = 0.0035). The effect of urban agriculture was marginally below significance (p = 0.0647), possibly because of reduced statistical power. Having netting in front of windows or doors was no longer significant (p = 0.3638), presumably because presence of nets was associated with a higher socioeconomic status (p<0.001).

Table 1. Summary of variables measured for children with and without malaria parasites, with results of univariate (Pearson chi-square or *t*) tests*

Variables	*Plasmodium*-positive blood slide (n = 261)	*Plasmodium*-negative blood slide (n = 1,496)	p value
Mean Hb, g/dL (SD)	10.17 (1.62)	10.94 (1.42)	<0.001
Hb <8 g/dL, %	11.3 (29/257)	3.3 (49/1,481)	<0.001
Mean age, months (SD)	36.44 (16.03)	32.92 (17.19)	<0.001
Mean socioeconomic score† (SD)	1.42 (0.99)	1.74 (0.98)	<0·001
Male (%)	123 (47.1)	739 (49.4)	0.498
Travel to village‡ (%)	17 (6.5)	93 (6.2)	0.855
Taken malaria medication in last 2 wk§ (%)	63 (24.1)	344 (23.0)	0.686
History of fever§ (%)	64 (24.5)	293 (19.6)	0.067
HH with reporting bed net use (%)	89 (34.1)	499 (33.4)	0.814
HH who spray weekly¶ (%)	71 (27.2)	435 (29.1)	0.537
HH with netting at windows/doors (%)	208 (79.7)	1,282 (85.8)	0.012
HH without ceiling (%)	77 (29.8)	382 (25.6)	0.147

*Hb, hemoglobin; HH, household.
†Composite measure of socioeconomic status used was the asset factor score of the World Bank for Ghana (www.worldbank.com/hnp).
‡Persons who had traveled to a rural (potentially malarious) area in the previous 3 weeks.
§In the last 48 hours, as reported by the caregiver.
¶Proprietary brands of insecticide aerosols.

In urban agricultural communities, GLMM analysis with parasitemia as the outcome was conducted with age group, distance to an urban agricultural site, socioeconomic status, and house effects. The Hb level was omitted because it was likely to be the result of malaria infection and its inclusion could obscure the effect of distance. Two of the districts, Mle (p = 0.021) and Kbu (p = 0.014), showed decreases in prevalence with distance from an urban agricultural site; the odds of infection were reduced ≈50% every 100 m from the site. However, these results need to be interpreted with caution because it is difficult to detect a putative decrease in prevalence with distance against the noise introduced by small, unidentified, often transitory, breeding sites. Their presence may explain why 2 sites, Rom (p = 0.043) and Dzor (p = 0.039), showed a significant increase in prevalence with distance, while 2 others, Air and Koto, showed a significant effect when distance in 100-m intervals was cross-tabulated with prevalence (p<0.001, Fisher exact test). Since unidentified breeding sites may also introduce unknown data structuring that cannot be incorporated into a GLMM, the probabilities obtained may be lower than are appropriate.

Conclusions

The parasitemia levels obtained in this study are worrisome because high-density urban African populations are not often considered particularly vulnerable to malaria infection. In other West African urban areas, malaria prevalence rates from 2% to 16% have been reported with large variation between communities (5,13). Recently, several authors focused attention on urban malaria (2,12) and stressed the need to investigate risk factors for urban malaria. In our study, the parasitemic children were more likely to be anemic, have a lower socioeconomic status,

Table 2. Summary statistics for variables measured in children in communities near urban agricultural sites and control communities, with results of univariate (Pearson chi-square or *t*) tests*

Variables	Children in urban agricultural communities (n = 1,223)†	Children in control communities (n = 534)	p value
Children with *Plasmodium*-positive slide, %	16.4 (200/1,223)	11.4 (61/534)	0.008
Mean Hb, g/dL (SD)	10.93 (1.46)	10.59 (1.46)	<0.001
Hb <8 g/dL, %	3.4 (41/1,215)	5.5 (29/529)	0.039
Mean age, months (SD)	33.3 (17.1)	33.8 (17.0)	0.601
Mean socioeconomic score‡ (SD)	1.78 (0.96)	1.49 (1.02)	<0.001
Travel to village§, %	7.9	2.4	<0.001
Taken malaria medication in last 2 wk¶, %	23.5	22.3	0.600
History of fever¶#, %	21.2	18.2	0.155
HH reporting bed net use, %	37.7	24.2	<0.001

*Hb, hemoglobin; HH, household. Control communities were those >1 km from an urban agricultural area.
†Number of children in the urban agricultural community group is higher because small plots of agriculture were discovered in 2 communities originally designated control sites. If these 2 communities were omitted from the analysis, similar results were obtained and significance remained the same except for children with moderate-to-severe anemia, which was no longer significant (p = 0.065) (data not shown).
‡Composite measure of socioeconomic status used was the asset factor score of the World Bank for Ghana (www.worldbank.com/hnp).
§Persons who had traveled to a rural (potentially malarious) area in the previous 3 weeks.
¶As reported by the caregiver.
#In the last 48 h.

and live in a community close to areas of urban agriculture. Since recent travel to a rural area did not affect outcome, local malaria transmission is indicated. Our entomologic studies in these study areas (unpub. data) have found *Anopheles gambiae* S form breeding in irrigation water at urban agricultural sites and resting at higher densities in houses in urban agricultural communities.

These findings are based on a point prevalence survey in the dry season. Although we continue to obtain data during the wet season, analysis of data indicates that the urban poor in Africa may be at higher risk for malaria than expected and that malaria can no longer be regarded only as a rural phenomenon. This finding is of great concern because in Africa the current urban population growth rate of 3.5% is >3 times the rural population growth rate, and by 2015 a total of 25 countries in sub-Saharan Africa will have urban populations larger than the rural populations (9). Although levels of transmission in urban areas may be lower than in contiguous rural areas, high population densities and possible lower immunity (6) may result in more disease impact in urban settings. Furthermore, although not the sole cause, irrigated urban agriculture may further increase the risk for malaria by providing suitable breeding sites. Further research on the interaction between type of urban agriculture and vector biology is needed because most African cities irrigate agricultural areas with water from polluted sources that is generally not favored by malaria vectors, although several studies have reported anophelines breeding in heavily contaminated water (14,15). The advantages of urban agriculture for alleviating poverty are numerous, but care must be taken that unregulated growth does not compromise its success. Integration of the activities of municipal authorities, agriculturalists, health professionals, and communities is essential to reduce the existing impact of malaria and to prevent future increases.

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West Nile Virus Detection in Urine

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We report West Nile virus (WNV) RNA in urine collected from a patient with encephalitis 8 days after symptom onset. Viral RNA was detected by reverse transcriptase–polymerase chain reaction (RT-PCR). Sequence and phylogenetic analysis confirmed the PCR product to have ≥99% similarity to the WNV strain NY 2000-crow3356.

West Nile virus (WNV) is a mosquitoborne flavivirus in the Japanese encephalitis serocomplex of the family Flaviviridae (1). Human disease is typically characterized by a mild, self-resolving, denguelike illness with the onset of fever and myalgia (2,3). In a small percentage of patients, primarily the elderly and immunocompromised, disease progresses to a more severe form with central nervous system (CNS) involvement, including encephalitis and meningitis (4,5). The death rate among patients with neuroinvasive disease in recent epidemics has averaged 10%. Among survivors, long-term neurologic sequelae may occur (6). Because WNV is a neurotropic virus, its pathology in the CNS has been the focus of many studies; therefore, little is known about WNV pathogenesis in organs other than the CNS. Studies of WNV in birds, dogs, and rodents have shown that the kidney is a site of replication (7–9); moreover, infectious WNV has been recovered from urine samples from experimentally infected hamsters as early as day 1 to day 52 postinfection (9). The purpose of this study was to determine whether WNV is similarly shed in the urine of humans with WNV infections. To our knowledge, this report is the first of WNV RNA detected in the urine of an infected patient with encephalitis.

The Study

A 65-year-old computer software engineer was admitted to a hospital in Phoenix, Arizona, on July 7, 2004, with fever, headache, and altered mental status evolving in the 7 days before admission. His cerebrospinal fluid (CSF) findings were consistent with viral encephalitis: leukocyte count 141 cells/mm^3, 27% polymorphonuclear cells, 73% lymphocytes; glucose 106 mg/L; protein 102 mg/L; and abnormal electroencephalogram results. The patient was treated empirically for 9 days with acyclovir, ribavirin, and interferon α-2B beginning on July 7, 2004. His fever resolved, followed by gradual improvement in strength and mental function. The patient recovered and was discharged.

CSF and serum samples were obtained on July 7 and 14, 2004. CSF tested positive for specific WNV immunoglobulin (Ig) M antibodies by using a capture enzyme-linked immunosorbent assay. Paired serum samples confirmed an acute WNV infection by showing a 4-fold rise in titer from acute-phase (July 7 [day 8 of illness; day of admission to hospital]) to convalescent-phase (July 14) sera on a 90% plaque reduction neutralization test (PRNT) (10). Antibody titers for acute-phase and convalescent-phase serum samples were 1:80 and ≥1:320, respectively. PRNT tests were negative on urine samples obtained on days 8 and 15 after symptom onset. CSF was unavailable for PRNT and isolation.

Attempts at virus isolation, by using Vero cells (green monkey kidney cells) and C6/36 cells (*Aedes albopictus*), from urine samples collected on days 8, 11, 12, 13, 14, and 15 after symptom onset were unsuccessful. Similarly, we were unable to isolate virus from serum samples collected on days 8 and 9 after symptom onset. Indirect immunofluorescence assays used to confirm culture results were negative.

RNA was extracted from 140 μL of freshly thawed urine by using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. To detect specific WNV gene sequences, we used the following primer sets: WNV 233, 640c and WNV 9483, 9794 that amplify the cap/prM gene and the NS5 gene regions, respectively (11). To generate an amplicon we used Qiagen One-Step RT-PCR Kit with the following thermocycling conditions: reverse transcription (RT) at 50°C for 20 min, 94°C for 15 min, and 55°C for 30 s; 40 cycles of polymerase chain reaction (PCR) at 95°C for 10 s, 56°C for 10 s, and 72°C for 15 s. The urine sample collected on day 8 of illness tested positive with the aforementioned primer sets. The urine specimens analyzed by RT-PCR on days 11, 12, 13, 14, and 15 tested consistently negative with both primer sets.

Amplicons generated with primer set WNV 233, 640c were purified by Qiagen Gel Purification Kit according to the manufacturer's instructions and sequenced on an automated sequencer (Model 373A, Applied Biosystems, Foster City, CA, USA). Sequencing results based on the capsid/prM region from the patient's day 8 urine confirmed the identity of WNV Arizona JW 2004 (GenBank accession no. DQ011267), which had 99.7% homology to

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the WNV strain NY 2000-crow3356 (GenBank accession no. AF404756.1) over the cap/prM region.

A phylogenetic tree was generated by comparing the WNV Arizona JW 2004 sequence against 34 other WNV strains by using a 356-bp sequence corresponding to nucleotide positions 260–615 in the cap/prM region (Figure). Previously published WNV isolates used in the analysis included the first WNV isolate from Uganda in 1937 (GenBank accession no. M12294), the Egypt 1951 laboratory strain (Eg101, GenBank accession no. AF260968), and several American isolates, such as the NY 2000-crow3356 isolate. After sequence alignment with ClustalW, a phylogenetic tree was constructed by using a maximum likelihood (ML) algorithm implemented in PAUP* (v. 4.0b10, Sinauer Associates, Inc., Sunderland, MA, USA). The ML tree was estimated by using the general-time reversible (GTR+I) model of nucleotide substitution, with the substitution matrix, base composition, and proportion of invariant sites (I) all estimated from the data. For tree topology support, we performed 1,000 bootstrapped neighbor-joining replicate trees under the ML substitution model described above and also generated posterior probabilities for each node by using Bayesian MCMC (Metropolis-Hastings Markov chain Monte Carlo) tree sampling (variable substitution rate by codon position, 4 chains of 2×10^6 generations sampled every 100 generations, burn-in of 2,000, and convergence assessed at effective sample size [ESS] >400) implemented in MrBayes v. 3 (12).

Four main geographic groupings are observed in the phylogram. As expected, the WNV Arizona JW 2004 sequence grouped with the other isolates from the United States. Isolates from Russia, Romania, France, and Italy formed a distinct cluster, as did 3 Kunjin virus isolates and their close relatives from China and Egypt (Figure). The WNV 1937 Uganda sequence, along with other sequences (lineage II), was characterized by having the longest branch length (Figure), which implies poor homology with the other isolates.

Contamination of samples within the laboratory is highly improbable. First, the positive control used in all tests was WNV strain Eg101, isolated from Egypt in 1951. Sequence results of this control confirmed its identity and showed 96.9% homology to the cap/PrM region. Second, at the time of testing, our laboratory did not possess any North American WNV isolates, including the WNV strain NY 2000-crow3356, to which the WNV Arizona JW 2004 sequence showed 99.7% homology. Finally, sample contamination during RNA extraction and RT-PCR procedures was unlikely because we used a continuous single-sample RNA extraction, and RT-PCR was accompanied by a negative water control. Results from the continuous single-sample test confirmed previous results: the patient's urine

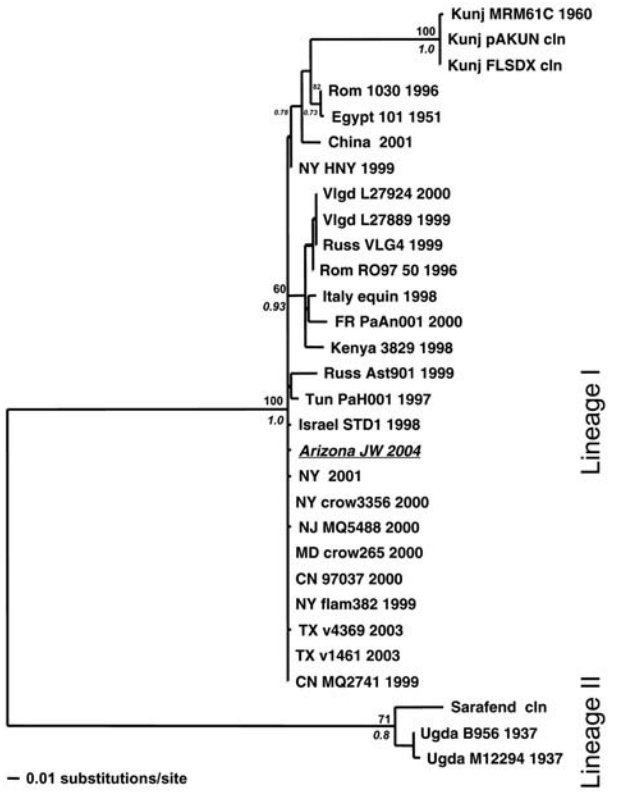

Figure. Maximum likelihood (ML) tree showing the phylogenetic relationships between West Nile virus (WNV) urine sample Arizona JW 2004 (italicized and underlined) and previously published WNV strains based on capsid/prM gene junction (356 bp). Samples are coded by location, strain, and year of isolation. Locations include France (FR); Kunjin (Kunj); Romania (Rom); Russia (Russ); Tunisia (Tun); Uganda (Ugda); Volgograd, Russia (Vlgd); and the US states of New York (NY), Texas (TX), New Jersey (NJ), Maryland (MD), and Connecticut (CN). Support indicated above and below nodes are bootstrap values (1,000 neighbor-joining replicates using the ML model of evolution) and Bayesian posterior probabilities (Bayesian MCMC [Metropolis-Hastings Markov chain Monte Carlo tree sampling] for 4 chains length 2×10^6, sample frequency 100, with a 2,000-tree burn-in), respectively.

on day 8 after symptom onset tested positive for WNV RNA by RT-PCR, and the water control was negative. No blood was visible in the day 8 urine sample. A contemporaneous serum sample collected on day 8 was negative by both RT-PCR and virus isolation.

Conclusions

This report is the first of WNV RNA detected in urine from a patient with encephalitis. St. Louis encephalitis virus (SLEV), a related neurotropic flavivirus, has been reported in human urine. SLEV antigen was detected by indirect immunofluorescence, electron microscopy, and immune electron microscopy in 12 patients during the

1976 outbreak in the United States (13). Furthermore, experimental animal studies have shown that certain flaviviruses are shed in the urine. A study on Japanese encephalitis virus infection in a mouse model showed viral shedding in urine; however, viral shedding did not necessarily correlate with isolation of virus from the kidney (14). In a recent study, infectious WNV was isolated from hamster urine 52 days after initial infection despite the development of high antibody titers against WNV (9). Based on the above reports on flavivirus shedding in humans and experimental animals and our detection of WNV RNA in human urine, we believe WNV may be shed in human urine during the course of infection.

A rapid diagnostic test for flaviviral infection in humans is of clinical interest. Historically, flavivirus infections have been diagnosed by serologic tests or virus isolation (15). Several molecular techniques are available for diagnosis (11), but these tests are not readily accessible in many community medical facilities where disease is commonly reported. We believe the development of a rapid diagnostic test for human flaviviral infections is warranted.

The implications of our finding remain unclear. The presence of WNV RNA in the day 8 urine sample but not subsequent urine samples suggests that neutralizing antibodies in the blood may prevent virus excretion in the urine. WNV would likely be excreted in urine during the viremic phase of illness. Thus, future studies on WNV in human urine should emphasize early collection and testing. In addition, the effect of interferon and ribavirin on the recovery of WNV from the urine remains unknown. Studies currently under way will provide additional information on how often and for how long WNV can be found in urine samples from patients with clinical and subclinical WNV infections.

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Posttraumatic Stress after SARS

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Posttraumatic stress disorder (PTSD) can arise in patients with medical illness. We used 2 Chinese self-report measures to examine features of PTSD, anxiety, and depression in 131 survivors of severe acute respiratory syndrome at 1 month and 3 months after discharge from the hospital. Risk factors associated with psychological distress were identified.

In the outbreak of severe acute respiratory syndrome (SARS) in Hong Kong in 2003, a total of 1,755 people were infected, and 299 died. Among the infected, 386 were healthcare workers (1). According to the literature, a life-threatening physical illness can lead to symptoms associated with posttraumatic stress disorder (PTSD) after recovery. The prevalence rates of PTSD are 1%–5% for childbirth and 14%–59% for a life-threatening situation in an intensive care unit (ICU) (2). Predictors of PTSD identified in previous studies included aspects of the trauma itself, emotional support, and invasiveness of the medical intervention. Severity of illness was not correlated with the development of PTSD (2).

Previous studies showed that 10%–35% of SARS survivors reported having features of anxiety, depression, or both at 1 month after discharge (3–6). Repeated measures of the effect at different time points beyond 1 month after discharge are needed to understand the psychological sequelae related to SARS and enrich the understanding of the long-term psychological functioning of survivors of life-threatening infectious disease.

The Study

This study examined the psychological sequelae related to SARS at 1 month and 3 months after discharge from hospital. According to previous studies on posttraumatic stress, 3 categories of risk factors were postulated. The first category included pre-SARS variables: sex, age, education level, family income, availability of emotional support as indicated by the number of persons with whom one could talk and share worries, and whether one was a healthcare worker. The second category included parameters for severity of disease and treatment regimen: lowest level of blood oxygen saturation (SaO_2) during hospitalization, duration of hospitalization for SARS treatment, whether treatment in ICU was required, and total steroid dosage used during hospitalization. The third category was SARS-related psychological and social variables: whether the participant knew anyone who was suspected or confirmed to have SARS, whether the participant knew anyone who died of SARS, and rating for subjective sense of threat.

The assessment materials printed in Chinese were mailed to 476 SARS patients l month and 3 months after they were discharged from the hospital. Of the 476 SARS survivors contacted, 25 were healthcare workers. One hundred ninety-five (41%) respondents returned the completed questionnaires at 1 month after discharge; characteristics and survey results for the psychological adjustment of these respondents were previously documented (5). A total of 131 (28%) respondents responded at both l month and 3 months after discharge. Our study was based on the data for these 131 respondents. No significant difference was seen between the 3-month respondents and nonrespondents for all the variables examined at 1 month.

Among the 131 participants, ages were 18–84 years (mean 41.82, standard deviation [SD] 14.01); 57 (44%) were men, 74 (56%) were women. Fourteen (11%) were healthcare workers, 4 (3%) had a history of psychiatric consultation, 12 (9%) had other chronic diseases, and 15 (11%) required treatment in the ICU. The lowest level of SaO_2 during hospitalization was 79%–96% (mean 91.59%, SD 3.29). The total steroid dosage used for treatment ranged from 0 to 86,900 mg (mean 14,120.28 mg, SD 12,254.95).

Fifty-seven participants (44%) personally knew someone who was suspected or confirmed to have SARS. Fourteen (11%) knew someone who died of SARS. Regarding the number of persons with whom they could talk and share their worries, 6 participants (5%) indicated no one, 68 (52%) indicated 1–2, 41 (31%) indicated 3–4, and 16 (12%) indicated $\geq$5. For the rating on subjective sense of threat caused by the disease, 11 participants (8%) reported "not at all," 37 (28%) reported "a little," 43 (33%) reported "moderate," 28 (21%) reported "quite serious," and 12 (9%) reported "extremely serious."

The measures used in the study include the Chinese versions of the Impact of Event Scale – Revised (IES-R) (7,8) and the Hospital Anxiety and Depression Scale (HADS) (8–10). Based on research on PTSD and the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (11), intrusion, avoidance, and hyperarousal were identified as the primary domains of measurement on the IES-R. Scoring was based on previous studies that indicated a mean subscale score of 2, representing a moderate level of distress, is the appropriate cut-off point (7,8).

The Chinese HADS (8–10) is a self-report instrument designed to detect symptoms related to anxiety and

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depression. As in previous studies, the marker for a moderate level of distress (i.e., the subscale score of 11) was used as the cutoff point for HADS subscale scores (8–10).

Repeated measures analysis of variance (ANOVA), with time (1 month vs. 3 months after discharge) as the within-subject factor, was used to examine the change of symptom severity. The 1-month scores for IES-R intrusion (mean 1.12, SD 0.73), hyperarousal (mean 1.05, SD 0.79), and HADS anxiety (mean 5.87, SD 3.89) were significantly higher than the 3-month scores for IES-R intrusion (mean 0.91, SD 0.73), $F_{1, 130} = 18.52$, $p<0.001$), hyperarousal (mean = 0.85, SD = .74), $F_{1, 130} = 13.96$, $p<0.001$, and HADS anxiety (mean 5.19, SD 4.48), $F_{1, 130} = 5.23$, $p<0.05$).

At 1 month after discharge, the number of participants who surpassed the subscale cutoff was 16 (12%) for intrusion, 12 (9%) for avoidance, 19 (15%) for hyperarousal, 17 (13%) for anxiety, and 23 (18%) for depression. At 3 months after discharge, 13 participants (10%) surpassed the subscale cut-off for intrusion, 11 (8%) for avoidance, 12 (9%) for hyperarousal, 18 (14%) for anxiety, and 17 (13%) for depression. As PTSD is characterized by the presence of all 3 symptom clusters (i.e., intrusion, avoidance, and hyperarousal), the percentage of participants who passed the cutoff for all IES-R subscales was examined. Among the 131 participants, 6 (4%) at 1 month and 7 (5%) at 3 months postdischarge had all 3 IES-R subscale scores above the cutoffs.

Results of multivariate analysis of variance indicated significant difference in the combined dependent variables between participants with and without a history of psychiatric consultation (Wilks = 0.83, F $test_{5, 125} = 5.18$, $p<0.001$, effect size 0.17). Results in univariate F tests suggested that having a history of psychiatric consultation, being a healthcare worker, and knowing someone who had SARS were associated with various IES-R and HADS scores (Table 1).

Results of Pearson correlations (Table 2) showed that the level of SaO_2, the number of persons with whom one could talk and share worries, and the rating on perceived threat were significantly related to various IES-R and HADS subscale scores. To determine which variables had the greatest effect on symptom severity at 3 months after discharge, standard multiple regressions were conducted with IES-R and HADS subscale scores as the criterion variables. The lowest level of SaO_2 during hospitalization, the rating for subjective sense of threat, and the number of persons with whom one could talk and share worries were entered into the regression model as predictor variables. Overall, the amount of total variance accounted for in individual IES-R and HADS subscale scores by these variables was significant. The lowest level of SaO_2 during hospitalization was the most significant predictor for intrusion and avoidance scores. High level of perceived threat was the most significant predictor for hyperarousal and the HADS anxiety scores. The number of persons with whom one could talk was the most important predictor for the HADS depression score.

Conclusions

The occurrence rate of PTSD features for SARS survivors is in the middle of the range reported in previous samples of other medical diseases (2). For most SARS survivors, a significant decrease in symptom severity from l month to 3 months after discharge was identified.

The significant predictive value of SaO_2 as an index of disease severity in this study suggests that direct physiologic measures may be more sensitive as indexes of

Table 1. Results obtained for predictors with significant group difference in IES-R and HADS subscales (N = 131)*

	IES-R intrusion		IES-R avoidance		IES-R hyperarousal		HADS anxiety		HADS depression	
	M (SD)	F	M (SD)	F	M (SD)	F	M (SD)	F	M (SD)	F
History of psychiatric consultation										
Yes (n = 4)	2.31 (0.80)	17.24§	1.71 (0.084)	5.73‡	2.33 (0.80)	18.70§	11.50 (4.12)	8.61‡	9.50 (5.25)	4.21†
No (n = 127)	0.86 (0.68)		0.84 (0.70)		0.80 (0.68)		5.00 (4.36)		5.03 (4.25)	
Healthcare worker										
Yes (n = 4)	1.28 (0.89)	4.26†	1.16 (0.73)	2.43	1.35 (0.77)	7.61‡	7.14 (4.94)	2.99	6.85 (4.12)	2.39
No (n = 127)	0.86 (0.69)		0.84 (0.72)		0.79 (0.71)		4.96 (4.39)		4.97 (4.33)	
Know someone to have SARS										
Yes (n = 4)	1.01 (0.66)	2.09	1.00 (0.75)	2.98	0.96 (0.75)	2.42	6.07 (3.93)	3.89	6.12 (4.24)	4.96†
No (n = 127)	0.83 (0.76)		0.77 (0.70)		0.76 (0.72)		4.52 (4.78)		4.44 (4.28)	

*IES-R, Impact of Event Scale – Revised; HADS, Hospital Anxiety and Depression Scale.
†$p<0.05$.
‡$p<0.01$.
§$p<0.001$.

Table 2. Summary of multiple regression analyses of IES-R and HADS subscales (N = 131)*

Predictor variables	B	ß	sr^2	R	R^2	Overall F
Regression analysis to predict IES–R intrusion score						
Subjective threat	0.12	0.18	0.03†	0.22‡		
Emotional support	–0.17	–0.18	0.03†	–0.21‡		
SaO_2	–0.04	–0.22	0.04†	–0.26‡	0.13	$F_{3, 127} = 6.57$§
Regression analysis to predict IES–R hyperarousal score						
Subjective threat	0.13	0.19	0.04†	0.22‡		
Emotional support	–0.14	–0.15	0.02	–0.17†		
SaO_2	–0.02	–0.13	0.02	–0.16†	0.09	$F_{3, 127} = 4.15$†
Regression analysis to predict IES–R avoidance score						
Subjective threat	0.15	0.22	0.05‡	0.26‡		
Emotional support	–0.17	–0.19	0.03†	–0.22‡		
SaO_2	–0.05	–0.24	0.06‡	–0.29§	0.17	$F_{3, 127} = 8.62$§
Regression analysis to predict HADS anxiety score						
Subjective threat	0.73	0.18	0.03†	0.20‡		
Emotional support	–0.77	–0.13	0.02	–0.15†		
SaO_2	–0.16	–0.12	0.01	–0.15†	0.07	$F_{3, 127} = 3.43$*
Regression analysis to predict HADS depression score						
Subjective threat	0.24	0.06	0.00	0.09		
Emotional support	–10.03	–0.18	0.03†	–0.20†		
SaO_2	–0.18	–0.14	0.02	–0.16†	0.06	$F_{3, 127} = 20.91$†

*IES-R, Impact of Event Scale – Revised; HADS, Hospital Anxiety and Depression Scale, Subjective threat, subjective sense of threat; emotional support, number of persons one could talk to and share worries with; SaO_2, lowest level of blood oxygen saturation during hospitalization.
†$p<0.05$.
‡$p<0.01$.
§$p<0.001$.

disease severity than other indexes, which could be confounded by other factors (e.g., treatment regimen). Our findings imply that mobilization of resources for emotional support may enhance resilience of SARS survivors. SARS survivors who were healthcare workers, knew someone who had SARS, or had a history of psychiatric consultation had a higher risk for psychological distress and may warrant early and focused support services.

Our study was limited by the low response rate and small sample size of certain groups of participants (e.g., healthcare workers and patients with history of psychiatric consultation). As criterion A in DSM-IV for PTSD, which focuses on the nature and personal response involved in the traumatic experience, was not specifically assessed, the occurrence rate of PTSD could not be taken as a prevalence estimate for PTSD in a straightforward manner. Such findings could better be substantiated by clinical interviews. The study is limited by the availability of a comparison group. Since SARS is a new disease, the psychological effect of some possible long-term physical outcomes related to the disease and treatment regimen (e.g., avascular necrosis) that were not discovered until recently were not captured.

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EID Covers Online
www.cdc.gov/ncidod/EID/cover_images/covers.htm
Image created by Curt Wommack

Evaluating Antibiograms To Monitor Drug Resistance

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We used hospital antibiograms to assess predominant pathogens and their patterns of in vitro antimicrobial resistance in central Illinois, USA. We found a lack of information about national guidelines for in vitro antimicrobial susceptibility testing and differences in interpretation among laboratories in the region.

A number of databases are available in the United States to monitor antimicrobial resistance at a national level (1). The academic and educational value of these databases is particularly useful for microbiologists and infectious disease clinicians. However, databases are unlikely to prove useful in improving antimicrobial use in the communities for a number of reasons: 1) most antimicrobial drug prescriptions in the community are written by primary care physicians, 2) most primary care physicians do not use these resources, and 3) industry-generated data are often used to highlight a particular antimicrobial drug. Even for infectious disease clinicians, national databases serve as a general guide, particularly during the initial or presumptive phase of antimicrobial therapy when culture results are not available.

For tertiary care/referral hospitals, a substantial percentage of patients are transferred from community hospitals outside the local area. Under these circumstances, resistance surveillance data from these areas would help select presumptive therapy or change existing therapy. Very often, when patients have been treated with empiric antimicrobial drugs, the culture results at the tertiary care institutions may be negative or do not detect the infecting organism. To overcome these difficulties and improve the outcome of serious infections in the referral area for our institution, we monitored resistance patterns in the region and generated a regional antibiogram, which will be shared with all participating hospitals.

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The Study

A packet was sent to the clinical microbiology laboratories of the 77 hospitals in the area. It included a letter describing the project, a questionnaire on hospital characteristics and laboratory testing methods, and a request for existing antibiograms from the most recent period for which completed data were available. We used only antibiograms from January 2001 to June 2002. From the antibiograms, the numbers of isolates tested and number of susceptible isolates were added for each antimicrobial agent from all hospitals for each region (Appendix Table 1, available from http://www.cdc.gov/ncidod/EID/vol11no08/05-0135_app1.htm) and for all regions combined.

The proportion of responding hospitals was 53%; all major academic centers participated. Data from 10 hospitals were excluded, 7 because the aggregated antibiograms did not include the number of isolates tested and 3 because the antibiogram data predated January 2001. Thirty-one hospitals that were included in the final analysis represented the 4 regions as follows: 16 (42%) of 38 hospitals in the central region, 6 (43%) of 14 in the west, 4 (40%) of 10 in the south, and 5 (33%) of 15 in the southwest. Of the hospitals included, 16% did not send a cumulative antibiogram but instead sent their data as a monthly report for a period from 3 months to 1 year. Our research team generated cumulative antibiograms for these hospitals.

The proposed guidelines for analyzing and presenting cumulative antimicrobial susceptibility data were published by the Clinical and Laboratory Standards Institute (formerly NCCLS) in 2002. The M39-A document provides a standardized means of data extraction for all drugs tested and outlines the most appropriate way to present the data (2).

In our discussions with laboratory personnel, we found that many laboratories are unaware of these guidelines, and laboratories that use the document find that adhering to all recommendations is difficult. Many laboratories lack a microbiology supervisor with insight into the clinical relevance of the results they generate. For example, a laboratory reported 4% vancomycin resistance in *Streptococcus pneumoniae*, but the laboratory staff was not able to explain this finding or recognize the clinical implications. Also 2 of the hospitals reported 2 vancomycin-intermediate *Staphylococcus aureus* in their antibiogram. However, the isolates were not available for verification, and the laboratory staff was not aware of the implications of this finding. The staff did not know that such findings should be reported to the Illinois Department of Public Health and the Centers for Disease Control and Prevention.

In all regions, *Escherichia coli* was the most commonly isolated organism, followed by *S. aureus*. Coagulase-negative staphylococci, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* were among the 5 most frequently reported species (Appendix Tables 1 and 2 [available from

http://www.cdc.gov/ncidod/EID/vol11no08/05-0135_app2.htm]). The 10 most frequently reported species in our study are generally comparable to those found in the SENTRY survey conducted by Pfaller et al. (3).

Of the *S. aureus* isolates tested in the central, west, south, and southwest regions, 27%, 53%, 34%, and 42%, respectively, were resistant to methicillin. Of the hospitals that reported speciation of enterococci, *E. faecalis* was susceptible to vancomycin at 91%–99%. The vancomycin resistance among *E. faecium* was 32%–73%. However, hospitals from the southwest area reported enterococci other than *E. faecalis* as *Enterococcus* spp. only. The unusually low susceptibility of *E. faecium* in our study may be attributed to specimen duplication.

In the central Illinois region, the susceptibility of *S. pneumoniae* to penicillin was 64%–75%, and 52%–77% of isolates were susceptible to erythromycin. The susceptibility of common gram-positive bacteria in our study appears to be lower than reported national averages (3). Although antibiogram surveillance and active surveillance yield comparable results (4), national data may not be directly comparable to our findings because national data used for comparison results from active surveillance with different reporting periods. In addition, geographic factors must be taken into consideration (4–7).

Conclusions

In spite of expertise and resources available in the United States, the use of antimicrobial drugs in day-to-day practice is suboptimal and directly responsible for multidrug resistance in a number of common pathogens. The factor that converts antimicrobial therapy from "empiric" to "rational" is in vitro susceptibility testing and reporting. However, if these tests are either not conducted or conducted poorly, they are not useful clinically and may create a false sense that therapy is rationally guided. Given the differences and shortcomings we reported among laboratories in a region, national recommendations are either unknown or not followed. Use of expertise, cooperation, and collaboration at the regional levels may be the simplest and most useful public health measures to optimize the usefulness of diagnostic microbiology in managing infectious diseases. Antimicrobial drug use guidelines, if they are based on consistent, reproducible, and comparable data between different laboratories, will produce better outcomes. A master antibiogram for a region would allow a tertiary care institution to consider resistance patterns in hospitals referring patients and to select appropriate "presumptive" antimicrobial therapy or change drugs in nonresponding patients. We hope that the concept of "empiric antimicrobial therapy" would be changed to that of "presumptive antimicrobial therapy" based on host factors, common pathogens, and known susceptibility patterns in any given region.

This study has helped us identify serious shortcomings in susceptibility testing methods and reporting, and we hope to address these issues through a regional advisory group. Even if following all the recommendations in M39-A are not possible, the second best option may be to have all regional laboratories adhere to the same subset of recommendations. Antimicrobial resistance data generated by this approach will have better day-to-day application than will data generated by large national databases. The data will also be useful in monitoring resistance trends in a region over time and assessing the effects of interventions to reduce antimicrobial resistance. We recognize the shortcomings of the data presented in this article but believe them to be the basis for improvement at a fundamental level.

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Influenza A H5N1 Detection

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We developed a sensitive and rapid real-time reverse transcription-polymerase chain reaction (RT-PCR) assay to detect influenza A H5N1 virus in clinical samples. This assay was evaluated with samples from H5N1-infected patients and demonstrated greater sensitivity and faster turnaround time than nested RT-PCR.

The first outbreak of highly pathogenic influenza A H5N1 virus in humans occurred in Hong Kong in 1997 and 6 people with confirmed infection died (1). In February 2003, 2 persons who had traveled to Fujian Province in China were hospitalized in Hong Kong with H5N1 infection (2). Early in 2004, an influenza A H5N1 outbreak emerged in Vietnam and Thailand that caused death in humans and epidemics in the poultry industry (3–5). The recent recurrence of influenza A H5N1 prompted us to highlight the need for a highly sensitive, accurate, and rapid diagnostic test for the infection. Such a test would be important, not only in infection control but also to facilitate early antiviral therapy. Conventional diagnostic tools, cell culture, and serologic testing require from 2 days to 2 weeks for results; thus, they are less useful in making therapeutic and infection control decisions. On the other hand, commercially available rapid antigen tests such as Directigen Flu A+B (Becton Dickinson, Sparks, NJ, USA) or Binax NOW (Binax Inc., Portland, ME, USA) are rapid and simple, but subtyping of viruses is not feasible. Molecular diagnosis of influenza by reverse transcription-polymerase chain reaction (RT-PCR) provides a sensitive and rapid means for detection and has facilitated the typing and subtyping of viruses. Previously, researchers developed tests to detect H5N1 virus by using conventional RT-PCR (6–8) and confirmed the results by Southern blot analysis (6) or restriction fragment length polymorphism–based strategy (8). Although real-time RT-PCR based on the avian H5 gene was developed, the assay has not been evaluated on human clinical specimens (9). We developed a highly sensitive, rapid, and accurate real-time RT-PCR assay to directly diagnose influenza A subtype H5 in human clinical samples. When we evaluated this system using clinical samples from patients infected with H5N1 in Hong Kong and Vietnam, we found it was more sensitive and faster in detecting the virus than the nested RT-PCR that we used previously (3).

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The Study

Our real-time RT-PCR is a multiplex assay that employs a mixture of 2 sets of inhouse designed primers and dual-labeled fluorescent probes that specifically target 2 different regions of the HA gene of H5N1. The primer and probe sets were designed by using the Primer Express software program (Applied Biosystems, Foster City, CA, USA). Both primer and probe sets designs were based on some of the sequences of the recent Vietnam H5N1 strains (e.g., A/Viet Nam/1194/2004). Multiple alignments of previous and recent H5N1 strains were performed to minimize primer mismatch. Multiplex performance was maximized by selecting primers and probes with uniform melting temperatures and minimal cross-hybridization potential. The primer and dual-labeled fluorescent probe sequences are shown in Table 1. This assay is a 2-step, real-time RT-PCR system. Viral RNA is reverse transcribed with random hexamers, followed by real-time PCR. Briefly, viral RNA was extracted from 140 μL clinical specimens in phosphate-buffered saline or viral transport medium with a viral RNA mini kit (Qiagen, Hilden, Germany) and a final elution with 60 μL AVE buffer. Reverse-transcriptase reactions contained 4.2 μL RNA extract, 2 μL 10× PCR buffer (Applied Biosystems), 2.5 μM random hexamer primers (Applied Biosystems), 20 units RNase inhibitor (Applied Biosystems), and 1 μL (50 U) MuLV reverse transcriptase (Applied Biosystems).

Reverse-transcriptase reactions were performed at room temperature for 10 min, then at 42°C for 30 min and at 95°C for 5 min. Five microliters of the cDNA was then used for amplification in the real-time PCR assays. Real-time PCR was carried out with a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). The real-time PCR reactions (total volume 20 μL) contained 2 μL LightCycler DNA Master Hybridization Probes reaction mix (Roche Diagnostics GmbH), 3 mmol/L magnesium chloride, 250 nmol/L each of the 4 primers, and 125 nmol/L each of the dual-labeled fluorescent probes. The thermal profile used was initiated at 95°C for 10 min (preamplification hot start), followed by 50 cycles of PCR at 95°C for 10 s (denaturation), 56°C for 15 s (annealing), and 72°C for 12 s (extension). At the end of each annealing step, the fluorescent signal of each reaction was measured at a wavelength of 530 nm with the LightCycler fluorimeter. Precautions were taken to prevent cross-contamination of PCR (10).

To test for cross-reactivity, RNA was extracted from isolates or persons with human influenza A H1, H3, H9 subtypes; influenza B; human CoV 229E and OC43; respiratory syncytial virus; rhinoviruses; and enteroviruses. The

Table 1. Primers and dual-labeled fluorescent probes of the H5 real-time reverse transcription–polymerase chain reaction assay

Primer/probe	Target	Primer/probe sequence (5′ to 3′)	Nucleotide position*
H5-1F		TGCCGGAATGGTCTTACATAGTG	266–288
H5-1R	HA† gene	TCTTCATAGTCATTGAAATCCCCTG	347–323
H5-1P		f†-AGAAGGCCAATCCAGTCAATGACCTCTGTTA-xp†	290–320
H5-2F		GTGGCGAGCTCCCTAGCA	1615–1632
H5-2R	HA gene	TCTGCATTGTAACGACCCATTG	1695–1674
H5-2P		f-TGGCAATCATGGTAGCTGGTCTATCCTTATGG-xp	1634–1665

*Based on some of the Vietnam H5N1 strains (e.g., A/Viet Nam/1194/2004).
†HA, hemagglutinin; f, 6-carboxyfluorescein; xp, carboxytetramethylrhodamine.

RNA was then tested by the described real-time RT-PCR. The results showed that the assay was specific for the H5 subtype. The detection sensitivity of the real-time assay was compared with that of 2 other assays by analyzing a serial 10-fold dilution of viral stock with 10^8 tissue culture infective dose $(TCID)_{50}$/mL of rhabdomyosarcoma cell–culture fluid of a recent H5N1 isolate (A/Viet Nam/1194/2004). The results showed that our real-time RT-PCR assay was the most sensitive of the 3 assays, 10-fold more sensitive than nested RT-PCR (Table 2). We analyzed 18 archived respiratory samples collected ≤10 days after onset of illness from 13 confirmed H5N1–infected patients from Hong Kong in 1997 and 2003 and 10 samples collected on an unknown onset day of illness from 5 confirmed H5N1–infected patients from Vietnam in 2004. All samples were confirmed as H5N1 positive by virus isolation and characterization. Our findings demonstrated that the detection rate of the RT-PCR assay was 100% (28 of 28 samples), whereas that of the nested RT-PCR was 89% (25 of 28 samples). This finding indicates that the real-time assay shows similar or greater sensitivity than the nested RT-PCR. Furthermore, the H5 viral load of the archived clinical samples was determined by a standard curve obtained by plotting from the same series of 10-fold dilutions of virus stock. Viral load analysis showed that the concentration of H5N1 RNA in those clinical specimens ranged from 10^1 to 10^6 $TCID_{50}$/mL. Based on the serial sample analysis of 2 patients, the viral load of patient 1 increased 5-fold from day 0 to day 3, whereas the viral load of patient 2 dropped 3-fold from day 4 to day 7 (Figure). Because a limited number of samples were analyzed, the peak of viral load cannot be determined. Studies have reported that the viral load in nasopharyngeal aspirate of the H5N1 patients was lower than in those with H3N2 infection in 2003 (11). To determine whether the viral load of H5N1 patients in 1997 and 2004 is different from that of H3N2 patients, clinical specimens from these 2 groups of patients were analyzed by using a quantitative real-time RT-PCR assay that targeted the M gene of influenza A (12). Similarly, viral load was determined by a standard curve obtained by plotting from series of 10-fold dilutions of a H3N2 virus stock. The mean viral load of 22 patients with H3N2 virus infection was 1.5×10^6 $TCID_{50}$/mL, whereas the mean viral load of H5N1 patients was 1.6×10^5 $TCID_{50}$/mL in both 1997 and 2004. Thus, 10-fold lower viral loads were observed in H5N1 patients in both 1997 and 2004 (*t* test, $p<0.05$). These data are consistent with previous data that showed rapid diagnostic methods for influenza are less sensitive for H5N1 detection, likely due to lower viral loads.

Conclusions

In this study, we demonstrated that our new multiplex real-time RT-PCR assay that specifically targets 2 different regions of the H5 gene is more sensitive than nested RT-PCR and even more sensitive than real-time RT-PCR with a single set of primers and probes (unpub. data). Our data prove that the assay is specific for H5 subtype and capable of detecting and quantifying H5 RNA in clinical samples from patients obtained during different outbreaks (1997, 2003, and 2004). Unlike nested RT-PCR, the real-time assay not only reduces the risk for contamination but also reduces turnaround time to 1–2 hours, 3 times faster than the nested RT-PCR. In conclusion, our study demonstrates that our real-time RT-PCR assay is rapid, specific, and relatively sensitive for directly detecting influenza A subtype H5 virus and may be useful in routine diagnostic testing.

Table 2. Detection sensitivity of polymerase chain reaction and rapid antigen test for H5N1*

	Dilution†					
Test	10^3	10^4	10^5	10^6	10^7	10^8
Rapid antigen test‡	+	+	–	–	–	–
Conventional nested RT-PCR	+	+	+	+	–	–
Real-time RT-PCR	+	+	+	+	+	–

*RT-PCR, reverse transcription–polymerase chain reaction.
†Serial 10-fold dilution with a concentration of 10^8 50% tissue culture infective doses/mL of rhabdomyosarcoma cell culture fluid.
‡Binax NOW; +, positive; –, negative.

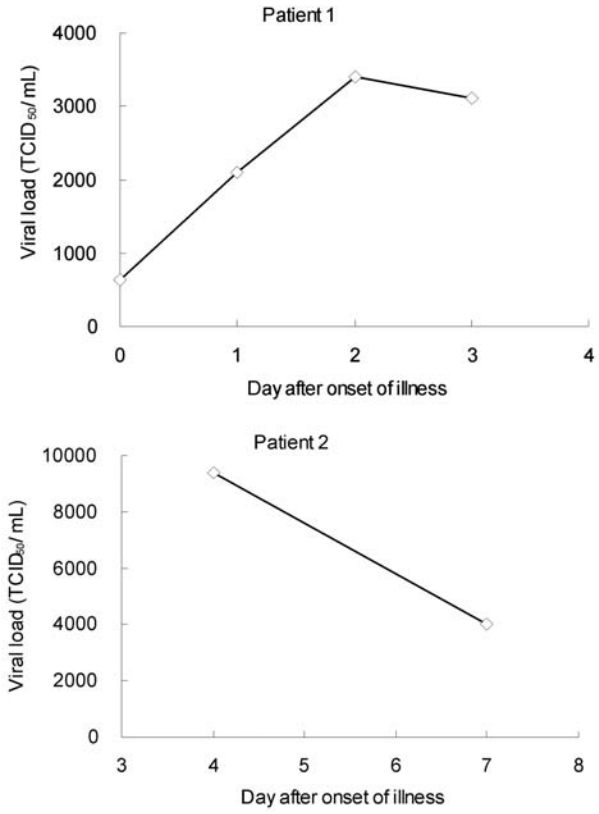

Figure. Serial quantitative analysis for influenza A H5N1 virus in respiratory samples from 2 patients with H5N1 in 1997 by quantitative real-time reverse transcription–polymerase chain reaction assay. $TCID_{50}$, 50% tissue culture infective dose.

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Drug-resistant Diarrheogenic *Escherichia coli*, Mexico

Teresa Estrada-García,* Jorge F. Cerna,*† Leova Paheco-Gil,‡§ Raúl F. Velázquez,† Theresa J. Ochoa,¶ Javier Torres,† and Herbert L. DuPont¶#**

Diarrheogenic *Escherichia coli* isolates from 45 (73%) of 62 hospitalized patients were resistant to common antimicrobial drugs. Sixty-two percent were multidrug resistant, and >70% were resistant to trimethoprim-sulfamethoxazole and ampicillin. Ciprofloxacin and cefotaxime were uniformly active. Effective and safe oral agents are needed to treat children with bacterial diarrhea.

The best characterized diarrheogenic *Escherichia coli* (DE) groups include enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAEC), and Shiga toxin–producing (STEC) *E. coli*. STEC is also known as verotoxin-producing or enterohemorrhagic *E. coli*. Except for slow-fermenting sorbitol STEC (as O157:H7), DE are not routinely sought as stool pathogens in clinical laboratories worldwide, perhaps because rapid and sensitive laboratory techniques are lacking. However, DE are a leading cause of children's diarrhea in developing countries (1), and some are increasingly being recognized as important enteropathogens in developed countries (1,2). To establish the prevalence and resistance patterns of these microorganisms in hospitalized children in Mexico, we conducted a prospective study. *E. coli* strains were analyzed by using 2 comprehensive multiplex polymerase chain reaction (PCR) assays, and strains harboring DE genes were analyzed for their antimicrobial resistance patterns.

The Study

Two groups of children <5 years of age hospitalized for acute diarrhea were studied: 1) 285 children enrolled from March 2000 to February 2001 at 3 main hospitals of Mexico City, Instituto Mexicano del Seguro Social (IMSS); and 2) 145 children enrolled from February to October 2004 at the Children's Hospital in Villahermosa, Tabasco, Hospital del Niño, Secretaría de Salud (SS). The institutional review boards of IMSS and SS approved these studies, and parental informed consent was obtained for each patient. Children were included if they had ≥3 loose stools in 24 hours or an episode of bloody diarrhea. Children were excluded if they had received previous antimicrobial drug treatment. A total of 430 Mexican children hospitalized for acute diarrhea (<14 days) were included, 222 (52%) were male and 321 (77%) were ≤2 years of age. Stool diagnostic evaluations were done by standard laboratory procedures, including culture for *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, and *Campylobacter* spp.; enzyme-linked immunosorbent assay or latex agglutination test for rotavirus; and microscopy for *Entamoeba histolytica*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Isospora* spp., and *Giardia lamblia*. In addition, from all 430 stool samples, 5 lactose-fermenting colonies and 5 sorbitol-nonfermenting colonies with shapes resembling that of *E. coli* were selected from standard and sorbitol MacConkey agar plates, respectively and speciated biochemically. A total of 2,010 *E. coli* strains from 430 patients were selected, and all were analyzed by a multiplex PCR (3) that detects the following pathogenic genes: heat-stable and heat-labile enterotoxins (*st*, *lt*) for ETEC, intimin (*eae*A) and bundle-forming pilus (*bfp*) for EPEC, Shiga toxin 1 and 2 (*stx*1, *stx*2) and intimin (*eae*A) for STEC, and invasion-associated loci (*ial*) for EIEC. STEC from patients were further characterized by the expression of the O157 lipopolysaccharide antigen and enterohemolysin gene (*hly*A) by using latex particle agglutination kit (Oxoid Limited, Basingstoke, UK) and PCR, respectively. Moreover, all *E. coli* strains from the Villahermosa study were analyzed by a second multiplex PCR that detects 3 plasmidborne virulence genes (*aap*, *agg*R, and *aat*A) from EAEC (4). Both PCRs were developed at the Department of Molecular Biomedicine, Centro de Investigación y de Estudios Avanzados (CINVESTAV). In the present study, pathogenic EAEC were defined as those harboring the 3 plasmidborne genes, *aap*, *agg*R, and *aat*A, as previously described (4). This definition may be stringent, but for the antimicrobial susceptibility analysis, we wanted to include only pathogenic DE when possible. Finally, *E. coli* strains positive for any DE gene were analyzed for their antimicrobial susceptibility by disk diffusion, according to the Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines (5).

DE were identified in 62 (14%) of 430 patients, the second highest proportion after that of rotavirus (41%). Other pathogens isolated were *Shigella* spp. (9%), *Salmonella*

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spp. (3%), *Cryptosporidium* spp. (0.9%), and *Campylobacter* spp. (0.7%). Of the 2,120 analyzed strains, 170 (8%) were positive for at least 1 DE gene. As shown in Table 1, ETEC was the most prevalent DE group isolated in patients, closely followed by EAEC (only characterized in the Villahermosa study) and then by atypical EPEC (aEPEC [*eae*A+ *bfp*–]). Most STEC strains were *stx*2+ or *stx*1+ *eae*A+, none expressed the O157 lipopolysaccharide antigen, and only 1 contained the *hly*A gene.

Antimicrobial resistance patterns for patients that had DE included 65% resistant to trimethoprim-sulfamethoxazole (TMP-SMX) and 73% to ampicillin (Table 2). Resistance to ≥3 antimicrobial drugs (multidrug resistance) was 58%. The antimicrobial resistance patterns for the strains were similar to the ones described above for the patients. Thus, of the 170 strains 105 (62%) were multidrug resistant, 145 (85%) were resistant to tetracycline, 124 (73%) to ampicillin, 127 (75%) to TMP-SMX, 29 (17%) to chloramphenicol, 4 (2%) to gentamicin, and none to ciprofloxacin and cefotaxime. Most isolated strains per patient showed similar susceptibility. Comparison of resistance patterns between patients belonging to different DE groups showed that aEPEC was significantly less resistant ($p<0.05$, chi-square test) to ampicillin and TMP-SMX than ETEC and EAEC.

Conclusions

Diarrheogenic *E. coli* prototypes cause high rates of persistent diarrhea (6–8), which has been associated with malnutrition, growth impairment, and death, in developing countries (1,6,8). From the 62 patients with DE, ETEC was the most prevalent group (27% of cases), showing that ETEC continues to be a major health problem in developing countries (6). EAEC accounted for 26% of cases, although it was characterized at only 1 site, and a stringent definition of pathogenic EAEC was used. EAEC prevalence may be even higher, and it may be responsible for much acute diarrhea requiring hospitalization in children, as recently shown in the United States (2). Unexpectedly, 21% of patients harbored aEPEC strains; the pathogenic role of this emerging *E. coli* group is still unclear, but it has been associated with acute (9) and persistent diarrhea (10). In addition, those aEPEC strains showed significantly less resistance ($p<0.05$, chi-square test) to ampicillin and TMP-SMX than ETEC and EAEC strains, which suggests that aEPEC strains may have recently been acquired in Mexico. Finally, 18% of patients harbored STEC non-O157 strains, showing its role in acute diarrhea that requires hospitalization in Mexico. Together these observations highlight the role of DE in children's diarrhea that requires hospitalization and stress the importance of seeking DE in children's stools by using multiplex PCR technology. We have also shown that specific and sensitive multiplex PCR technology (3,4) that recognizes a diversity of loci in *E. coli* may be cost effective, which would allow clinical laboratories worldwide to identify these pathogens.

Since some DE infections appear indistinguishable from viral gastroenteritis, isolation and identification of DE strains could allow caretakers to provide appropriate treatment for pathogen-specific illness. Oral rehydration therapy (ORT) in children with dehydrating forms of diarrhea has reduced death rates worldwide. ORT, however, does not shorten duration of illness and shedding, whereas antimicrobial therapy may be of value for some forms of DE diarrhea (11). Antimicrobial therapy may be indicated in children with DE diarrhea that is promptly identified and in children with persistent diarrhea. We have shown that most DE strains that cause diarrhea in hospitalized children in Mexico are resistant to TMP-SMX and ampicillin, drugs commonly used to treat pediatric diarrhea. This resistance pattern is an emerging problem for DE strains isolated from children in other developing countries (12) and for other enterobacteria worldwide (12–14). All strains were sensitive to ciprofloxacin and cefotaxime; however, ciprofloxacin and other quinolones are not approved for children because of the risk of damage to immature joints (14), and most parenteral third-generation cephalosporins (e.g., cefotaxime) are administered only in a hospital setting. These results show the need for new, affordable, and safe oral antimicrobial drugs to treat enterobacterial infections in children.

Table 1. Diarrheogenic *Escherichia coli* (DE) isolated from patients with diarrhea

DE group*	No. patients by DE group (%)	No. strains† by DE group	Strain genotypes and no. positive strains by gene(s)
ETEC	17 (27)	50	*lt* = 29, *st* = 17, *st-lt* = 4
EAEC	16 (26)	56	*aap-agg*R-*aat*A = 56
aEPEC	13 (21)	31	*eae*A = 31
STEC	11 (18)	22	*stx*2 = 9, *stx*1-*eae*A = 9, *stx*1 = 3, *stx*1-*eae*A-*hly*A = 1
EPEC	3 (5)	7	*eae*A-*bfp* = 7
EIEC	2 (3)	4	*ia*l = 4
Total	62	170	

*ETEC, enterotoxigenic *E. coli*; EAEC, enteroaggregative *E. coli;* aEPEC, atypical enteropathogenic *E. coli;* STEC, Shiga toxin–producing *E. coli;* EPEC, enteropathogenic *E. coli;* EIEC, enteroinvasive *E. coli*.
†All diarrheogenic *E. coli* strains isolated from patients with diarrhea; in general, 5 *E. coli* strains were isolated from each patient.

Table 2. Diarrheogenic *Escherichia coli* resistance patterns*†

No. patients	Tet, n (%)	Amp, n (%)	TMP-SMX, n (%)	Chlor, n (%)	MDR, n (%)
ETEC (n = 17)	16 (94)	15 (88)	12 (71)	2 (12)	11 (65)
EAEC (n = 16)	15 (94)	13 (81)	14 (88)	3 (19)	11 (69)
aEPEC (n = 13)	6 (46)	5 (38)	5 (38)	2 (15)	5 (38)
STEC (n = 11)	9 (81)	8 (72)	7 (63)	4 (36)	7 (63)
EPEC (n = 3)	3 (100)	3 (100)	2 (67)	0	2 (67)
EIEC (n = 2)	1 (50)	1 (50)	0	1 (50)	1 (50)
Total (n = 62)	51 (82)	45 (73)	40 (65)	12 (19)	36 (58)

*All isolates were susceptible to ciprofloxacin and cefotaxime. One STEC (9% of STEC or 2% of all isolates) was resistant to gentamicin; all other isolates were susceptible.
†Tet, tetracycline; Amp, ampicillin; TMP-SMX, trimethoprim-sulfamethoxazole; Chlor, chloramphenicol; MDR, multidrug resistant; ETEC, enterotoxigenic *E. coli*; EAEC, enteroaggregative *E. coli;* aEPEC, atypical enteropathogenic *E. coli;* STEC, Shiga toxin–producing *E. coli;* EPEC, enteropathogenic *E. coli;* EIEC, enteroinvasive *E. coli*.

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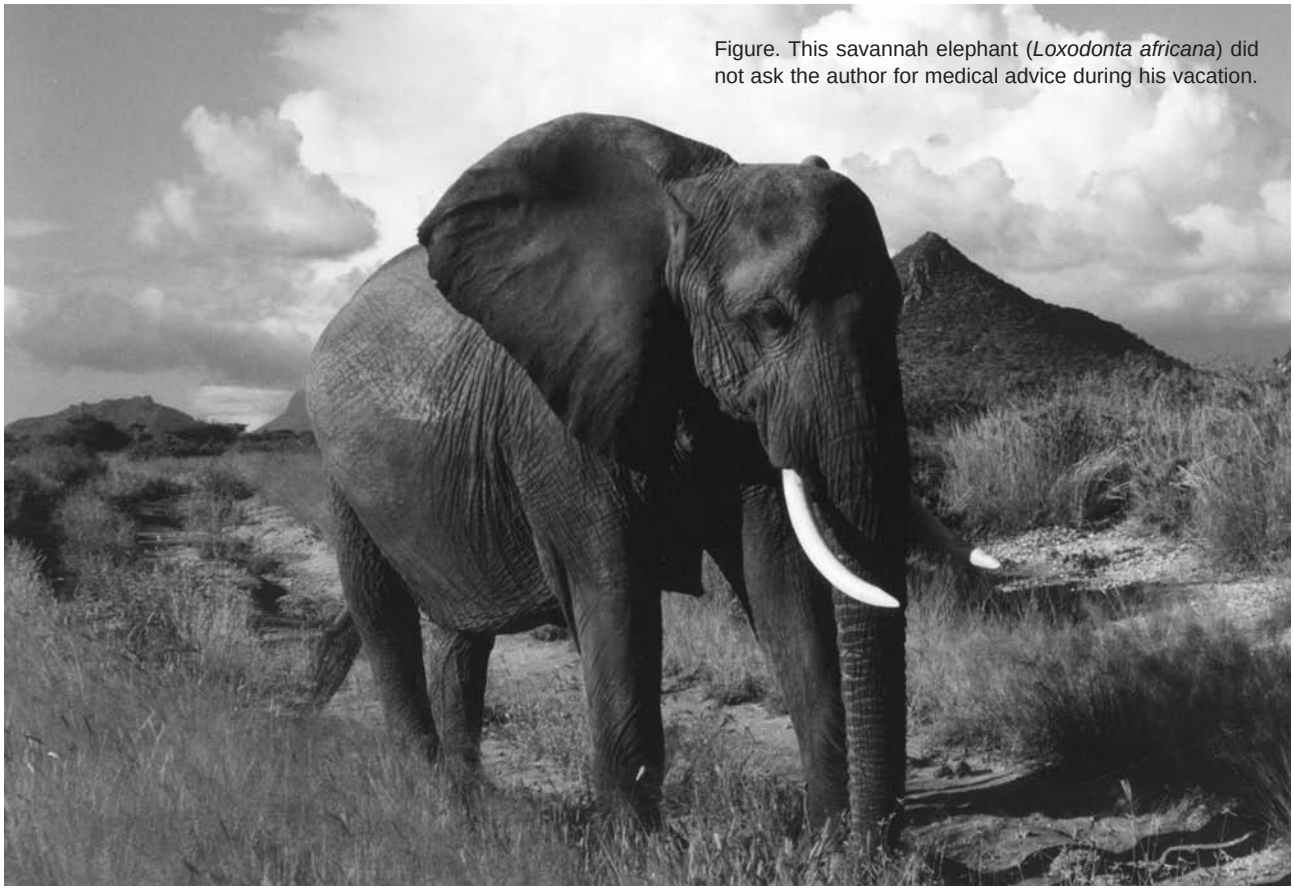

Figure. This savannah elephant (*Loxodonta africana*) did not ask the author for medical advice during his vacation.

Photograph courtesy of Edyce Winokur.

Trepid and Intrepid Travelers

Eric V. Granowitz*

When I proposed travel to Africa for our honeymoon, Edy wrinkled her nose. "Why?" she asked.

"It will be wonderful!" I exclaimed. "The people are different. The animals are different. Even the countryside is different. It will be a fantastic getaway. We'll have a great time. Can you think of anyplace better to go?"

"Martha's Vineyard," she answered. "Someplace nearby. I don't want adventure. I just want to spend time with you." Sensing my disappointment, she added, "Why not go to Africa for our first anniversary?"

After the wedding and a wonderful trip to Martha's Vineyard, we relocated. I joined a new infectious diseases division, and Edy worked as a self-employed, real estate title examiner. We began planning our safari to Kenya and Tanzania. Edy would see the flamingos of Lake Nakuru, I the wildlife on the Serengeti Plain.

In the months preceding our departure, a New York Times article on political violence in coastal Kenya precipitated anxious telephone calls to the State Department and the US embassy in Nairobi. We ordered DEET, permethrin, a water purification device, and oral rehydration salts. I cringed a little when I wrote a nonrefundable check for the trip. Edy cringed a lot when we received our yellow fever, meningococcal, and hepatitis A immunizations. Mary Jo, the nurse in our travelers' health clinic, went over a list of dietary precautions. When Edy heard from a friend about mefloquine's neuropsychiatric side effects, I could not palliate her fears. Mary Jo was able to counsel her.

The week before departure, Edy sprayed our clothes with permethrin. Another call to the State Department confirmed no recurrence of political violence. We began

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taking mefloquine. Because I had a history of anaphylaxis to bee venom, I had a prescription filled for an epinephrine autoinjector.

In Nairobi we were greeted by the touring company. A representative drove us through shanty-lined roads to an elegant country club. Next morning, we met our tour group: Noel, an Australian with a quick smile and no previous travel experience, who was embarking on a yearlong trip around the world; Bob, a broad-shouldered Mormon in a sailing T-shirt, who resembled "Mr. Clean"; Paula and Dan, a wealthy couple from Southern California. For someone with an impressive travel résumé, Dan appeared anxious. The source of his anxiety was Paula. While her eyes were filled with life, her body was not. Her ill-fitting wig, pale complexion, and unsure gait reminded me of patients on the oncology ward at the hospital. To avoid being identified as the tour physician, I introduced myself as assistant professor at a New England university.

While the rest of us tried to get over jetlag, Edy and Dan braved a thunderstorm to take a shuttle downtown. The next morning, I awoke to find Edy with her hands folded over her abdomen and a worried expression on her face. "Ice cream sundae," she admitted, when I asked what she had eaten in Nairobi. After a dose of ciprofloxacin and loperamide, Edy made her way to the jeep. Her eyelids drooped when the guide announced the 8-hour drive to our destination. Although Edy made it to the game park uneventfully, her dining partner was not as fortunate. Despite antimicrobial treatment for probable salmonellosis, Dan had fever and explosive diarrhea. Noel experienced recurrent emesis and diarrhea, probably from brushing his teeth with tap water. Forced by these circumstances to reveal my identity as an infectious disease specialist, I now became "the doctor." Dan and Noel recovered uneventfully, but my vacation changed irrevocably.

Most of our meals were now punctuated by, "Can I eat this?" The query was directed at me. "Because this is a 5-star hotel," I responded, "that's probably okay. But generally, if the food hasn't been cooked, boiled, or peeled, don't eat it." The follow-up question was, "Would you eat it?" Being conservative, I smiled and shook my head when they pointed to the salad.

When our tour group grew, the questions multiplied. People asked about vaccinations, although almost everyone had been properly immunized. There was a case of doxycycline-associated photosensitivity in a woman who did not apply sunscreen. Two people stopped malaria chemoprophylaxis because of perceived adverse effects. Another woman told me she was glad a doctor was on the trip so she didn't have to be so careful with food. I was changing, from wanting to help to becoming resentful. My companions seemed unwilling to follow simple precautions, yet expected care when their "indiscretions" made them ill.

A new companion, Lester, got travelers' diarrhea. I suggested bottled water and beverages without ice cubes. Before I could even ask, Edy reached into her knapsack and gave Lester ciprofloxacin and loperamide. That evening, I heard Lester's wife screaming my name. I arrived to find Edy's patient unconscious and held upright by his friends. After we moved Lester into the Trendelenburg position, he quickly regained consciousness. The next morning, Lester was off for a ride in a hot air balloon.

Six months before our trip, Paula had undergone a stem cell transplant for non-Hodgkin's lymphoma. She had since experienced recurrent emesis and anemia unresponsive to erythropoietin, now complicated by lightheadedness and near syncope. When Dan asked if they should abandon the tour, I recommended they return home. They remained on safari. Eventually, hematocrit of 13 at an altitude of 5,000 ft proved too much for Paula. She was flown to the hospital for emergency transfusion.

Finally, our trip concluded, Edy and I settled in for the flight home. My thoughts were a menagerie of lions, elephants, rhinoceroses, giraffes, oryxes, ostriches, and vultures. I relived the experience of watching a pack of hyenas tear the entrails out of a wild-eyed wildebeest. In spite of every effort to free itself, the wildebeest could not escape. Similarly, even on vacation, I could not escape being a doctor. My personal life had once again been compromised by my professional responsibilities.

I fell asleep. Some time later, Edy roused me with a nudge. "I was proud of the way you took care of people," she declared. This was the first time Edy had seen me practice medicine, and she had enjoyed watching me attend to our group. Her comments reminded me that caring for others was a privilege.

Before I could express these thoughts, Edy continued, "This was a great vacation. Where do you want to go next year? Thailand? It would be wonderful. The people are different. The animals are different. Even the countryside is different. It will be a fantastic getaway. We'll have a great time. Can you think of anyplace better to go?"

"Martha's Vineyard," I said.

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Real-time Polymerase Chain Reaction To Diagnose Lymphogranuloma Venereum

To the Editor: An outbreak of rectal lymphogranuloma venereum (LGV) has been detected in the Netherlands among men who have sex with men (1–4). More cases of LGV in other European countries such as Belgium, France, and the United Kingdom have been reported, and the first cases have been detected in the United States as well. This infection is encountered not only by clinicians who treat sexually transmitted diseases but also by gastroenterologists. Both the European Surveillance of Sexually Transmitted Infections (http://www.essti.org) and the Centers for Disease Control and Prevention (http://www.cdc.gov) are working on outbreak warning and response systems to increase the awareness and the direct management of the LGV outbreak (5,6).

Different approaches have been described to diagnose LGV infections (Figure). The first 3 approaches have serious disadvantages: cell culture is rarely available in routine diagnostic settings, polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis (usually nested PCR approaches are used) needs post-PCR restriction enzyme profiling, and sequencing requires additional analyses of sequence data to identify the *Chlamydia trachomatis* serovar responsible for infection. In addition, all 3 techniques are time consuming (at least 1–4 days to get a result), laborious, and require specially trained personnel in a sophisticated laboratory setting. Therefore, we developed a real-time PCR approach (TaqMan and Rotorgene) that can easily identify LGV strains in 2 hours with equipment that is available in almost all diagnostic settings.

We used the polymorphic membrane protein H gene (*pmp* gene) as a PCR target because it has a unique gap in LGV strains of *C. trachomatis*, compared to other serovars, which makes it highly specific. The following primers and probes were selected: LGV-F 5′ CTG TGC CAA CCT CAT CAT CAA 3′, LGV-R 5′ AGA CCC TTT CCG AGC ATC ACT 3′, and LGV MGB-probe 6-FAM-CCT GCT CCA ACA GT. Real-time PCR conditions (20-µL format) for TaqMan were as follows: 2× TaqMan Universal Mastermix (Applied Biosystems, Foster City, CA, USA), 18 pmol each primer, 0.2 µmol/L probe, and 2 µL (LGV L2) DNA or clinical sample; 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. Conditions for Rotorgene were as follows: 10× buffer (Hoffman-La Roche Ltd, Basel, Switzerland), 10 pmol each primer, 0.04 µmol/L probe, 2 µL (LGV L2) DNA or clinical sample; 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 sec at 95°C and 1 min at 60°C. By using a previously described serial dilution of LGV L2 (7), sensitivity was assessed as 0.01 inclusion-forming units for both real-time PCR assays.

To determine specificity, we tested different *C. trachomatis* serovars and serovariants A, B, Ba, C, D, Da, D-, E, F, G, Ga, H, I, Ia, I-, J, Jv, K, L1, L2, L2b, L3, *C. muridarum* (MoPn), *C. pneumoniae*, *C. pecorum*, *C. psittaci*, and 32 other microorganisms that normally reside in the human perianal and urogenital region and in the oropharynx. These organisms included gram-positive and gram-negative bacteria and yeast: *Acinetobacter baumannii*, *Campylobacter jejuni*, *Candida albicans*, other yeast, *Enterobacter agglomerans*, *Enterococcus faecalis*, *Escherichia coli*, *Streptococcus* spp., *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Mycoplasma* spp., *Neisseria meningitidis*, *Pasteurella* spp., *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Shigella sonnei*, *Staphylococcus aureus*, and others. Only LGV strains L1, L2, L2b, and L3 tested positive in both the TaqMan and Rotorgene assays, which shows the analytical specificity of real-time PCR.

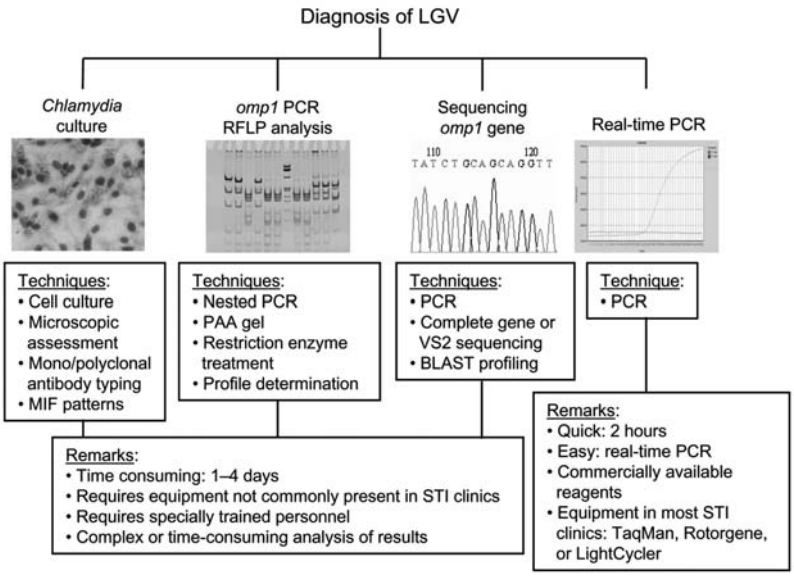

Figure. Diagnosis of lymphogranuloma venereum. MIF, microimmunofluorescence; STI, sexually transmitted infection; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; PAA, poly acrylamide; BLAST, basic local alignment search tool.

Subsequently, we determined in a blinded setting the presence of LGV in a selected group of patients (clinical spectrum and epidemiology described elsewhere [8]) according to *C. trachomatis*–positive rectal swab (Chlamydia 2SP Collection & Transport Kit [Quelab] by commercially available PCR (COBAS AMPLICOR, Hoffman-La Roche Ltd). By using the 2 reference standard techniques to type *C. trachomatis* serovars (PCR-based RFLP of the *omp1* gene or sequencing the variable segment 2 [VS-2] of the *omp1* gene) (9,10) with DNA isolated from rectal swab specimens (standard isopropanol DNA isolation method), we identified 28 of 125 men as LGV-positive. These 28 samples were also positive in both the TaqMan and Rotorgene assays. We also identified 2 additional LGV infections, which were initially typed and then retested as single-strain infections with serovars E and D by both PCR-based RFLP analysis and VS-2 sequencing. This discrepancy is most likely due to a double infection, which will, in most cases, result in the preferential amplification of 1 strain in the *omp1* PCR and PCR-based sequencing methods; in the TaqMan and Rotorgene assays, only LGV strains can be amplified. Whether this outbreak is partially technically driven must be assessed in the future by retrospectively investigating the presence of these LGV infections in men who have sex with men and the presence of the L2b strain in the past, since at present only LGV infections from 2003 to 2005 have been investigated.

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SARS Vaccine Protective in Mice

To the Editor: Less than a year after the identification of the severe acute respiratory syndrome coronavirus (SARS-CoV) (1), 3 independent laboratories reported protection from SARS-CoV challenge in animal models using a DNA vaccine or recombinant forms of the modified vaccinia Ankara or a parainfluenza virus, encoding the spike gene (2–4). Their protective efficacies are encouraging because they provide proof that a SARS-CoV vaccine is feasible. However, vaccines based on those technologies are not licensed for human use, and recommendation and licensing will likely take many years. We have developed an inactivated virus vaccine that induces neutralizing antibodies and protects against SARS-CoV challenge.

The vaccine was produced as described elsewhere (5). Briefly, the SARS-CoV (strain FRA, GenBank accession no. AY310120) was grown in Vero cells, inactivated with β-propiolactone (BPL), and complete inactivation was confirmed by 2 consecutive passages on Vero cells. Inactivated virus was purified by column chromatography followed by sucrose gradient centrifugation. The fraction containing virus was dialyzed

against phosphate-buffered saline pH 7.2, and total protein content was determined by using the Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Immunogenicity of the vaccine was tested by immunizing BALB/c mice at 0, 2, and 4 weeks with 5 μg of inactivated virus combined with the adjuvant MF59, an oil squalene-in-water emulsion (6) approved for human use in Europe for an influenza vaccine. Ten days after the third immunization, serum samples were tested for the presence of SARS-CoV spike protein–specific antibodies by using enzyme-linked immunosorbent assay, and high titers ($1–3 \times 10^4$) of anti-SARS-CoV spike immunoglobulin (Ig) G antibodies were detected. IgG subclass determination indicated a predominant Th2-type immune response similar to that observed in BALB/c mice vaccinated with a UV-inactivated SARS-CoV plus alum (7).

Efficacy of the inactivated virus vaccine was also assessed in a BALB/c mouse model of SARS-CoV infection (8). Animal studies were approved by the National Institutes of Health Animal Care and Use Committee and were conducted in an animal biosafety level 3 facility. SARS-CoV replicates in the respiratory tract of BALB/c mice following intranasal infection with 10^4 50% tissue culture infectious doses ($TCID_{50}$) of virus. Generally, virus titers peak within 2 days after infection and are cleared within 7 days (8). BALB/c mice were immunized at 0, 2, and 4 weeks with 5 μg of BPL-inactivated SARS virus with or without the MF59 adjuvant. Mice were also immunized with 4 different control preparations: phosphate-buffered saline, adjuvant MF59 alone, and 5 μg of BPL inactivated influenza A virus vaccine with or without MF59. Serum samples were collected 2 weeks after each dose, and assayed for their ability to neutralize SARS-CoV (8). After 2 vaccine doses, SARS-CoV neutralizing antibodies were detected only in the group of mice immunized with the BPL-inactivated SARS virus vaccine plus MF59 adjuvant (1:91). Two weeks after the third dose, the BPL-inactivated SARS virus vaccine without MF59 induced neutralizing titers of 1:64, while the adjuvanted vaccine elicited neutralizing titers >1:600 (Table).

Mice were challenged intranasally at this point with 10^4 $TCID_{50}$ SARS-CoV (Urbani strain, GenBank accession no. AY278741). Nasal turbinates and lung tissues were analyzed for infectious virus 2 days later (Table). SARS-CoV titers in mice from the control groups were $\approx 10^6$ $TCID_{50}$ virus/g of lung tissue and $\approx 10^3$ $TCID_{50}$ virus/g of nasal turbinate tissue. Complete protection from virus replication was observed in mice that received the MF59 adjuvanted SARS-CoV vaccine. Immunization with the nonadjuvanted vaccine resulted in complete protection of the upper respiratory tract and a significant reduction (30,000-fold) of viral titers in the lower respiratory tract compared to the control groups. The incomplete protection of this group was attributed to a single animal that contained detectable infectious virus in the lung.

Accelerated or enhanced virus replication or disease in immunized persons is a concern in developing any vaccine. This may be particularly true for SARS-CoV vaccines since adverse effects have been reported for one animal coronavirus vaccine, feline infectious peritonitis virus (9). Additionally, some in vitro experiments were performed with pseudotyped lentiviruses that expressed the spike glycoprotein derived from SARS-like virus isolated from civets. In these experiments, the presence of antibodies that neutralized most human isolates of SARS-CoV

Table. Immunogenicity and efficacy of β-propiolactone (BPL)–inactivated severe acute respiratory syndrome coronavirus (SARS-CoV) vaccine in mice against subsequent challenge with live SARS-CoV

				Virus replication upon challenge‡			
	Neutralization titer†			Lungs		Nasal turbinates	
Immunogen*	2 wk post 1st dose	2 wk post 2nd dose	2 wk post 3rd dose	No. infected/ no. tested	Mean (± SE) virus titer§	No. infected/ no. tested	Mean (± SE) virus titer§
PBS	<1:8	<1:8	<1:8	4/4	6.3 ± 0.3	3/4	2.8 ± 0.35
MF59	<1:8	<1:8	<1:8	4/4	6.1 ± 0.13	3/4	3.0 ± 0.58
Influenza A (5 μg)	<1:8	<1:8	<1:8	4/4	6.3 ± 0.07	3/4	2.9 ± 0.36
Influenza A (5 μg) + MF59	<1:8	<1:8	<1:8	4/4	6.0 ± 0.19	4/4	3.0 ± 0.11
BPL-SARS-CoV (5 μg)	<1:8	<1:8	1:64	1/4	2.0 ± 0.0¶#	0/4	≤1.8 ± 0**††
BPL-SARS-CoV (5 μg) + MF59	<1:8	1:91	1:645	0/4	≤1.5 ± 0¶**	0/4	≤1.8 ± 0**††

*The indicated immunogens or control preparations were administered to mice by subcutaneous injection on 3 occasions 2 weeks apart; PBS, phosphate-buffered saline.
†Neutralization titers were determined as described (8).
‡Mice were challenged with 10^4 50% tissue culture infectious doses ($TCID_{50}$) SARS-CoV intranasally.
§Virus titers are expressed as $\log_{10}$ $TCID_{50}$/g of tissue.
¶$p<0.00001$ in a 2-tailed Student *t* test, compared to titers seen in mice that were immunized with PBS.
#Indicates the titer of a single animal. The remaining 3 mice had no detectable levels of virus.
**Virus not detected; the lower limit of detection of infectious virus was 1.5 $\log_{10}$ $TCID_{50}$/g in a 10% wt/vol suspension of lung homogenate and 1.8 $\log_{10}$ $TCID_{50}$/g in a 5% wt/vol suspension of nasal turbinates.
††$p = 0.025$ in a 2-tailed Student *t* test, compared to titers seen in mice that were immunized with PBS.

demonstrated enhanced entry into renal epithelial cells (10). In our studies, we did not find enhanced virus replication in the respiratory tract of vaccinated mice upon SARS-CoV challenge. However, since mice are a model of SARS-CoV infection but not disease, the issue of disease enhancement will have to be carefully evaluated if and when an appropriate animal model in which this phenomenon can be demonstrated becomes available.

In summary, an inactivated SARS-CoV vaccine, produced with a technology that has a safety record established by immunizing hundreds of millions of persons, protects mice from challenge with SARS-CoV. The vaccine adjuvanted with MF59 elicits neutralizing antibodies (titer 1:91) after only 2 doses. We conclude that the vaccine described here has desirable properties, and our data support further development and plans for clinical trials.

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Echinococcosis, Ningxia, China

To the Editor: Ningxia is the smallest provincial autonomous region on the Loess Plateau in central China, with a population of ≈5.5 million persons composed of 35 ethnic and various religious groups. The natural environmental conditions and socioreligious ethnic status in Ningxia, particularly the southern region, are conducive to sheep farming, an important part of the agricultural economy. Both cystic echinococcosis (CE) and alveolar echinococcosis (AE) (1) are endemic in the northwest part of China; high prevalences have been reported in several provinces (1), including Gansu (2,3) and the Xinjiang Uigur Autonomous Region (4). Little information is available about the extent of human echinococcosis in Ningxia; this is the first report of a provincial investigation for both human CE and AE there.

We conducted a retrospective survey of clinical records from 7 local county hospitals and 4 other hospitals in Yinchuan to determine the epidemiology of human echinococcosis in southern Ningxia. All surgical and clinical records were checked, and diagnoses were confirmed based on imaging, surgical reports, and histopathologic reports. Data concerning age, sex, domicile, ethnicity, occupation, year of diagnosis, cyst or lesion numbers and anatomical location, type and duration of anthelminthic treatment (if given), and the nature and number of surgeries performed for echinococcosis were recorded for each confirmed CE and AE patient.

From 1985 to 2002, a total of 2,216 cases of echinococcosis were recorded, most of which were due to CE (96%). The incidence of combined CE and AE from 1994 to 2001 was 7/100,000 persons for southern Ningxia, compared with 1/100,000 persons for Yinchuan in the north

(Table). Human AE cases were reported only from the 3 southern counties of Xiji, Haiyuan, and Guyuan; CE cases occurred throughout Ningxia, with a mixed endemic focus for both human CE and AE in the south. A variable distribution of AE and CE cases from 1994 to 2001 was evident from hospital records. Both AE and CE were recorded in Xiji (6/100,000 persons), Guyuan (5/100,000 persons), and Haiyuan (11/100,000 persons) counties. CE incidences for Tongxin (13/100,000 persons) and Pengyang (5/100,000 persons) were substantially higher than for Longde (0.5/100,000 persons) and Jingyuan (0.15/100,000 persons). This heterogeneous distribution of echinococcosis may reflect different patterns of parasite transmission in different areas of Ningxia. CE and AE incidence (Table) were compared for 1994 and 2001 by chi-square test. Apart from a substantial increase in Haiyuan ($p<0.05$) and a substantial decrease in Xiji ($p<0.05$), no other substantial incidence changes were apparent (Table).

Patients' ages ranged from 1 to 80 years for men and from 3 to 77 years for women (mean age 35.7 years). The patient sex ratio was 0.72 (916 men, 1,268 women), whereas the population sex ratio was 1.05, indicating significant differences in echinococcosis case numbers between men and women ($p<0.05$). Farm laborers accounted for 66.1% (1,464/2,216) of cases, students 12.4% (275/2,216), workers and self-employed 5.2% (116/2,216), village leaders 4.8% (106/2,216), teachers and housewives 0.9% (21/2,216), butchers 0.2% (4/2,216), and others 0.1%. A comparison of the ethnic composition ratio (5) with an average incidence from 1994 to 2001 showed a substantial difference between the Hui and Han nationalities in Yinchuan, Haiyuan, and Pengyang, and for Ningxia as a whole. No substantial difference was shown in Longde, Tongxin, Guyuan, and Xiji for the 2 ethnic groups by incidence risk ratios with 95% confidence intervals (Table).

Radical surgery to remove CE cysts was performed for 83.3% of patients; only 7.3% of patients received combined albendazole or mebendazole chemotherapy pre- and post-surgery. Recurrences of CE cysts that required surgery were high (30%), suggesting inadequate surgical and medical care. Most AE patients received only chemotherapy treatment and were at an advanced stage of infection.

The retrospective data of human AE and CE infection rates in the current study suggest that disease transmission was more pronounced 10–15 years earlier. The landscape of woods and scrub cover was greater then, domestic sheep and dog populations were larger, and red fox and rodent species densities were likely higher, providing optimal transmission conditions for both *Echinococcus granulosus* and *E. multilocularis*.

The incidence data (2001) for human CE and AE are likely underestimated for several reasons. Access to medical treatment for villagers is problematic and many asymptomatic cases (6) would be excluded. An age and sex bias also exists, as generally older persons and women receive most of the limited medical attention. Furthermore, the high surgical costs for treatment (CE and AE), especially in the past 10 years, coupled with poor economic development, preclude access to treatment in many rural communities. Because abdominal ultrasound examinations for CE and AE can identify clinically silent infections (7,8), comprehensive community-based studies are needed to identify asymptomatic or early-stage cases.

Our study provides accurate data to determine the true prevalence and incidence of CE and AE, which contributes to better treatment outcomes. These data can help determine the

Table. Average incidence of human echinococcosis (AE/CE combined), 1994–2001, and incidence compared for ethnicity (Han/Hui) in southern Ningxia and Yinchuan City*

			Ethnicity comparison							
	1994–2001		Hui (1994–2001)			Han (1994–2001)				
Location (population)	Cases	Incidence†	%‡	Cases	Incidence†	%‡	Cases	Incidence†	IRR	95% CI
Ningxia										
Tongxin (328,700)	353	13.42	81.49	291	13.58	18.48	62	12.75	0.94	0.71–1.25§
Haiyuan (344,000)	305	11.08	70.58	195	10.04	28.52	110	14.01	1.40	1.10–1.77
Guyuan (470,700)	226	6.00	43.23	108	6.63	56.70	118	5.52	0.83	0.64–1.09§
Xiji (403,100)	198	6.14	52.00	89	5.30	47.99	109	7.04	1.33	0.99–1.77§
Pengyang (231,000)	101	5.46	29.77	46	8.36	70.23	55	4.24	0.51	0.34–0.76
Longde (197,300)	8	0.50	8.98	1	0.70	91.01	7	0.53	0.76	0.1–16§
Jingyuan (81.301)	1	0.15	96.50	1	0.15	3.48	0	0	—	§
Subtotal (2,056,101)	1,192	7.24	—	—	—	—	—	—	—	—
Yinchuan (1,614,700)	165	1.27	18.36	59	2.48	79.88	106	1.02	0.41	0.3–0.57
Total (3,670,801)	1,357	4.62	33.88	790	7.94	65.47	567	2.94	0.37	0.33–0.41

*AE, alveolar echinococcosis; CE, cystic echinococcosis; IRR, incidence risk ratio; CI, confidence interval.
†Cases/100,000/year.
‡Ethnicity composition ratio (%) at each location.
§Comparison between ethnicity composition ratio and incidence not significant.

reasons for the heterogeneity of disease distribution in Ningxia to better prepare for future echinococcosis control strategies throughout the region.

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Mycobacterium neoaurum Contamination

To the Editor: In reviewing "Rapidly Progressive Dementia due to *Mycobacterium neoaurum* Meningoencephalitis," by Heckman et al. (1), I found, contrary to the authors' conclusion, that *M. neoaurum* was more likely a contaminant than a cause. First, within the granulomatous brain lesions, the strongest evidence for the authors' conclusion, no acid-fast bacilli were isolated or identified on special stains; thus, the Koch postulates were not satisfied. Rather, the lesions were likely rheumatoid nodules. Longstanding rheumatoid arthritis commonly causes granulomalike rheumatoid nodules. I did a PubMed search using "rheumatoid nodule in the brain" and 7 articles were found (2,3). A "rheumatoid endarteritis" search found 25 articles. Heckman et al. failed to exclude or discuss this possibility.

Second, *M. neoaurum* is a rare environmental mycobacterium that grows in ≤2 days on sheep blood agar and is not difficult to culture. As the authors stated, there have been 8 reports of this organism, 7 isolated from blood and 1 from urine. The blood isolates were associated with either central venous catheter or intravenous drug use. Thus, *M. neoaurum* is of low virulence and unlikely to cause spontaneous infection in tissue unless inoculated accidentally, perhaps. Third, polymerase chain reaction (PCR) is exquisitely sensitive and prone to contamination. The problem is worse when bacterial DNA is amplified by using highly conserved primers. The PCR reagents, from the Taq polymerase (of bacterial origin) to water, contain sufficient, despite minute quantity, bacterial DNA to be amplified (4). Although direct sequencing of the amplicon is often blurry because of its low quantity and mixed content, when cloned, each amplicon may be ligated to the vector and proliferates and gets sequenced later.

Therefore, I believe the presence of *M. neoaurum* DNA, not the organism itself, represented contamination. Generally, drawing cause-disease conclusion based on PCR sequencing needs vigilance to satisfy the modified Koch postulates (5).

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In Response: In response to our report on a case of rapidly progressive dementia (1,2), Dr. Han argues that *Mycobacterium neoaurum* was "more likely a contaminant than the cause" and that the actual cause of death was most likely rheumatoid pachymeningitis. Dr. Han bases his argument on the absence of positive acid-fast stains or mycobacterial cultures and his assessments that the identification of *M. neoaurum* DNA was due to contamination and that the pathologic findings represented rheumatoid nodules.

The inability to stain or culture an organism in this case is not unusual, as paucibacillary mycobacterial infections, such as tuberculous lymphadenitis and leprosy, are common (3,4). Though the possibility is not inconceivable, environmental contamination is unlikely, because tissue samples were positive with *M. neoaurum*–specific primers, whereas controls containing identical reagents but no tissue were not.

Dr. Han expresses a valid concern that rheumatoid pachymeningitis was not given due consideration. Rheumatoid pachymeningitis is a rare complication of rheumatoid arthritis, in which patients may exhibit headache, cranial neuropathies, focal deficits, seizures, or cognitive dysfunction (5,6). Rheumatoid pachymeningitis usually, but not exclusively, occurs in patients with long-standing rheumatoid arthritis characterized by erosive disease and extra-articular manifestations, although the systemic disease may be quiescent when neurologic complications arise. Cerebrospinal fluid analysis is generally nonspecific. Magnetic resonance imaging may show prominent meningeal enhancement. Pathologic features may include vasculitis, rheumatoid nodules, and meningeal inflammation, with the latter 2 features being most common (5). The dura may demonstrate inflammation with fibrinoid necrosis (6). We reviewed the pathologic specimens of this case and confirmed the presence of abundant giant cells, endarteritis proliferans, and, most notably, extensive caseation necrosis typical of mycobacterial infection. We found no evidence of rheumatoid nodules, dural inflammation, or fibrinoid necrosis.

Though this case does not satisfy Koch postulates, neither do most novel infectious diseases. Substantial international efforts were required to satisfy the postulates in the case of SARS (7). In this case, the identification of DNA from a "rare environmental mycobacterium" in a patient with overwhelming pathologic evidence of mycobacterial infection provides strong, though not foolproof, evidence of a possible causal role.

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Yersinia pestis Genotyping

To the Editor: Drancourt et al. (1) report the development of an original genotyping system for *Yersinia pestis* based on intergenic spacer sequencing. However, the approach appears to rely upon the characterization of polymorphisms due to tandem repeat variation. Eight spacers are used in the report, 7 of which contain tandem repeats, and the sequence variability used to produce the typing data and

the strain clustering result from variation in the number of tandem repeats (and incorrect data analysis produces a dendrogram with 34 branches from only 19 different isolate types). Three of the spacers and associated polymorphisms were previously reported. Spacers YP3 and YP5 are, respectively, ms38 and ms56 (2); spacer YP10 is M61 (3). YP3 is later used to investigate ancient DNA samples, and 3 amplification products are described in detail. The sequences are compared to modern sequences by BLAST analysis, which is not relevant for tandem repeats. Instead, the Figure shows how internal variation within the array can be coded to facilitate interpretation. In this collection, Orientalis strains are "abcdeeef," whereas Antiqua strains from Africa are "abcdeef." All these different codes can be deduced one from the other by simple duplication and deletion events, with no need to invoke point mutations. The codes for all 3 ancient samples are identical to the Orientalis code "abcdeeef."

In conclusion, the data presented by Drancourt et al. do not appear to support their claim. They did not invent a new genotyping method but used the well-known multiple locus variable analysis (MLVA) number of tandem repeats approach. The finding that the "genotype Orientalis was involved in all three pandemics" is not valid since the Orientalis type is defined by a biochemical assay, resulting in all known Orientalis strains from a 93-bp glycerol-3-phosphate dehydrogenase microdeletion (4,5), which was not investigated here.

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A

'dictionary'

```
a : --G---G--------A
b : --G---G-T-------
c : -----T-T----G---
d : ------------G---
e : ACCAGCTCCAACAATT
f : -T---T-T--------
g : -----T----------
h : -----T------G---
```

B

internal coding

Medievalis	abceef
Antiqua	abcdeef
Antiqua (Asia)	abcdceef
Orientalis	abcdeeef
#202 (Justinien)	abcdeeef
#283	abcdeeef
#292	abcdeeef
Ypseu	abcdgdhhhef
Microtus	abcdceef

Figure. A) sequence-to-code correspondence (1 letter per 16-bp repeat unit). Differences from repeat unit "e" are shown. B) Tandem repeat arrays were coded accordingly. All sequences were obtained from Genbank (Ypseu: *Yersinia pseudotuberculosis* IP32953; Microtus: "*Y. microtus*" Chinese strain #91001).

In response: We thank Dr. Vergnaud for his response (1). Since the time of our publication (2), 2 articles related to our paper were either submitted or published. One (3) reported identification of *Yersinia pestis*–specific genes in teeth from patients who died during the Justinian plague; another proposed identification of *Y. pestis* strains by using variable numbers of tandem repeats analysis (VNTR) (4). The authors concluded that isolates could easily be compared in their database by using 7 markers. As opposed to work with cultures where ample, high-quality DNA template is available, successful amplifications with 7 different primer sets cannot be achieved by using DNA extracted from ancient teeth (5). By comparing genome sequences, we evaluated short intergenic spacers that were more divergent. Divergences included mutations, deletions, and duplications (VNTR). Phylogenetically, an entire repeat unit has the same weight as that of a single nucleotide polymorphism. By sequencing, we have identified all events (single nucleotide polymorphism and VNTR). Sequencing is more versatile for use in strain identification (5),

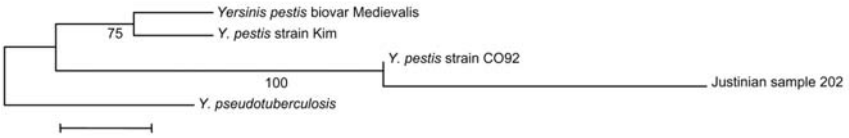

Figure: Unrooted tree showing the phylogenetic relationships between the sequence obtained from the YP3 spacer from Justinian sample 202 and that from the genomes of *Yersinia pestis* strain CO92 (GenBank accession no. AJ414159), *Y. pestis* biovar Medievalis (AE017139), *Y. pestis* strain Kim (AE013993), and *Y. pseudotuberculosis* (BX936398). DNA sequences were aligned by using the ClustalW software, version 1.81 (2). Deletions were considered single events. A distance matrix was constructed by using the Kimura-2 parameter, and the phylogenetic tree was inferred by using the neighbor-joining method in the Mega2 software package. The scale bar represents a 0.5% nucleotide sequence divergence. Bootstrap values are indicated at the nodes (2).

allows distinction at the species level, and can be applied directly on clinical and forensic samples.

The discovery of a unique sequence is critical to authenticate results in such controversial areas as paleomicrobiology (5). Fortunately, we have identified a unique sequence that contains several mutations. These mutations do not exclude this strain from being *Y. pestis* (see Figure). Additionally, we doubt that our conclusions would have been accepted had we simply used the VNTR, demonstrating only an amplicon of the right size on a gel.

In conclusion, our results have been validated by others. The sequence is original and, therefore, authentic. Dr. Vergnaud agrees that the results we presented did represent a sequence associated with the Orientalis biovar. This finding may end the controversy.

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Pandemic *Vibrio parahaemolyticus* O3:K6, Europe

To the Editor: *Vibrio parahaemolyticus* is a halophilic member of the genus *Vibrio* that inhabits temperate and tropical marine environments worldwide. Strains that produce the thermostable direct hemolysin or the thermostable direct hemolysin-related hemolysin, which are encoded by *tdh* and *trh* genes, respectively, are considered pathogenic. While almost all clinical strains have these virulence factors, these strains represent <1% of all environmental strains.

Recently, *V. parahaemolyticus* infections have increased globally; they are usually associated with eating raw or undercooked seafood. *V. parahaemolyticus* is the leading cause of seafood-associated bacterial gastroenteritis in the United States (1) and causes approximately half of the foodborne outbreaks in some Asian countries (2). In 2001, the Scientific Committee on Veterinary Measures Relating to Public Health of the European Commission concluded that *V. parahaemolyticus* outbreaks are rarely reported in Europe (3). Because the risk of *V. parahaemolyticus* infection is extremely low in Europe, the organism has been excluded from the European Network for Epidemiologic Surveillance and Control of Communicable Diseases and from Microbiologic Surveillance System for Infectious Gastroenteritis. *V. parahaemolyticus* is also excluded from the European applicable microbiologic requirements for shellfish-harvesting areas and ready-to-eat seafood.

However, data obtained after an exhaustive review of clinical journals published in Spain and from unreported cases of *V. parahaemolyticus* infections identified at Spanish hospitals have shown that *V. parahaemolyticus* infections in Spain are more common than previously assumed. This organism was isolated from patients with gastroenteritis in Barcelona (1986, 1987, and 1999), Zaragoza (1993), and Madrid (1998 and 2000). In Galicia (northwestern Spain) alone, where most Spanish shellfish are produced, 84 cases of *V. parahaemolyticus* infection were identified retrospectively from hospital records from 1997 to 2000. A single outbreak of 64 cases in 1999 was associated with oyster consumption (4). Most Spanish clinical isolates were serotype O4:K11, and pulsed-field gel elec-

trophoresis (PFGE) analysis demonstrated these to be a unique clone distinct from Asian and American clinical strains (5).

In July 2004, a *V. parahaemolyticus* outbreak of 80 illnesses occurred in A Coruña, Spain. All the case-patients attended weddings in the same restaurant. *V. parahaemolyticus* was isolated from stool samples of 3 patients. The outbreak isolates were characterized by serotyping, polymerase chain reaction (PCR) for species-specific genes (Vp-*toxR* and *tlh*), virulence-related genes (*tdh* and *trh*), and group specific (GS)-PCR (a PCR method to detect the pandemic clone). Two isolates belonged to the serotype O3:K6, while the remaining isolate was O3:K untypeable. All 3 isolates had the *toxR*, *tlh*, and *tdh* genes, lacked the *trh* gene, and were positive for the GS-PCR assay to detect pandemic strains. These results unequivocally linked the outbreak isolates to the O3:K6 pandemic clone of *V. parahaemolyticus*. To confirm the relationship with the pandemic clone, the outbreak isolates were additionally subjected to DNA fingerprinting analyses. PFGE and arbitrarily primed PCR analyses showed that these isolates exhibited a pattern indistinguishable from those of pandemic strains from Asia. The epidemiologic investigation associated with the outbreak identified the boiled crab eaten in the restaurant as the most probable source of the infection. Live crabs were imported to Spain from the United Kingdom, processed under unhealthy conditions, and stored at room temperature for several hours before they were eaten. All the seafood eaten at the weddings was harvested in Europe, and no imported food was eaten or handled in the restaurant.

Pandemic O3:K6 clone of *V. parahaemolyticus* appeared in Asia around 1996 (6). Since its emergence, it has accounted for most *V. parahaemolyticus* infections in Asia. It spread to the United States in 1998 (7) and more recently to Chile (8), where it has caused hundreds of infections, resulting in the first *V. parahaemolyticus* pandemic in history (9). We report the first evidence that it has been introduced to Europe. The emergence of this virulent serotype in Europe is a public health concern and emphasizes the need to include *V. parahaemolyticus* in microbiologic surveillance and reexamine control programs for shellfish-harvesting areas and ready-to-eat seafood.

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Q Fever and the US Military

To the Editor: Q fever is a zoonotic disease caused by the rickettsialike organism *Coxiella burnetii*. The disease has a worldwide distribution and can infect many different species, although cattle, sheep, and goats are the primary reservoirs (1). Transmission to humans usually occurs by inhaling dust or aerosols from infected animals, and approximately half of infected persons manifest clinical symptoms. In acute Q fever infection, the 3 main sets of symptoms are flulike syndrome, pneumonia, and hepatitis (2,3).

Q fever has military relevance not only in its potential use as a bioterrorism agent, but also because of the risk for natural infection in deployed military personnel. Thousands of cases of Q fever have been seen in military personnel since the disease was first reported in the 1930s (4). Since the most common mode of transmission is airborne, personnel do not need to have direct contact with infected animals to be exposed.

C. burnetii was first recognized as an infectious disease threat to US military troops serving in Iraq in 2003 during a pneumonia outbreak investigation. Nineteen cases of severe pneumonia, including 2 deaths, occurred from March 1 to August 20 (5). A case was defined as occurring in a patient with bilateral alveolar infiltrates that required intubation and mechanical ventilation. This investigation involved extensive serologic testing for possible infectious causes of pneumonia, including *C. burnetii*. Of 19 patients with severe pneumonia tested for *C. burnetii*, 3 had positive antibody titers by immunofluorescence assay (IFA). No other infectious cause was confirmed for the remaining cases of pneumonia. Although *C. burnetii* was not determined to be the cause of the pneumonia outbreak, the finding of 3 patients with positive antibody titers launched an effort to ascertain other cases of Q fever among military personnel who served in Iraq during that time.

Approximately 62 cases of pneumonia, both severe and nonsevere, occurred in Iraq from March 1 to August 20, 2003. A pneumonia case was defined as occurring in a patient with a chest radiograph suggesting pneumonia and ≥1 of the following symptoms: fever, cough, or shortness of breath. The Defense Medical Surveillance System (DMSS) was queried to determine how many patients had both predeployment and postdeployment serum samples available for Q fever testing. The Army Medical Surveillance Activity, which operates DMSS, also maintains the Department of Defense Serum Repository and stores serum from service members after mandatory HIV testing and deployment processing (6). Predeployment sera must be collected within the year before deployment.

Twenty-two soldiers had predeployment and postdeployment sera available; samples were tested for phase I and phase II antibody to Q fever by using IFA. Results showed 5 additional soldiers in whom pneumonia was diagnosed while serving in Iraq and who seroconverted to *C. burnetii* before postdeployment serum draws (Table). All predeployment antibody titers for both immunoglobulin (Ig) G and IgM were negative in these 5 soldiers, with an IFA titer of 1:16 as a cutoff.

The initial 3 Q fever patients ascertained through the pneumonia outbreak investigation were extensively interviewed for possible exposures. All 3 patients first experienced symptoms while in northern Iraq and reported contact with domestic animals, including dogs, cats, sheep, goats, and camels. Two of the patients reported tick bites within 30 days before becoming ill, and 1 reported drinking raw sheep's milk. The 5 other patients who became ill with pneumonia also first sought care while in northern Iraq. Predeployment sera from these 3 patients were also tested for *C. burnetii* by IFA, and all samples were negative for both IgG and IgM.

Extremely limited information is available on Q fever disease prevalence in Iraq, either in animals or humans. Iraq is primarily an agricultural country, and nomadic herding takes place countrywide, except in the northernmost regions and along the eastern border, where adequate land is available for grazing livestock. The most common livestock in Iraq are cattle, sheep, and goats (7). Although herds of infected animals may exist in any region of Iraq, larger concentrations of livestock may exist in northern areas, where land is suitable for ruminants to graze. This concentration could lead to a higher risk for transmission to humans because the chance of contact with infected animals would be greater.

These data indicate the potential importance of *C. burnetii* as an infectious disease threat to US military troops in Iraq. Healthcare providers should include Q fever in their differential diagnosis of community-acquired pneumonia and consider adding doxycycline to a combined antimicrobial drug regimen to presumptively treat severe pneumonia. Future studies to be completed include case ascertainment to locate US troops who were infected with Q fever while in Iraq and in whom pneumonia or other clinical manifestations of illness may have developed.

Table. Postdeployment serum antibody titers to phase II antigen for Q fever in 8 US military personnel who served in Iraq, March 1–August 20, 2003*

Patient	IgG	IgM
1	1:1,024	Negative
2	1:128	Negative
3	>1:1,024	1:512
4	1:256	1:256
5	1:512	>1:1,024
6	1:512	1:512
7	1:64	1:64
8	>1:1,024	>1:1,024

*All predeployment titers were negative for immunoglobulin (Ig) G and IgM.

Research was conducted in compliance with the Animal Welfare Act and

other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition.

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Anaplasma phagocytophilum, Sardinia, Italy

To the Editor: *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*), a tick-transmitted pathogen that infects several animal species, including humans (involved as accidental "dead-end" hosts), is the causative agent of human granulocytic anaplasmosis (HGA). It is a pathogen of veterinary importance responsible for tickborne fever of ruminants and for granulocytic anaplasmosis of horses and dogs (1,2). HGA was first described in the United States in 1994 (2) and is emerging in Europe (3). Although only 2 human cases have been reported in Italy (4), serologic and molecular findings have shown *A. phagocytophilum* infections in dogs and *Ixodes ricinus* ticks (5). Incidence, prevalence, and public impact of HGA and horse granulocytic anaplasmosis are, therefore, unknown for this geographic area. From 1992 to 1996, an average rate of 13.4 cases/year/100,000 inhabitants of tick bite–related fever of unknown etiology has been reported on the island of Sardinia, Italy, which is considerably higher than the corresponding national average value of 2.1 cases/year/100,000 inhabitants. Moreover, 117 cases of tick bite–related fever, whose etiology remains obscure, have been reported from 1995 to 2002 in the central west coast area of the island. Local newspapers occasionally report deaths as a result of tick bites, although no HGA-associated deaths have been documented in Europe.

This study investigated *A. phagocytophilum* in Sardinia. From 2002 to 2004, veterinarians based on the central west coast of the island were instructed to collect EDTA blood samples when a suspected case of tick bite–related fever was found at their clinics. A total of 70 blood samples were collected from 50 dogs and 20 horses that showed tick infestation and symptoms consistent with tickborne disease, such as fever, anorexia, jaundice (only in horses), anemia, myalgia, and reluctance to move. Genomic DNA was extracted from the buffy coat obtained by centrifugation of 2 to 4 mL of blood, as previously described (6). Furthermore, DNA was extracted from 50 *Rhipicephalus sanguineus* ticks removed from 30 dogs. Primers EphplgroEL(569)F (ATGGTATGCAGTTTGATCGC), EphplgroEL (1193)R (TCTACTCTGTCTTTGCGTTC), and EphgroEL(1142)R (TTGAGTACAGCAACACCACCGGAA) were designed and used in combination to generate a heminested polymerase chain reaction (PCR) for the selective amplification of 573 bp of the *groEL* gene of *A. phagocytophilum*. The final 50 μL PCR volume of the first PCR round contained 5 μL of the DNA extraction, primers EphplgroEL (569)F and EphplgroEL(1193)R, and HotMaster Taq DNA polymerase (5u/μL, Eppendorf) according to the manufacturer's basic protocol (Eppendorf AG, Hamburg, Germany). Heminested PCR was performed by using 5 μL of each of the first PCR products and primer EphgroEL (1142)R. To confirm the PCR diagnosis, amplicons were digested with the *Hin*dIII restriction endonuclease (predicted digestion pattern: 3 fragments of 525 bp, 21 bp, and 27 bp). *Anaplasma phagocytophilum* DNA was obtained from strain NCH-1 and used as positive control in PCR reactions. Sequences were obtained by cloning the PCR products into the pCR2.1-TOPO vector (Invitrogen S.R.L., Milan, Italy) and using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), according to the protocols supplied by the manufacturers. Sequences (AY848751, AY848747) were aligned to the corresponding

region of other species belonging to *Rickettsiales* by using ClustalX (7). Genetic distances among species were computed by the Kimura 2-parameters method by using MEGA, and were used to construct bootstrapped neighbor-joining trees (8).

Of 120 DNA samples, 1 tick, 3 dog, and 3 horse samples generated the predicted band of 573 bp representative of the *groEL* gene of *A. phagocytophilum*. *Hin*dIII digestions confirmed PCR diagnosis (see Appendix Figure, available at http://www.cdc.gov/ncidod/eid/vol11no08/05-0085_app.htm). Two different *groEL* sequence types were obtained from 1 dog and 1 horse and confirmed by BLAST (http://www. ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html) queries as *A. phagocytophilum groEL* sequences (average identity 99%; average E value = 0), indicating that sequences did not reflect contamination. Bootstrapped neighbor-joining trees confirmed the identity of the new sequences obtained, which are closely related to HGA strains isolated in Europe and the United States (Figure).

The molecular approach applied in this study established *A. phagocytophilum* in an area of Sardinia characterized by a high prevalence of tick bite–related fever in humans and animal species. To our knowledge, this is the first evidence of *A. phagocytophilum* in Sardinian dogs and horses and the first documentation of infection in Italian horses caused by pathogenic strains. Therefore, these findings suggest the emergence of *Anaplasma phagocytophilum* in Italy. *Ixodes ricinus* ticks are indicated as vectors transmitting *A. phagocytophilum* in Europe. Although only 0.3% of 4,086 ticks collected in 72 sites of Sardinia (9) have been identified as *Ixodes*, other tick species are better represented on the island (*Rhipicephalus*, 67.2%; *Haemaphysalis*, 24.1%; *Dermacentor*, 4.9%). *A. phagocytophilum* in 1 *Rhipicephalus sanguineus* could indicate a role of this tick in the epidemiology of HGA. Finally, these data indicate the presence of a potential threat to human and animal health and suggest activation of further epidemiologic surveillance and controls.

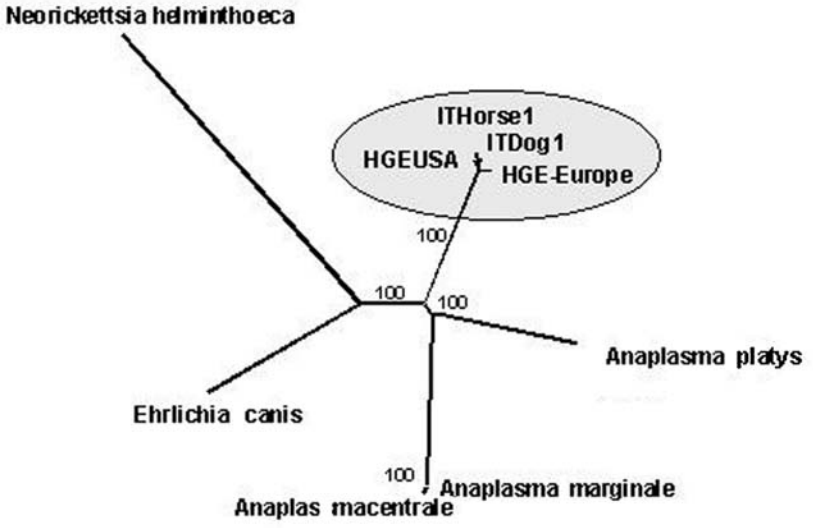

Figure. Bootstrapped neighbor-joining tree of several species belonging to Rickettsiales and identification of the strains isolated during the study as *Anaplasma phagocytophilum.* Strains associated to Sardinian *groEL* variants are closely related to European and American pathogenic human granulocytic anaplasmosis strains. Numbers indicate statistically supported bootstrap values.

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Williamsia muralis Pulmonary Infection

To the Editor: Bacteria of the genus *Williamsia* are mycolic acid–containing actinomycetes of the suborder Corynebacterineae (1). This suborder also includes the genera *Gordonia*, *Mycobacterium*, *Nocardia*, *Corynebacterium*, *Rhodococcus*, *Dietzia*, *Skermania*, *Tsukamurella*, and *Turicella* (2,3). Within the genus *Williamsia*, only 2 species have been reported: *Williamsia muralis*, isolated from a daycare center (4), and *W. maris*, isolated from the Sea of Japan (5). One important aspect shared by both species is their apparent lack of pathogenicity, since they have been isolated only from environmental samples.

An 80-year-old woman, whose medical history included allergy to penicillin and high blood pressure, was admitted to the cardiothoracic intensive care unit at Juan Canalejo Hospital Complex in La Coruña, Spain, because of a loss of consciousness following an aortic valve replacement. Physical examination showed a systolic murmur and an echocardiogram showed aortic stenosis. Transaortic peak pressure was 100 mm Hg, and the aortic valvular area was 0.3 cm^2. A biologic valve prosthesis (Mitroflow 21, Sorin Group Canada, Ltd., Burnaby, British Columbia, Canada) was inserted under the cardiopulmonary bypass.

Forty-eight hours later, the patient had paroxysmal atrial fibrillation and a temperature of 39°C, with severe hemodynamic and respiratory impairment. She was intubated and intravenous drugs were administered. Blood and urine cultures were requested. Central venous pressure lines were changed, and cultures were obtained. Empiric treatment with levofloxacin, amikacin, and teicoplanin was started for the patient. One of 2 blood cultures was positive for *Staphylococcus epidermidis*, as were cultures from femoral and jugular venous lines. Although considered a contaminant, we observed that *S. epidermidis* was susceptible to empiric antimicrobial drugs.

One week later, a chest radiograph showed bilateral alveolar infiltrates suggestive of pulmonary edema (Figure). To rule out infection, bronchoscopy and protected specimen brush were conducted. An unidentified gram-positive bacillus was cultured from the brush sample. Urine cultures were positive for *Candida kefyr*, but the patient showed no evidence of candidemia. An echocardiogram showed no evidence of infective endocarditis. Since the patient's condition did not improve, levofloxacin was replaced with imipenem, and treatment with fluconazole was initiated. However, the patient developed septic shock, adult respiratory distress syndrome, and oliguric acute renal failure, and died of multiple organ failure.

On direct examination, a Gram stain of the protected specimen brush sample showed numerous gram-positive bacilli. After incubation for 48 h in either an aerobic or capnophilic atmosphere, >1,000 CFU/mL were observed on Columbia agar plates containing 5% sheep blood (BD Stacker Plates, BBL, Franklin Lakes,

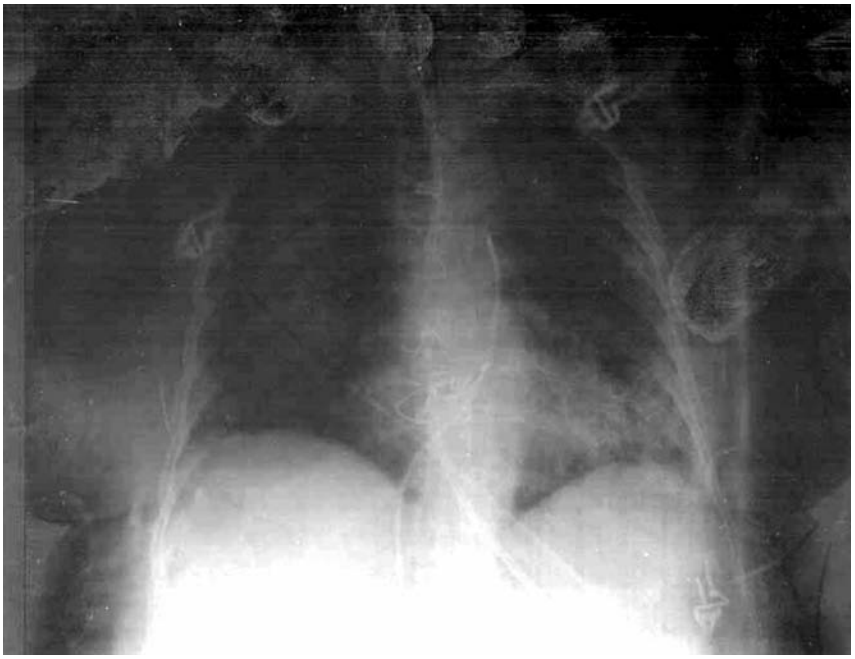

Figure. Chest radiograph of the patient showing bilateral alveolar infiltrates. Although pulmonary edema was the initial diagnosis, an infectious cause should be considered and, on the basis of sepsis, appropriate treatment initiated.

NJ, USA). These colonies were round, slightly convex, white to pale yellow, and 1–3 mm in diameter. Microscopic examination showed short gram-positive bacilli. A few colonies of coagulase-negative staphylococci were also isolated from the clinical sample.

We attempted biochemical identification of the gram-positive bacilli, but discordant results were obtained. Test results of cultures after 24 and 48 h with a commercial assay (apiCoryne, bioMérieux, Marcy l'Etoile, France) identified the bacilli as a *Rhodococcus* spp. (probability >98.2%). However, negative results by Kinyoun modified acid-fast staining and by the CAMP test, 2 features characteristic of *Rhodococcus equi*, aroused suspicion regarding the unusual properties of this isolate.

Genomic DNA was isolated from the bacilli and analyzed by polymerase chain reaction (PCR)–mediated amplification of 16S ribosomal DNA, purification of PCR products, and direct sequencing, as previously reported (6). The 16S rRNA gene sequence (1,438 bp) of the isolate (500/04; GenBank accession no. AY986734) showed 99% similarity with *W. muralis* (4). Other noteworthy similarity matches of the isolate were with *W. maris* (96%), *Gordonia* sp. (95%), *Nocardia transvalensis* (95%), and *Rhodococcus* sp. (95%).

Antimicrobial drug susceptibility patterns were determined by using a commercial assay (Trek Diagnostic Systems Ltd., East Grinstead, UK). Since no interpretive criteria exist for *Williamsia* spp., those previously reported for *Nocardia* spp. (7,8) were used for estimating breakpoints. Results showed the isolate was susceptible to amoxicillin-clavulanate, cefotaxime, imipenem, ciprofloxacin, tobramycin, gentamicin, and cotrimoxazole and resistant to ampicillin and erythromycin after incubation for 48 h in either air or a CO_2 atmosphere (GasPak CO_2 Pouch Capnophilic System, BD Biosciences, Sparks, MD, USA). Isolates of the genus *Williamsia* are currently recognized as environmental microorganisms (4,5). However, its potential as a pathogen in clinical infections has not been reported.

In summary, we report the isolation of >1,000 CFU/mL of *W. muralis* from a protected specimen brush sample of an 80-year-old woman. The number of colonies obtained, as well as features of the source of the clinical sample and the chest radiograph (clearly pathologic) at the time the isolate was obtained, strongly suggest that this microorganism was associated with lung infiltrates and poor prognosis, resulting in the death of the patient. The isolation of a few colonies of a coagulase-negative staphylococci may be considered irrelevant.

We also report the antimicrobial drug susceptibility pattern of *Williamsia* spp. Since no clinical findings for this genus have been reported, no clinical recommendations have been made regarding empiric treatment for infections with this microorganism. This is the first report of this bacterium as a potential human pathogen.

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Neisseria meningitidis Endotoxin and Capsule Transmission by Transplantation

To the Editor: Donor organs for transplantation are in extremely short supply. The increased number of patients with advanced liver disease requiring transplantation has resulted in the expansion of selection criteria for potential donors. Previously unacceptable or marginally acceptable donors, such as those who die from bacterial meningitis, are now donor candidates (1).

Several case reports and small, retrospective studies have shown that, with appropriate antimicrobial drug treatment, transplant recipients of organs from donors with bacterial meningitis do not have increased risk for infection (2,3) or reduced graft survival (3). However, endotoxemia in liver transplant recipients has been associated with graft failure and a high mortality rate (4,5), and in gram-negative bacterial infections, the physiologic effects of endotoxin may persist after adequate antimicrobial drug treatment. We report a case of liver transplantation in which the donor had brain death from meningococcal meningitis. This unique case provided an opportunity to study the serologic responses and clinical course of an organ recipient after liver transplantation from a donor who had died of *Neisseria meningitidis* infection. Our observations suggest that biologically relevant levels of antigens, including endotoxin, may have been transferred to the recipient.

A 57-year-old woman with progressive sclerosing cholangitis and cryptogenic cirrhosis received a liver transplant from a previously healthy 18-year-old man who died of serogroup C *N. meningitidis* meningitis. He had received antimicrobial drug therapy with ceftriaxone and ampicillin for 5 days before brain death was determined; cultures were negative, and the mildly elevated liver function tests, recorded on admission, had resolved, and no evidence of hepatic impairment was shown.

The transplantation surgery was prolonged (17 h) and technically difficult, requiring intraoperative blood products (≈25 U), prolonged postoperative mechanical ventilation, blood pressure support, and renal hemofiltration. Pathologic examination of the recipient's explanted liver showed secondary biliary cirrhosis. The recipient was given ceftriaxone before and after transplantation for 7 days. During postoperative week 4, she was also treated for *Pseudomonas aeruginosa* nosocomial pneumonia and pleural effusion caused by *Enterococcus*. She was extubated 3 weeks after transplantation and discharged 5 weeks after transplantation. No evidence of clinical infection with *N. meningitidis* was identified.

Pathologic examination of the explanted donor liver demonstrated focal acute subcapsular necrosis, large droplet fat accumulation, and mild chronic portal inflammation. The *N. meningitidis* isolated from the blood and cerebrospinal fluid of the donor was serogroup C by immunoprecipitation, and the lipooligosaccharide (LOS) immunotypes (determined by Brenda Brandt, Walter Reed Army Institute of Research, Washington, DC.) were L2, L3, L7, and L9.

Banked serum specimens, obtained from the recipient before the operation and every week for 5 weeks after the operation, were assayed for presence of antibodies to *N. meningitidis*. Elevated levels of immunoglobulin (Ig) G antibodies (11.2 μg/mL at week 2; 22.5 μg/mL at week 4) (6) to the group C meningococcal polysaccharide capsule were detected in recipient serum (Figure, panel A). Levels of IgM antibodies to LOS L9 rose sharply and peaked in the sample taken 2 weeks after the operation (Figure, panel B). Levels of IgM antibodies to L3 also rose slightly between weeks 2 and 4. IgG antibodies to the 4 LOS types and antibodies to outer membrane vesicles were elevated in the first week postoperation and then declined (data not shown) and may reflect antibodies present from intraoperative blood products. Antipolysaccharide antibodies to *Streptococcus pneumoniae* serotypes 14 and 23 and *N. meningitidis* serogroup A also rose between weeks 2 and 4 posttransplantation but were not above values expected in normal adult sera at any time. Bactericidal assays against the infecting strain could not be performed because of endogenous killing that was not complement mediated and was presumed to be caused by the presence of antimicrobial drugs in the serum samples.

The rise in IgM antibodies to LOS L9 and IgG antibodies to group C polysaccharide is consistent with a response to exposure to *Neisseria* antigens at the time of transplantation. With effective antimicrobial drug treatment, the recipient has little risk for bacteremia after transplantation of organs from donors dying of *N. meningitidis* infection (3). However, bacterial antigens, endotoxin, and cytokines could potentially be sequestered in a donor liver, especially when organ transplantation occurs within days of the bacteremic episode. Despite appropriate antimicrobial drug treatment of the donor and recipient, and the absence of any evidence of active infection of the recipient, these data suggest that proinflammatory endotoxin and capsular polysaccharide from *N. meningitidis* were transplanted with the donor liver. Although we cannot definitively associate these findings with the organ recipient's difficult intra- and postoperative course, this case raises the question of the role of proinflammatory responses to transplanted endotoxin in postoperative

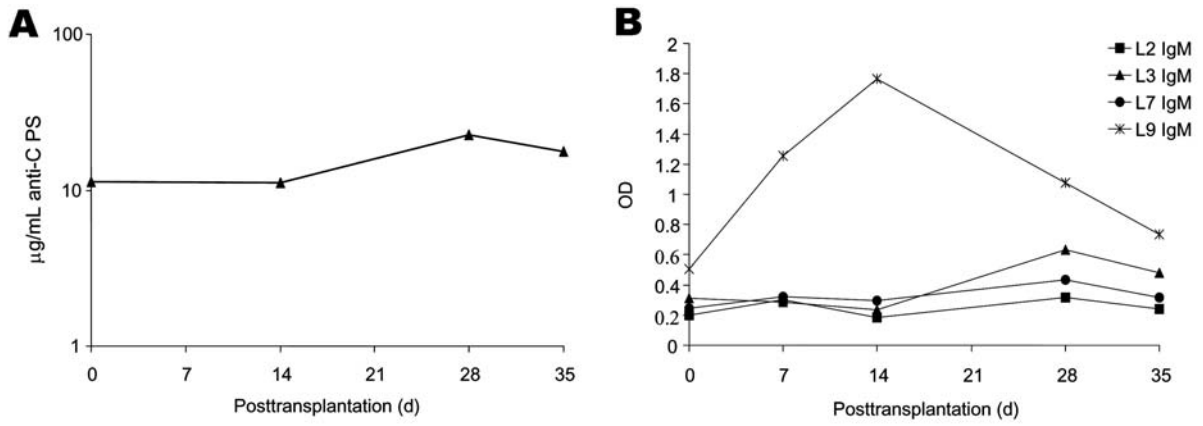

Figure. Pre-and posttransplantation serum antibodies as measured by enzyme-linked immunosorbent assay (ELISA). A) Immunoglobulin (Ig) G antibodies to *Neisseria meningitidis* serogroup C capsular polysaccharide (CPS) determined as described by Arakere and Frasch (7) with minor modifications. Samples were run in duplicate at 8 serial dilutions, and antibody concentrations were calculated relative to the standard reference serum lot CDC 1992 (courtesy of G. Carlone, Centers for Disease Control and Prevention, Atlanta, GA). B) IgM antibodies to *N. meningitidis* lipooligosaccharide (LOS) immunotypes (L2, L3, L7, L9). ELISA to detect antibodies to LOS immunotypes L2, L3, L7, or L9 was performed as described (8), with minor modifications, by using goat antihuman IgG (g-specific, Kirkegaard & Perry, Gaithersburg, MD, USA) or goat antihuman IgM (m-specific, Sigma, St. Louis, MO, USA) conjugated to alkaline phosphatase. Samples were run in duplicate at 4 serial dilutions. OD, optical density.

condition and graft dysfunction in this critically ill population (9,10).

Prospective studies identifying and quantifying endotoxin in the transplanted liver itself and in the recipient may be valuable in assessing the meaning of this finding. An assessment of endotoxin transfer will assist in further defining the risks associated with organ transplantation from donors with *N. meningitidis* infections and may lead to the consideration of additional interventions to mediate the effects of endotoxin exposure.

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Surveillance of Human Calicivirus in Spain

To the Editor: Human caliciviruses (HuCVs) are an important cause of acute viral gastroenteritis in young children worldwide (1,2). In Spain, norovirus infections are not subject to specific surveillance; few data exist about sporadic cases (3,4) and none about outbreaks across the country.

We have conducted a surveillance study of acute gastroenteritis epidemics to determine the prevalence of HuCV infections. Our goal was to gain insight into the epidemiology of these infections in Spain and consider new directions to prevent them and control improvements in food and water quality and sanitary practices.

From October 1998 to October 1999, a pilot prospective program was designed to study viral strains causing annual epidemics of severe diarrhea in children. A total of 822 stool specimens were obtained from children <4 years of age with sporadic gastroenteritis, who visited the emergency room of a hospital in Madrid. A gastroenteritis episode was defined as ≤3 looser-than-normal stools within a 24-h period. Clinical and epidemiologic information was collected. No pathogens were detected in fecal specimens from 292 children. A subset of 201 of these samples was tested for HuCVs.

Additionally, 741 fecal samples were collected from 135 outbreaks that occurred throughout Spain (13 of 17 geographic areas) from 2000 to 2002. An outbreak was defined with Kaplan criteria (5). Epidemiologic data (mode of transmission, setting and size of the outbreak, persons affected, persons at risk, attack rate, and age of affected persons) were recorded on a standardized form submitted to the National Microbiology Center.

Enteropathogenic bacteria in fecal specimens were examined by conventional culture procedures. Viruses (group A rotavirus, adenovirus, and astrovirus) were also examined by commercial enzyme immunoassay (Dako Diagnostics, Cambridgeshire, UK). In negative samples (1,033 of 1,563), viral RNA was extracted as described (3) and analyzed by reverse transcription–polymerase chain reaction (RT-PCR) for HuCVs by using JV12/JV13 primers (6). RT-PCR–negative samples were also reanalyzed with p289/290 (7) and NVp110-Nvp69 (8) primers pair, which detect sapovirus. HuCV-positive specimens were confirmed and genetically characterized by the reverse line blot hybridization method (9) and sequencing assays by using High Pure PCR Product Purification kit (Boehringer, Mannheim, Germany) and ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit (Perkin Elmer Biosystems, Foster City, CA, USA) on an automated sequencer (Applied Biosystems model 3700). For HuCV phylogenetic analyses, a multiple-sequence alignment was generated from the consensus sequence of each of the isolates and the reference strains by using ClustalX 1.8 methods. The nucleotide sequences were analyzed with the MEGA 2.1 analytical package by using neighbor-joining methods and Kimura 2-parameters algorithm. GenBank accession numbers for sequences described in this study are AY207341–AY207365.

In pediatric gastroenteritis cases, HuCVs were detected by RT-PCR in 63 specimens (31%). Twenty-nine of these were genotyped, and results of phylogenetic analyses are shown in the Table. The median age of children with HuCV infection was 15 months (range 1–47). In HuCV-positive cases, 83% were associated with vomiting, 32% with fever, 24% with mild dehydration, and 2% with severe dehydration. Hospitalization was required in 13% of the cases.

Additionally, noroviruses were detected in 85 (63%) of 135 outbreaks. Seventy- seven (91%) of them were segregated into genogroup II (Table). Detection rates within outbreaks ranged from 13% to 100%. The setting was provided for 82 (97%) of 85 norovirus outbreaks. Nursing homes (57%) were the most common setting, followed by schools (10%), camping and vacation destinations (7%), hospitals (6%), and restaurants and hotels (4% each). The attack rate was provided for 33 (39%) norovirus outbreaks, and the median number of persons affected was 35. The mode of transmission was provided for 30 (35%) of the norovirus-positive outbreaks; the most common mode of transmission was person-to-person contact (n = 15), followed by contaminated food (n = 10) and contaminated water (n = 5).

This report shows the importance and diversity of HuCVs circulating throughout Spain. Noroviruses particularly have been found as a main causative agent of sporadic pediatric cases and outbreaks. The lower prevalence of sapovirus is similar to that shown by other authors (2), perhaps because sapovirus causes milder symptoms than norovirus. Analysis showed the predominance of

Table. Human caliciviruses (HuCVs) and phylogenetic clusters found in sporadic pediatric cases and gastroenteritis outbreak

	Pediatric cases (N = 201) (%)	Outbreak incident (N = 135) (%)
HuCVs found by reverse transcription–polymerase chain reaction	63 (31)	85 (63)
HuCVs analyzed phylogenetically	29	83
Sapovirus	3 (10)	0
Norovirus*	26 (90)	85 (100)
GI-Desert Shield cluster	0	5
GI-Queens Arms cluster	1	1
GII-Hawaii cluster	0	8
GII-Leeds cluster	0	1
GII-Lordsdale cluster	23	63
GII-Meklsham cluster	0	5
GII-Mexico cluster	2	0

*GI, genogroup I; GII, genogroup II.

norovirus genogroup II and Lordsdale cluster as the main genotypes both in sporadic cases and outbreaks, also shown in other reports (1,2,6).

Our study confirms that noroviruses are the main cause of nonbacterial gastroenteritis outbreaks throughout Spain, as in other European countries (1,10). However, we consider that HuCV infections could be underdiagnosed because a substantial number of nonbacterial outbreaks are labeled of unknown etiology. The systematic application of sensitive techniques to detect these viruses, as well as a more systematic surveillance system for viral diarrhea, would provide broader knowledge of norovirus infection in Spain.

Acknowledgments

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Correction, vol. 11, no. 6

In "Community-acquired Methicillin-resistant *Staphylococcus aureus*, Uruguay" by Xiao Xue Ma et al., errors occurred on pages 973 and 974.

The first sentence of the abstract should read as follows: A novel, methicillin-resistant *Staphylococcus aureus* clone (Uruguay clone) with a non–multidrug-resistant phenotype caused a large outbreak, including 7 deaths, in Montevideo, Uruguay.

The first sentence of the article should read as follows: Since the 1990s, methicillin-resistant *Staphylococcus aureus* (MRSA) infections have been increasingly recognized in the community, and MRSA strains isolated from patients with community-associated cases have been called community-associated MRSA (CA-MRSA).

The first sentence of Figure 1 legend (p. 974) should read as follows: The monthly accumulation of cases of infections due to non–multidrug-resistant MRSA strains from January 2002 to October 2003.

The corrected article appears online at http://www.cdc.gov/ncidod/eid/vol11no06/04-1059.htm

We regret any confusion these errors may have caused.

Cellular Microbiology, 2nd ed.

Pascale Cossart, Patrice Boquet, Staffan Normark, and Rino Rappuoli, editors

ASM Press, Washington, DC
ISBN: 1-55581-302-X
Pages: 593, Price: US $119.95

The field of cellular microbiology is relatively new and incorporates aspects of microbiology and host cellular biology. The first edition of this text, published in 2000, was novel and well received. In general, this new edition is also well written and includes many of the most important recent advances in the field (e.g., microarrays and genome sequencing). The text deals almost exclusively with host cell responses elicited by interactions with pathogens. The editors are top researchers in the field of bacterial cellular microbiology, and they have brought together many new investigators to write chapters in their areas of expertise.

The book's first 2 chapters contain topical background information. These chapters thoroughly cover many of the basic concepts in molecular cell biology and introduce all of the various pathogens (bacterial, viral, and eukaryotic) currently being examined in the popular literature. The organization of the subsequent chapters typically alternates between topics in cell biology and bacterial pathogenesis. For example, chapter 11 describes assembly of the cellular cytoskeleton, while chapter 12 describes the mechanisms used by pathogenic bacteria to manipulate the cytoskeleton. Subsequent chapters provide good coverage of bacterial secretion systems, toxins, and their interactions with the host immune system. Generally, the figures, diagrams, and drawings are well chosen, and the tables contain sufficient detail to demonstrate critical points. This is particularly true for chapters 4–6, which describe the host cell surface properties, how pathogenic bacteria adhere to and enter the host cell, and ultimately how the pathogen induces various types of cell signaling. The final chapters focus on new methods of identifying virulence genes and the use of nonvertebrate hosts, such as plants and insects, to model mammalian infections.

This book has only a few drawbacks. For example, the first 2 introductory chapters are too detailed. In subsequent chapters, the emphasis is placed on bacterial pathogens; only 1 chapter is dedicated to viruses and none to eukaryotic pathogens (only the introductory paragraphs in chapter 1) or to nonpathogenic microorganisms of any kind.

This volume will be an important addition to the resources available to students and researchers in general cell biology or microbiology. Perhaps Internet interactive companion programs and accompanying CDs would be useful with future editions. Because the field is moving so quickly, the authors might consider more frequent updates.

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Biology of Disease Vectors – 2nd ed.

William C. Marquardt, editor

Elsevier Academic Press, Burlington, Massachusetts
Second Edition
ISBN: 0-12-473276-3
Pages: 785, Price US $99.95

This edition is a massive, 7-section, 57-chapter medical entomology reference text. The chapters are written by 72 experts from around the world and provide an understanding of disease vectors on a broad front, including biologic requirements of vectors, epidemiology, molecular biology, genetics, principles of control, and insecticide resistance. The text consistently emphasizes molecular biologic approaches to these topics.

This book begins by discussing the vectors themselves, with chapters on mites, ticks, true bugs, lice, fleas, mosquitoes, and various dipterans such as tsetse flies and sand flies. Line drawings and black-and-white pictures abound. The number of color photos is limited; those in the kissing bug/bed bug chapter and the flea chapter are especially beautiful. Subsequent sections delve into the physiologic and genetic basis of vector biology. The final 2 sections concern controlling insects and acarines and special (laboratory) methods associated with vectors. The last section, which deals with laboratory methods, is like a giant appendix in which updates are given for the care, maintenance, and experimental infection of various disease vectors, including notes on handling, housing, rearing facilities, containment, and safety issues.

One of the most helpful chapters for this reviewer was the one entitled, "Systematic Relationships among Disease Vectors," which defines

molecular systematics terminology and explains how phylogenetic relationships among species are inferred from molecular data. I wish every traditional taxonomist and systematist would read this chapter.

This book contains a few misspellings (e.g., the chapter title in the Table of Contents, Chapter 46), but no major errors. Its only weakness seems to be one of disunity. The title doesn't match the book's content, and the text is so comprehensive that it seems unfocused. There are chapters on chemical and genetic control of vectors, cell culture, and even research safeguards for transgenic mosquitoes. How these fit under the title biology of vectors was difficult to discern. Perhaps in future editions, the chief editor could split the book into several separate volumes, each with a more appropriate title.

Nevertheless, this book is an indispensable reference and a wonderful treasure trove of information about medical entomology. Its only flaws are organizational, not factual. The chief editor, section editors, and authors are to be congratulated on this scholarly work.

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Tuberculosis and the Tubercle Bacillus

Steward T. Cole, Kathleen Davis Eisenach, David N. McMurray, and William R. Jacobs Jr, editors

ASM Press, Washington, DC
ISBN: 1-55581-295-3
Pages: 584; Price: US $125.95

Mycobacterial infections, including tuberculosis (TB) and leprosy, are bacterial diseases of global importance. An estimated 2 billion people are infected with *Mycobacterium tuberculosis*. Control of TB is complicated by its ease of transmission, difficulty in administering the long-course chemotherapy regimens, and subsequent appearance of multidrug-resistant strains (MDR-TB). This situation is made even worse by the deadly combination of coinfections of HIV and *M. tuberculosis*. New approaches to the control of TB are urgently needed, including development of short-term antimicrobial regimens to minimize the appearance of drug resistance, new drugs to treat MDR-TB patients, and new vaccines with greater efficacy than BCG.

Tuberculosis and the Tubercle Bacillus has many contributors; chapters are provided by experts in many areas of TB research to bring together a comprehensive update of research development in the past decade. The publication of this book is necessary and timely, considering the current urgencies and growing interests of investigators from various fields.

The book is divided into 3 sections, each consisting of multiple chapters on various subjects. The first section focuses on clinical aspects of the disease, including the global impact of TB, clinical and epidemiologic features, as well as diagnosis and treatment. The second section deals with the bacteriology of *M. tuberculosis*, with chapters devoted to molecular genetics, genomics, cell wall structure and synthesis, and metabolism. The third section details the host-pathogen interaction, covering topics such as the intracellular survival of *M. tuberculosis*, host immune response, animal models, and vaccine development.

The book accurately reflects current knowledge of TB and recent research efforts and progresses to the control of the disease. The book flows smoothly from chapter to chapter. Each chapter is clearly written and appropriately referenced. The book focuses primarily on *M. tuberculosis*; research performed on other mycobacterial species is not discussed or only briefly mentioned. Nevertheless, at 584 pages, this book is easily read and is a useful reference for clinicians and basic scientists, including students, laboratory supervisors, and senior scientists.

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Herakles and the Stymphalian Birds (detail) (circa 6th century BC)
Athenian black-figured amphora (40.6 cm), The British Museum, London, UK

Ancient Myths and Avian Pestilence

Polyxeni Potter*

"These birds are the size of a crane and are like the ibis, but their beaks are more powerful, and not crooked like the ibis," wrote ancient Hellene traveler and writer, Pausanias (1). He was referring to large flocks of metal-clawed ornithes, which according to legend, roosted in the dense marshes around Lake Stymphalis in Arcadia, ravaging crops and the livelihood of neighboring villages (2). This scourge, he speculated, was not local. "The Arabian Desert breeds, among other wild creatures, birds…which are quite as savage against men as lions or leopards…. These fly against those who come to hunt them, wounding and killing them with their beaks" (1).

The flesh-eating predators further terrorized local inhabitants by dispatching against them razor-edged feathers like arrows. "All armor of bronze or iron that men wear is pierced by the birds," elaborated Pausanias (1). Pets of Ares, god of war, these birds were a public menace too great for the community to control, a challenge finally assigned, along with other "labors," to strongman of all time, Herakles.

Son of Zeus and mortal Alcmene, Herakles might have enjoyed the privileged life of a demigod. But, victimized by Zeus' jealous wife, Hera, he endured a mortal lot of labor and hardship, punctuated by periods of madness and aberrant behavior. Strong, resourceful, and gifted with magical defenses, he had to struggle, nonetheless, against nature that was deadly, unpredictable, and arbitrary. During his celebrated labors, he battled vicious beasts (among them Kerberos, the guard of Hades) and cleaned out the infamous Augean Stables, which housed the filthiest herd of cattle in Hellas. To attain immortality, he performed, as penance for his misdeeds, arduous service to the community, using his unparalleled strength to support his fellow humans.

The thick marsh habitat of the Stymphalian birds worked against Herakles. His bow and arrows failed, for he could neither see nor reach the birds through the dense vegetation. Only asked to drive them away, he abandoned efforts to eliminate the birds; instead, he conned them into leaving the area on their own. With a pair of krotala (metal rattles) made by Hephaestus, god of the forge, Herakles frightened the birds out of their refuge and chased them as they flew east to the Isle of Ares in the Black Sea.

The detail on this month's cover comes from a black-figured amphora, a ceramic vase popular in ancient Athens in the 6th century BC. Such vases were made of iron-rich clay and decorated with black silhouettes in mythical heroic scenes. Illustrations were incised and painted with a slip (liquid clay), which turned black during firing without oxygen (3).

The scene is full of action but contains no background clues. Hellenic myths focus on the here and now and its terrifying uncertainties and dilemmas. They address

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human concerns, not philosophical conceit. Their narrative blurs the boundaries of history and legend as heroes cross back and forth from fantasy to reality, often operating in geographic locations that can never quite be verified on a map.

Herakles cuts a powerful figure as he leans forward, aiming a sling at the birds. His body, draped with the impenetrable hide of the Nemean Lion, a trophy from his first labor, forms a barrier against the flock. The birds scatter in disarray, not laden with metal as the myth prescribes, but confused, half resting at the foot of the hero, half flapping their wings against each other, compromised by the lack of cover. These are beautiful birds, dotted and striped, with elegant long necks turned defensively inward. Yet, in some versions of the Stymphalian labor, the birds are harpies—half metal-feathered ornithes, half human heads with bronze beaks.

Herakles probably wished he had not stopped at chasing these Arabian birds away from Arcadia for, even in the small world of antiquity, geographic migration ruled. The birds surfaced again, during his sail with Jason and the Argonauts in search of the Golden Fleece, to be chased away again, this time by the sons of the North Wind.

Flawed humanity tested by overwhelming challenges rings true today. Heroic figures battling great odds excite our collective imagination. And public challenges (waste pollution out of control, avian pestilence) have changed little. Waterfowl, a benign species, were demonized in the Stymphalian myth, their hideous mien likely borne of human fear and helplessness, for who knows what pestilence they had inflicted on the community around the lake. And each time those birds flew to a new place, they had contact with other birds and opportunities for genetic reassortment, redistribution, and modification of pathogens throughout the migratory route.

Resistant to slings and arrows and prone to long-distance migrations, birds such as the ones on this cover's amphora persist beyond our ancestors' morbid imaginations. Not because of mythical metal paraphernalia but for their explosive potential as natural reservoirs and amplifying hosts of pathogens. Viremic migratory birds acting as introductory hosts may have brought West Nile virus to the Western Hemisphere, perhaps by infecting ornithophilic mosquitoes, which may have infected amplifying hosts and eventually humans (4,5).

Migratory waterfowl (ducks, geese) also carry flu viruses in their intestines and shed them in their secretions and excretions. As these waterfowl migrate around the globe, they introduce new flu strains into domestic poultry and swine. These strains can then amplify and mutate close to human populations, increasing the risk that the virus will recombine with local human strains to form a new virus with pandemic potential. Like the legendary harpies, these new strains, half human half avian, pose an immense public health challenge. We now know more about bird pestilence. West Nile virus infection and avian flu are just as ominous as razor-edged feathers. And while Herakles had krotala from the gods, we must work with human tools: repellants and pesticides, vaccines (6), antiviral drugs, or medical isolation and quarantine.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the September issue for the following topics:

Dead Crow Density and West Nile Virus Monitoring

Dengue Virus Type 3, Brazil, 2002

Trypanosomiasis Control, Democratic Republic of Congo, 1993–2003

Fluoroquinolone-resistant *Escherichia coli*, Indonesia

Persistence of Resistant *Staphylococcus epidermidis* after Single Course of Clarithromycin

Antiviral Therapy during Possible Influenza Pandemic

Simulated Anthrax Attacks and Syndromic Surveillance

West Nile Virus–Infected Mosquitoes, Louisiana, 2002

Legionellosis from *Legionella pneumophila* Serogroup 13

Malaria Attributable to the HIV-1 Epidemic, Sub-Saharan Africa

Molecular Epidemiology of SARS-associated Coronavirus, Beijing

History and Epidemiology of Malaria, Western Kenya Highlands

Protective Behavior and West Nile Virus Risk

Sensitivities of West Nile Virus Specimens

Endemic Tularemia, Sweden, 2003

Complete list of articles in the September issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

August 28–September 2, 2005
5th International Conference on Ticks and Tick-borne Pathogens
University of Neuchâtel
CH-2000 Neuchâtel, Switzerland
http://www2.unine.ch/ttp5

September 10–14, 2005
Infectious Diseases 2005 Board Review Course
The Ritz-Carlton, Tyson Corners
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Contact 201-883-5826 or dvalencia@cbcbiomed.com
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Santa Fe, NM, USA
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November 13–18, 2005
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December 5–9, 2005
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Hyatt Regency Hotel on Capitol Hill
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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
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The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

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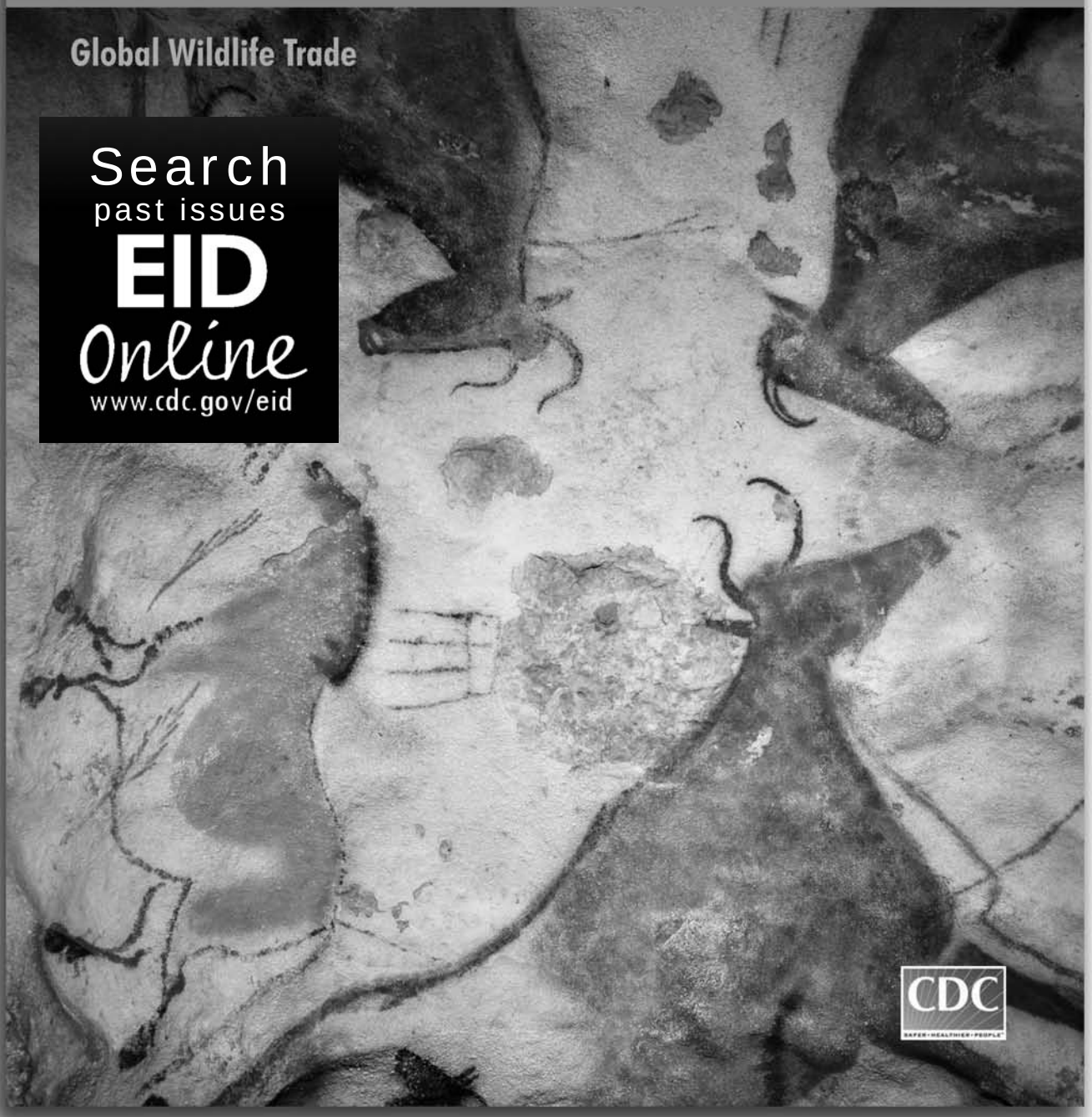

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Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 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.